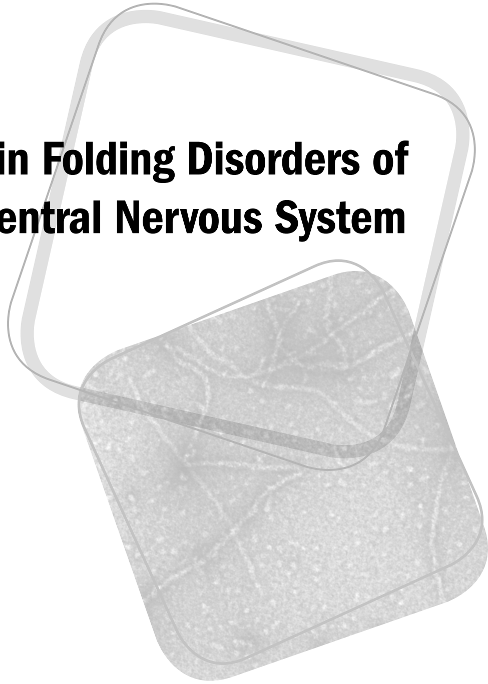
Protein Folding Disorders of the Central Nervous System



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Protein Folding Disorders of the Central Nervous System

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Published by

World Scientific Publishing Co. Pte. Ltd.

5 Toh Tuck Link, Singapore 596224

USA office: 27 Warren Street, Suite 401-402, Hackensack, NJ 07601

UK office: 57 Shelton Street, Covent Garden, London WC2H 9HE

Library of Congress Cataloging-in-Publication Data
Names: Ghiso, Jorge, editor. | Rostagno, Agueda, editor.
Title: Protein folding disorders of the central nervous system / edited by
Jorge Ghiso (New York University, USA), Agueda Rostagno (New York University, USA)
Description: New Jersey : World Scientific, 2017. | Includes bibliographical references.
Identifiers: LCCN 2017011131 | ISBN 9789813222953 (hc : alk. paper)
Subjects: | MESH: Central Nervous System Diseases--etiology | Proteostasis Deficiencies--
complications | Proteostasis Deficiencies--physiopathology
Classification: LCC RA644.P93 | NLM WL 301 | DDC 616.8/3--dc23
LC record available at https://lccn.loc.gov/2017011131

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

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Desk Editors: Kalpana Bharanikumar/T. Yugarani

Typeset by Stallion Press
Email: enquiries@stallionpress.com

Printed in Singapore

Preface

The proteins of a living organism, after synthesis at the ribosome, must properly fold into specific conformational states to successfully perform their biological function. To this aim, all cells possess complex mechanisms to efficiently regulate protein assembly into functionally active and optimally balanced states. In spite of these homeostatic mechanisms set in place, under some conditions, proteins fail to fold correctly, or to remain correctly folded in living systems, a failure that eventually leads to the accumulation of structurally abnormal pathogenic assemblies. The term "protein misfolding disorders" refers to an emerging complex group of chronic and progressive entities in which the pathogenesis of the respective diseases is driven by structural transitions in the native state of specific proteinaceous components into polymeric aggregates which generate poorly soluble tissue deposits.

Here, we took the challenge to organize a book focusing on diseases associated with protein misfolding in the central nervous system (CNS), which we felt needed to be addressed given the growing number of reports linking abnormal protein folding/aggregation to human disease, in general, and neurodegeneration, in particular. Although distinguished by disease-specific pathology and clinical presentations, these disorders share conspicuous similarities. They are mostly of sporadic origin and, since they are largely age-related, their relevance continues to rise as the number of individuals affected by these conformational disorders is expected to

increase as the world population ages. Current therapeutic strategies are only aimed at alleviating symptoms, and in most cases the normal physiological function of the proteins that aggregate in the respective diseases remain unknown. These proteins are, however, all intimately associated with the specific disorders, as mutations in the gene encoding the disease-linked protein, or its precursor, cause early-onset familial disease.

The book is divided into 14 chapters that provide a comprehensive, state-of-the-art perspective of the topic under discussion, bringing insights into the biological/biophysical mechanisms of protein folding and their relationship to CNS diseases linked to aberrant protein conformations. Individual chapters are dedicated to the most common neurodegenerative diseases associated with protein aggregation/fibrillization, focusing on the nature of the pathogenic species and the pathways involved in Alzheimer's, Parkinson's, and Huntington diseases as well as in amyotrophic lateral sclerosis, and prion diseases. A group of contributions is focused on the intracellular pathways and organelles affected by the different disease conditions and the transmissibility of protein misfolding, whereas another set of chapters is dedicated to novel strategies to therapy for these devastating diseases.

The combination of contributions compiled in this volume is expected to be of interest to the large audience of protein chemists, biochemists, and biophysicists from postgraduate level onward, as well as to clinicians, and all scientists with broad interests in aspects relating to structural biology, protein folding, and disease, as well as in the molecular and cellular aspects of disease pathogenesis in the CNS. We hope that this overview on this key biological problem will make this volume a resourceful source of information that bridges together different aspects of these complex problem.

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Chapter 1

Misfolding, Aggregation, and Amyloid Formation: The Dark Side of Proteins

Agueda Rostagno and Jorge A. Ghiso

1.1 Introduction

Protein misfolding disorders are an emerging complex group of chronic and progressive entities driven by structural transitions in the native state of specific proteinaceous components and the generation of polymeric aggregates that assemble into poorly soluble tissue deposits. In all these disorders, through mechanistic pathways still poorly understood, soluble proteins normally found in biological fluids change their conformation and form insoluble structures that accumulate as intra- and extracellular aggregates or as fibrillar deposits. The group comprises a wide range of diseases encompassing disorders that are either (i) localized to the central nervous system (CNS) and particularly associated with cerebrovascular compromise, neuronal vulnerability, and neurodegeneration; (ii) restricted to other individual organs, where their presence correlate with a specific organ dysfunction(s), e.g. the pancreas in type II diabetes or the heart in familial amyloidotic cardiomyopathy; or (iii) affecting multiple organs, as in the case of systemic amyloidosis. The group of disorders affecting the CNS, which is the focus of this book, is quite heterogeneous and, as illustrated in Table 1.1, includes conditions with dissimilar clinical manifestations — ranging from cognitive decline and dementia to severe motor deficits or to recurrent episodes of cerebral hemorrhage — as well as disease-specific

Table 1.1 Human diseases associated with disorders of protein folding in the CNS.

Disease Name	Primary Misfolded Protein(s)
AD	Aβ and Tau
Amyotrophic lateral sclerosis	Superoxide dismutase 1, TDP-43, Fused in sarcoma (FUS)
Dentatorubral-pallidoluysian atrophy	Atrophin-1 with polyQ expansion
Familial British dementia	ABri
Familial Danish dementia	ADan
Familial presenile dementia	Neuroserpin
Frontotemporal dementia and other tauopathies[a]	Tau
Hereditary cerebral amyloid angiopathies	Aβ, cystatin C, Gelsolin, Prion, and Transthyretin (TTR) mutants
HD	Huntingtin with polyQ expansion
Spinal and bulbar muscular atrophy	Androgen receptor with polyQ expansion
Spinocerebellar ataxias	Ataxins with polyQ expansion
Spinocerebellar ataxia 17	TATA box-binding protein with polyQ expansion
Spongiform encephalopathies[b]	Prion protein or Prion fragments
Synucleinopathies[c]	α-Synuclein

Notes: [a] Other tauopathies include cortico-basal degeneration, Pick's disease, progressive supranuclear palsy, traumatic brain injury, and tangle-only dementia.
[b] Include Creutzfeldt–Jakob disease, variant Creutzfeldt–Jakob disease, Gerstmann–Straussler–Scheinker disease, fatal familial insomnia, and kuru.
[c] Include Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy.

pathology [1–4]. The histological localization of the abnormally folded proteins differs among the different associated disorders, with protein aggregates accumulating in the extracellular space or within different sub-cellular compartments including the cytoplasm, the nucleus, or even the cell membrane [5]. In Huntington's disease (HD), for example, the aggregated polyglutamine protein huntingtin presents in the form of intranuclear inclusions; in prion diseases, the infectious prion particles are associated with the cell plasma membranes. In other cases, the localization of the protein aggregates is cytoplasmic, as in Parkinson's disease (PD), in which α-synuclein inclusions are found in neuronal Lewy bodies. In Alzheimer's disease (AD) — the most common of the protein folding disorders

associated to the CNS — intraneuronal deposits of hyperphosphorylated tau in the form of paired-helical filaments as well as extracellular amyloid plaques primarily composed of fibrillar amyloid β (Aβ) are the classical neuropathological features (Figure 1.1A). In spite of the differences among the biochemical nature of the deposited proteins, the extracellular or subcellular localization of the pathogenic aggregates, and the range of clinical manifestations, all these disorders share noticeable similarities: (a) they are largely age-related, currently incurable diseases, and mostly sporadic in origin; (b) the normal physiologic function of the proteins involved in the formation of aggregates/fibrillary deposits remains largely unknown; (c) mutations in the genes encoding these protein subunits or their respective precursors result in early-onset familial forms of the disease; (d) the presence of these heterogeneous deposits trigger similar pathogenic responses usually associated with local release of inflammatory mediators, oxidative stress, mitochondrial dysfunction, activation of the complement system, and initiation of cell death mechanisms ultimately resulting in cell damage, organ dysfunction, and eventually death.

1.2 Molecules associated with extra- and intracellular deposits of misfolded proteins

The number of proteins exhibiting aberrant misfolding and linked to disease in humans continues to expand. An important group of these molecules is constituted by those forming extracellular fibrillar deposits, structures generically known as *amyloid*. Indeed, as per the guidelines of the International Society of Amyloidosis, the location of poorly soluble fibrillar deposits in extracellular spaces is a requirement for a specific component to be considered as amyloid [6]. This characteristic has resulted in the latest inclusion of two molecules — α-synuclein and Tau — as new evidence has demonstrated that, in addition to the classical intracellular inclusions, fibrillar elements of these proteins are also present as extracellular deposits upon neuronal death. Overall, 36 different proteins and more than 100 genetic variants are known to be associated with systemic and localized forms of amyloidosis in humans at the present time. However, only a fraction of them are known to generate amyloid deposits in the nervous system — central (CNS) and peripheral (PNS) — with the vast majority forming multi-organ tissue deposits [1, 6, 7].

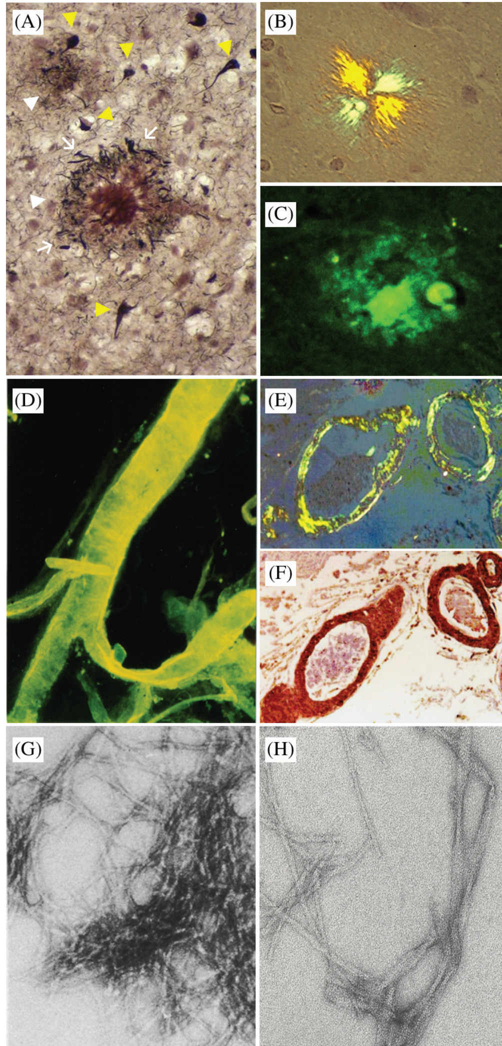

Figure 1.1 Misfolded protein deposits in AD. (A) Amyloid plaques (white arrowheads), intracellular neurofibrillary tangles (yellow arrowheads), and dystrophic neurites (white arrows) highlighted by silver stain; (B) apple-green birefringence of a parenchymal plaque viewed under polarized lights after Congo red staining; (C) classic yellowish-green fluorescence of a parenchymal plaque stained with Thioflavin S; (D) amyloid deposits in an isolated leptomeningeal vessel stained with Thioflavin S; (E) vascular deposits visualized by Congo red staining; (F) immunohistochemistry of a parallel section showing the vascular lesions stained with specific anti-Aβ antibody; (G) amyloid fibrils negatively stained and visualized by electron microscopy; (H) paired-helical filaments negatively stained and visualized by electron microscopy.

When affecting the CNS, the typical clinical expressions resulting from the structural changes of these proteins are neurodegeneration and/or cerebrovascular disease, whereas for those few targeting the PNS, the classic phenotypic outcome is polyneuropathy. The latter group is most commonly linked to multiple mutations in the transthyretin gene, although other amyloid proteins that have been associated with peripheral neuropathy include immunoglobulin light chain and the recently described genetic variants of apolipoprotein A1, gelsolin, and β2-microglobulin [8, 9]. Notably, among the many amyloid subunits manifesting with dementia and/or cerebral hemorrhage, several also exhibit multi-organ, albeit clinically silent, amyloid deposits, e.g. ABri, ADan, or ACys (Table 1.2). The

Table 1.2 Protein subunits associated with amyloid deposition in the CNS and PNS.

Amyloid	Precursor Protein	Chr #	S/R[a]	A/H[b]	PNP[c]	CNS	CAA
AαSyn[d]	α-Synuclein	4	R	A	No	Yes	No
AαSyn	α-Synuclein variants	4	R	H	No	Yes	No
Aβ	APP	21	R	A	No	Yes	Yes
Aβ	APP variants	21	R	H	No	Yes	Yes
Aβ2M	β2 microglobulin	15	S	H	Yes	No	No
ABri	BRI2 variant	13	S	H	No	Yes	Yes
ACys	Cystatin C variant	20	S	H	No	Yes	Yes
ADan	BRI2 variant	13	S	H	No	Yes	Yes
AGel	Gelsolin variants	9	S	H	Yes	Yes	Yes
AL	Immunoglobulin light chain	2/22	S	A	Yes	No	No
ApoA1	Apolipoprotein A1 variants	11	S	H	Yes	No	No
APrp^{Sc}	Prion protein	20	R	A	No	Yes	No
APrp	Prion protein variants	20	S	H	Yes	Yes	Yes
ATau[d]	Tau	17	R	A	No	Yes	No
ATTR	Transthyretin	18	S	A	Yes	No	No
ATTR	Transthyretin variants	18	S	H	Yes	Yes	Yes

Notes: [a] Multi-organ, systemic deposition (S), or to restricted/localized deposition (R).
[b] Acquired/sporadic (A) or hereditary (H).
[c] Polyneuropathy (PNP) indicates deposition at the peripheral nerves.
[d] These proteins are a new addition to the list of amyloid proteins and were recently incorporated upon demonstration that besides being part of intracellular inclusions, both proteins accumulated in extracellular deposits upon cell death.

only exception at the moment to the lack of clinical manifestations associated with the systemic deposits appears to be the newly reported PrP variants in which, in addition to the neurodegenerative clinical phenotype, patients show gastrointestinal symptoms and peripheral neuropathy [9].

It is important to highlight that in spite of the differences in the primary structure of the proteins composing the amyloid tissue deposits, all the unrelated subunits share common physical, structural, and tinctorial properties. Indeed, the very name amyloid — coined by Virchow in 1854 — originated in the Latin *amylum* which describes the starch-like iodine-staining properties of these deposits. In general, all amyloids are highly polymerized and poorly soluble protein assemblies, properties that prevent their efficient physiologic removal *in vivo* and require the use of strong detergents, harsh acid conditions, or concentrated chaotropes to extract them from the tissue deposits. Typically, the amyloidogenic proteins are structurally rich in β-pleated sheet conformations, exhibiting an apple-green birefringence when observed under polarized light following Congo red staining (Figures 1.1B and 1.1E), a yellow-green fluorescence when stained with thioflavin S (Figures 1.1C and 1.1D), and a fibrillar structure by electron microscopy displaying morphologically indistinguishable bundles of long twisted filaments (Figure 1.1G).

Other important group of proteins linked to disorders of protein folding is constituted by those that are found in the form of intracellular protein inclusions (Table 1.3). Strictly speaking, these deposits are not considered amyloid, failing one of the main criteria for inclusion in the group, the ability to form *extracellular* fibrils/aggregates. Irrespective of their intracellular localization, some of these protein inclusions share some properties with amyloid deposits. In this sense, neurofibrillary tangles — formed by hyperphosphorylated tau (Figure 1.1H) — exhibit a fibrillar structure, a cross β-sheet X-ray diffraction pattern, and are able to bind Congo red exhibiting green birefringence under polarized light. Similarly, Lewy bodies in the brains of PD patients with dementia consist of fine amyloid-like fibrils, which sometimes stain with Congo red and exhibit birefringence under polarized light. In other cases, the precise nature of the protein aggregates is still debatable. For example, in the case of TDP-43 inclusions, there is still conflicting information regarding whether the inclusions consist of fibrillar assemblies or whether they represent another type of misfolded aggregates [10]. While some reports

Table 1.3 Proteins associated with intraneuronal inclusions.

Misfolded Protein	Inclusion Name	Associated Diseases
Actin	Hirano bodies	Neurodegenerative disorders
α-Synuclein	Lewy bodies	PD
Ferritin	Not specified	Neuroferritinopathy
FUS/TLS[a]	Not specified	ALS[b], FTLD[c]
Huntingtin w/polyQ	Huntington bodies	HD
Neuroserpins	Collins bodies	Familial presenile dementia
Tau	Neurofibrillary tangles	AD, tauopathies
TDP-43[d]	Not specified	ALS, FTLD, others[e]

Notes: [a] Fused in sarcoma/translocated in liposarcoma.
[b] Amyotrophic lateral sclerosis.
[c] Frontotemporal lobar degeneration.
[d] TAR DNA binding protein, 43 kDa.
[e] Initially TDP-43 pathology was thought specific for ALS and a subset of FTLD cases, but is now known to be present in many neurodegenerative conditions, including AD, PD, diffuse Lewy body disease, and many others [17].

indicate the presence of TDP-43 positive, 10–20 nm wide filaments in the absence of Congo red and Thioflavin S binding, suggesting a non-amyloid structure [11–13], other evidence indicates the presence of a widespread Thioflavin S staining in the TDP-43 inclusions present in lower motor neurons of sporadic ALS cases, suggesting on the contrary an amyloid-like structure [14].

1.3 Protein folding and misfolding: Gauging the nature of the pathogenic species

It is well established that following synthesis on the ribosome, the proteins of a living organism must properly fold into a specific conformational state to successfully perform their biological function. As a consequence, all cells possess a complex system of mechanisms aimed to maintain protein homeostasis, known as proteostasis, efficiently regulating protein synthesis and assembly into functionally active and optimally balanced states [15]. Within the cells, folding takes place in a complex and highly crowded environment and begins as the nascent chain emerges from the ribosome

as a highly disordered, random coil state molecule. Proper folding, a process that becomes more increasingly challenging with the size and complexity of each respective protein, requires the aid of many families of auxiliary proteins, primarily molecular chaperones that either reduce the chance of aggregation following synthesis, allow aggregated proteins to refold, or target misfolded molecules for degradation [16]. In spite of these homeostatic mechanisms set in place, under some conditions, proteins fail to fold correctly or to remain correctly folded in living systems, a failure that eventually leads to the accumulation of structurally abnormal pathogenic assemblies that associate with specific disease processes.

There is considerable *in vitro* data indicating that the process of self-assembly toward the formation of amyloid fibrils proceeds in a nucleation-dependent polymerization mechanism. Above a critical concentration below which polymerization does not occur, monomers self-assemble to form dimers, oligomers, and polymers with kinetics that are characteristic of each amyloid subunit. In addition to differences in rate and degree of polymerization, all molecules exhibit a variable lag phase that can be minimized or even eliminated by the addition of pre-formed aggregates. In this sense, *in vitro* experiments indicate that the process of fibril formation is substantially accelerated when nonfibrillar solutions are seeded with preformed fibrils, a mechanism that has been correlated with the infectivity of prion diseases [18]. From the thermodynamic point of view, this process occurs in a two-stepwise reaction involving an initial slow lag period characteristic of each amyloid subunit which reflects the energy barrier necessary for the formation of a nucleation "seed", followed by a rapid fibril propagation and aggregation state [19, 20]. Using kinetic studies, the participation of seeding/nucleation mechanisms has been demonstrated in an array of different proteins, among them PrP, huntingtin, α-synuclein, Aβ, and ABri [21–23].

Mounting evidence indicate that cellular dysfunction and cell death in neurodegenerative diseases appear to be mainly caused by a subset of intermediate conformations rather than by the final fibrillar assemblies [24–26]. Indeed, increasing evidence seems to indicate that — at least in some cases — sequestration of oligomers into large insoluble deposits is protective [27], although it is unlikely that these deposits are entirely harmless [15]. The complex transition of a protein from its functional

soluble state to the amyloid/fibrillar conformation typically involves multiple precursor species (Figure 1.2). Many studies, based on microscopy, mass spectrometry, and single-molecule optical methodologies, have revealed that the initial stages of the aggregation process involve the formation of a heterogeneous array of oligomeric species, followed by protofibrillar structures, which are much smaller in length and width than those of mature fibrils [28–30]. The majority of the studies supporting the formation of pathogenic conformers have emerged from research on Aβ, which claim as the culprit of the amyloid pathogenesis a number of highly toxic aggregate species, including diffusible, oligomeric forms that lack ordered fibrillar topology [3]. In spite of the many studies, these toxic species remain structurally ill-defined and vary significantly with the dissimilar experimental conditions of the different publications but, in

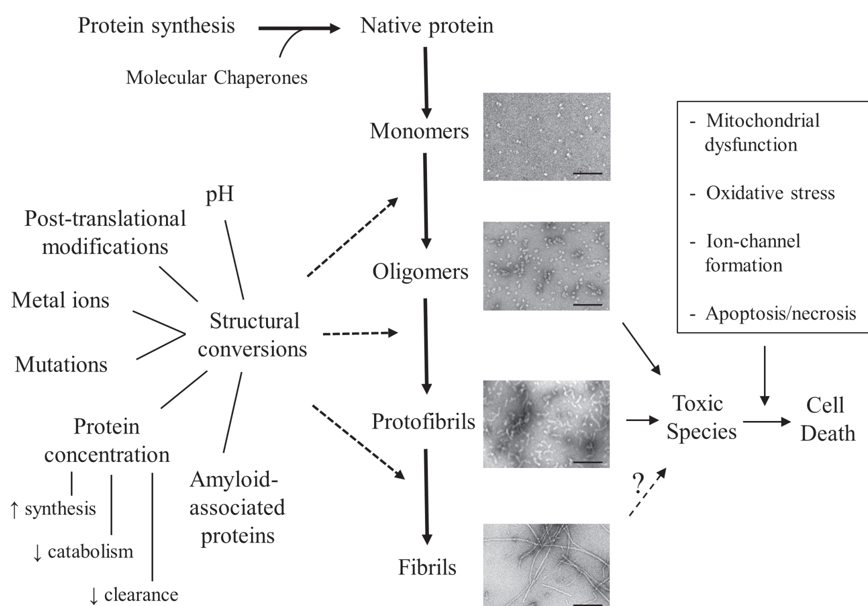

Figure 1.2 Schematic representation of the Aβ misfolded pathway resulting in oligomerization and amyloid fibril formation. The figure illustrates the major conformational modifiers promoting the structural misfolding conversions and some of the consequent pathogenic mechanisms triggered by the misfolded species that lead to cellular dysfunction and death, according to the current knowledge in the field.

general, they are thought to expose hydrophobic amino acid residues on unpaired β-strands that provide sites for aberrant interactions with other subunits favoring, in this way, the formation of aggregates or allowing interactions with other proteins or cellular membranes [3, 5, 15, 31].

The association of intermediate oligomeric assemblies with cellular distress and disease pathogenesis has been mostly studied for Aβ. Notwithstanding, piling evidence continues to emerge indicating that this is also the case for many, if not all, of the proteins linked to protein folding diseases in the CNS. In the case of tau, also a key pathogenic protein in AD whose primary function is to facilitate the assembly and maintenance of microtubules in neuronal axons, during the disease process the protein becomes modified, loses its affinity for microtubules, and accumulates as aggregates in the cell body. Albeit, it was originally thought that the assembly of the protein into the large neurofibrillary tangles constituted the pathological structure of the protein, more recent work indicates that smaller, soluble oligomeric forms of tau are best associated with neuron loss and memory impairment [29, 32] and can potentially drive the disease process [33]. Small oligomers and protofibrillar forms of a different protein, α-synuclein, a presynaptic neuronal component associated with PD, have likewise been reported as a key factor implicated in neuronal death [33, 34]. The potent toxicity of α-synuclein oligomers, similar to that described for the Aβ assemblies, mediates disruption of cellular homeostasis and neuronal death, through effects on various intracellular targets, including cellular membrane permeability, as well as mitochondrial and synaptic function [31, 34–37]. Furthermore, secreted α-synuclein soluble oligomers, likely released in a calcium-dependent manner by exosomes [38], not only may impact neuronal homeostasis and lead to neighboring neuronal death, but they may also exert deleterious effects through seeding of aggregation, thus possibly contributing to disease propagation [37].

1.4 Modulation of fibril formation: Lessons from cerebral amyloidosis

A growing number of genetic, structural, and environmental factors are known to play critical roles in the modulation of the *in vitro* and *in vivo* misfolding and aggregation of amyloidogenic subunits [39–42] which, in

turn, translate into a direct cytotoxic effect not only through caspase-dependent pathways or the formation of ion-like channel structures, but also through the induction of oxidative stress, mitochondrial dysfunction, and pro-inflammatory mechanisms. Among the many elements capable of enhancing amyloidogenesis, one of the most widely recognized for its relevance in disease pathogenesis is the occurrence of mutations in the precursor proteins. Other factors that also modulate the fibrillogenic propensity of pro-amyloidogenic proteins include changes in environmental pH and protein concentration, presence of specific metal ions and amyloid-associated proteins, as well as the existence of numerous post-translational modifications (Figure 1.2).

1.4.1 *Mutations*

The presence of mutations in the amyloidogenic subunits, a common feature among systemic and localized forms of amyloidosis, is heavily associated with the process of aggregation/fibril formation. In general terms, individual mutations have been demonstrated to correlate with structural changes that destabilize native structures by increasing β-sheet content and produce shorter lag phases and accelerated kinetic rates of fibrillization, features which typically correlate with the early onset observed in these familial diseases. The presence of genetic mutations results in diverse clinical phenotypes, translating either in early-onset cognitive impairment or in cerebrovascular manifestations specifically associated with cerebral amyloid angiopathy (CAA) that may or may not evolve into episodes of cerebral hemorrhage. The molecular changes resulting from the presence of specific mutations are also very diverse depending on whether the mutated residues are located within the amyloid subunits themselves, within the respective precursor proteins, or in the components of the proteolytic processing machinery that generate the individual amyloid molecules. When the genetic alterations affect the amyloid subunit, the changes usually consist of single point mutations that modify the structure and tendency to aggregate of the native molecule, as seen in genetic variants of Aβ, Cystatin C, or Gelsolin [43]. Notably, and for reasons not well understood, some of these variants are more vasculotropic than others and predominantly associate with cerebrovascular clinical

phenotypes. For example, a single point mutation at position 68 of the cysteine-protease inhibitor Cystatin C [44–46] — the first mutation described and characterized from a CNS amyloid deposit [45, 47] — is linked to early-onset CAA in patients with hereditary CAA Icelandic type, an autosomal dominant disorder characterized by massive amyloid deposition in cerebral small arteries and arterioles that clinically manifest with recurrent episodes of cerebral hemorrhages often with fatal outcome [48]. A similar case is observed in mutations located within the Aβ molecule which are also primarily associated with cerebrovascular deposition and microvessel dysfunction [43]. Particularly relevant is the Aβ region comprising residues 21–23 of the molecule, considered a "hot spot" for mutations due to the high number of genetic variants reported in this area. Mutations within this amino acid cluster typically show strong vascular compromise and primarily associate with CAA, hemorrhagic strokes, and dementia [43].

Genetic alterations affecting the precursor proteins of the amyloid subunits have different effects depending on the site affected by the mutation. For example, a mutation could create a premature stop codon in the precursor protein with the generation of a shorter molecule with different characteristics than its parent protein as it is exemplified by the mutation at position 163 of the *PRNP* gene that creates a premature stop codon generating a shorter PrP molecule that lacks the glycosylphosphatidylinositol (GPI) anchor and associates with CAA pathology [49]. In other cases, a genetic defect could abolish a normally occurring stop codon resulting in a longer precursor and the generation of molecules not present in the normal population, as the *BRI2* alterations in familial British and Danish dementias. In familial British dementia, a single point mutation replaces the normally occurring stop codon for arginine, elongating the *BRI2* open-reading frame by 11 amino acids [50]. Physiologic proteolytic processing of this extended precursor near its C-terminus generates the ABri peptide that forms the amyloid deposits in these patients. A similar effect originated in a different genetic defect on the same gene takes place in familial Danish dementia. In this case, the presence of a 10-nucleotide duplication-insertion one codon before the stop codon produces a frameshift in the *BRI2* gene and the elongation of the open-reading frame of

which the last 34 C-terminal amino acids compose the ADan subunit found associated with amyloid deposits in this disorder [51].

Genetic defects located in the precursor molecule but outside the amyloid subunit typically have an effect in the proteolytic processing of this precursor protein. In this sense, mutations in the Aβ precursor gene *APP* at the sites surrounding the β- and γ-secretase cleavage sites affect the quality and/or the quantity of the generated Aβ peptide. Genetic defects affecting the β-secretase site, as seen in the Swedish double mutation, increases the amount of Aβ generated. In cases in which the γ-secretase site is compromised — either by the presence of one of the multiple *APP* mutations surrounding the C-terminus of the Aβ sequence or by alterations in some of the genes codifying for components of the γ-secretase complex (e.g. *PSEN1* or *PSEN2*) — the outcome is an increased production of the longer and more amyloidogenic Aβ42 species and a clinical association with dementia phenotypes.

1.4.2 *Protein concentration: Effect of enhanced synthesis versus downregulated clearance*

Elevated levels of amyloidogenic proteins play an important role in the pathogenesis of systemic amyloidosis in which increased synthesis of different amyloidogenic subunits have been extensively documented. In the case of the cerebral amyloidosis where sporadic forms of AD are the most frequent, enhanced synthesis could be only demonstrated in few specific instances associated with genetic alterations. One of them is Down's syndrome in which an extra copy of the chromosome 21 produces an endogenous overexpression of the precursor APP and a concomitant overproduction of the amyloidogenic Aβ peptides. Interestingly, along with the enhanced expression of APP, triplication of chromosome 21 also causes overexpression of BACE-2 — the enzyme involved in the processing of the Aβ N-terminus — which may also contribute to the increased Aβ production [52]. In Down's syndrome, the characteristic cerebral lesions of AD appear at childhood, with virtually all carriers of the trisomy 21 developing AD neuropathology by the age of 40 years [53]. Patients with trisomy 21 show significantly higher Aβ plasma levels than age-matched controls [54, 55], and those individuals with more elevated

plasma levels of both Aβ40 and Aβ42 had a higher incidence of dementia [54], pointing out for a pathogenic role of the enhanced amyloid protein production. Similar effect is seen in association with certain APP mutations resulting in early-onset familial AD [56]. The role of elevated Aβ concentrations for amyloidogenesis is also stressed by the successful generation of multiple lines of transgenic animals in which the overexpression of various mutated genes promote the expression of increased quantity of Aβ peptides [57, 58]. Many comparable models have been reported, using various mutant forms of both APP and PS1, both singly and in combination. Aβ deposits are achieved by increasing APP overexpression, whereas the presence of PS1 mutations not only contributes to the Aβ load, but also to the early onset of the deposits [59].

Although no elevated circulating concentrations or overall enhanced production of Aβ have been found in sporadic AD, it is obvious that there is an increased brain accumulation of the protein aggregates under disease conditions. Current evidence points out, as important elements in the disease pathogenesis, to a dysregulation in the homeostatic mechanisms that maintain the steady-state levels of Aβ influencing the delicate balance between the rate of synthesis, dynamics of aggregation, and the rate of brain efflux. For the majority of AD cases, which are of late onset and of sporadic origin, the cause of this imbalance is unclear, but new findings indicate that an impaired brain efflux plays a critical role in amyloid formation and the pathogenesis of the disease [60]. The actual mechanisms involved in this deficient brain removal are under intense scrutiny but still remain undefined. Glial phagocytosis, local enzymatic degradation, efflux through the BBB, transport to the cerebrospinal fluid (CSF), peri- and paravascular drainage along with the more recently described glymphatic system, and meningeal lymphatic vessels are among the mechanisms under current investigation and with demonstrated participation in brain Aβ removal [61–67]. Notably, many factors regulate brain Aβ clearance and exert important changes in the physiological removal of the protein. One of the most relevant is age, which affects the functioning of the cerebral vasculature and the efficiency of CSF circulation with a decreased effectiveness in the removal of toxic substances, waste products, and metabolites, including those of Aβ. Other important element in the effective brain Aβ removal is the degree of peptide aggregation which

negatively influences the brain efflux and transport to the CSF, linking once more the process of protein folding to the pathogenesis of AD [68]. Recent clearance studies from our group employing wild-type mice intracerebrally injected with ^{125}I-Aβ1-40 showed higher brain retention and an ~3 times lower efflux to the CSF for oligomeric Aβ in comparison to the values observed for the monomeric form, adding another layer of pathogenic significance to the formation of the oligomeric assemblies. Through a delayed brain removal, these species — once formed — have higher capability and a longer time frame to exert their pathogenic activity. Furthermore, since the process of multimerization is concentration dependent, the persistence of oligomeric forms of Aβ within the brain is likely to exacerbate the assembly of higher-molecular mass species and/or recruit soluble forms of Aβ into multimolecular species in a nucleation/seeding effect, an event increasingly recognized as a significant contributor to the pathogenesis of neurodegenerative disorders [69, 70]. Targeted mass spectrometric analysis of the mouse CSF collected at different time points after the intracerebral Aβ injections consistently demonstrated that Aβ is cleared to the CSF not only as a full-length peptide, but also in the form of numerous C-terminally truncated fragments, indicative of catabolic cleavage by Aβ-degrading brain resident enzymes. Comparative analysis of the Aβ catabolic footprints with the short time elapsed after the intracerebral injection clearly indicated a preferential early removal of the more soluble C-terminally degraded fragments in comparison to the full-length Aβ1-40, providing support for an important role of enzymatic degradation in the fast and efficient brain Aβ clearance. Along this line, the higher retention exhibited by Aβ oligomeric species may well relate not only to a decreased efficiency in the efflux of these species *per se*, but also to a lower susceptibility of these assemblies of being cleaved by brain resident enzymes, as evidenced in our recent *in vivo* studies [68] and confirmed *in vitro* by the decreased potential of the matrix metalloproteinases MMP-2 and MMP-9 to cleave oligomeric forms of Aβ [71].

1.4.3 *Acidic pH*

A growing number of dissimilar proteins associated with various systemic and cerebral amyloid diseases form fibrils readily under mild acidic conditions

(pH ~4.7–5.5), among those exhibiting brain deposits: transthyretin, ABri, Aβ, and PrP [23, 72]. In the last two instances, it was demonstrated that the acidic pH resulted in an increment of the β-sheet structure and an enhanced exposure of hydrophobic patches on the surface of the molecule that correlated with the tendency for fibrillization [73–75]. Although extreme conditions of pH generally favor the formation of aggregates through denaturation mechanisms, in some cases the production of amyloid-like fibrils *in vitro* at low pH is also known to take place, as in the case of the variant of cystatin C associated with familial cerebral hemorrhage [76].

The remarkable behavior of the ABri peptide at different pH conditions is a perfect example of the complexity of the fibrillization process and how it is highly influenced by the microenvironment. In the 3.1–4.3 pH range, ABri adopts almost exclusively a random structure and a predominantly monomeric aggregation state as visualized by analytical ultracentrifugation. At neutral pH (7.1–7.3), the peptide shows limited solubility and produced spherical and amorphous aggregates with predominantly β-sheet secondary structure, whereas at the slightly acidic pH of 4.9, spherical aggregates, intermediate-sized protofibrils, and larger-size mature amyloid fibrils are detected by atomic force microscopy. With aging at pH 4.9, these protofibrils undergo further association and eventually elongate forming mature fibrils [23]. It is important to remember that acidic conditions within cells have serious consequences and that the maintenance of cytosolic pH within physiological ranges is required for normal neuronal activity, the reason why cells have developed different buffering strategies [77]. Notably, many cellular pathways found altered under specific neurodegenerative conditions are associated with cytoplasmic pH acidification, e.g. oxidative stress, initiation of apoptosis, and metabolic changes in ATP consumption to name a few. Survival and function of certain organelles are known to be severely affected by intracellular pH acidification. Mitochondrial morphology and trafficking [78] as well as relocation of lysosomes from perinuclear neuronal localization to peripheral zones [77] have been described. Based on the enhancement of fibrillization under acidic conditions, a putative role in amyloidogenesis was suggested for acidic lysosomes [75, 79] although it is still not clear how intracellular processing in these subcellular compartments may lead to extracellular amyloid deposition.

1.4.4 *Presence of metal ions*

The role of metal ions is becoming increasingly more relevant in a large number of pathologies associated with fibrillization of different proteins including Aβ, Tau, and α-syn [80–82]. It has been particularly well studied in the case of AD in which Zn^{2+}, Fe^{3+}, and Cu^{2+} — elements found in elevated concentrations in brain lesions [20, 83] — have been demonstrated to significantly contribute to the aggregation/fibrillization process [81, 84]. Among these findings, Zn^{2+} ions have been shown to induce Aβ aggregation in a pH-dependent manner [85], with comparable effects also reported for Cu^{2+}, Ni^{2+}, and Fe^{3+} [86]. Current lines of investigation suggest that the presence of Zn^{2+} affects both the nucleation and the aggregation stages of Aβ fibrillization inducing an instantaneous β-structural transition and, in the case of preformed fibrils, causing their aggregation into large precipitating masses [20]. Based on the current knowledge indicating that metal ions are powerful kinetic accelerators of fibril formation, they are actively being pursued as potential target for therapeutics aiming at modulating amyloid deposition [87]. In addition to their potential contribution to fibrillogenesis, metal ions are also intricately linked to the detrimental effects induced by oxidative stress mechanisms, since the chemical origin of the majority of reactive oxygen species (ROS) arise from the reaction with the redox-active metals copper and iron [88].

1.4.5 *Post-translational modifications*

Amyloid subunits and their degradation products are well known to sustain a number of post-translational modifications which largely contribute to the heterogeneity of the amyloid deposits. Among the many modifications identified in cerebral amyloidosis, phosphorylation, isomerization, racemization, oxidation, and N-terminal cyclation occurring in conjunction with proteolytic fragmentation are the most relevant [89–98]. Hyperphosphorylation is the common post-translational modification of intracellular tau composing the paired helical filaments accumulating as neurofibrillary tangles in several neurodegenerative disorders [99]. In the case of Aβ, oxidation of methionine at position 35 has been widely documented in AD [100–103], and Aβ peptides containing Met35 sulfoxide are able to induce oxidative stress and are more cytotoxic than the unmodified counterparts [104].

Another modification also frequently found in Aβ is the isomerization and racemization of the aspartyl residues at positions 1 and 7 which has been reported in more than half of the residues in some AD cases [89]. Similar modifications were reported in the Iowa variant of familial AD [105, 106] in which detailed biochemical studies demonstrated the enhanced proclivity of the isoAsp-modified molecules for oligomerization/fibrillization with a concomitant exacerbation of mitochondria-mediated cell death pathways [96].

A frequent post-translational modification is the formation of N-terminal pyroglutamate (pE) which has been largely studied in the case of Aβ. The loss of one negative charge incurred by this modification results in an increase in β-sheet content, greater hydrophobicity, and enhanced aggregation propensity of the molecule, with N-terminal cyclation providing additional resistance to proteolytic degradation [107, 108]. Indeed, it has been demonstrated that N-terminal-modified species progressively accumulate in the brain at the earliest stages of AD even before the appearance of clinical symptoms, suggesting that they may constitute potential seeding elements and play an important role in the formation of pathological amyloid aggregates [109]. The structural alterations introduced by the N-terminal cyclation correlate with the increased toxicity displayed when compared to full-length Aβ, further supporting the importance of this modification for the mechanism of disease pathogenesis and providing additional targets for therapeutic interventions [110–115]. Interestingly, these pE Aβ derivatives are not major Aβ species in AD biological fluids. Something similar occurs in familial British and Danish dementias, two diseases with striking neuropathological and phenotypic similarities to AD where the heavily dominant species in the amyloid deposits — ABri and ADan, respectively — also bear pE at the N-terminus, whereas circulating ABri and ADan peptides feature only glutamate at their N-terminus [95, 116, 117]. Since the formation of pyroglutamate from glutamate or glutamine — a reaction commonly catalyzed by glutaminyl cyclase [118, 119] — is chemically stable and poorly reversible, the presence of glutamate-only species in the circulation indicates either that the formation of N-terminal pE takes place at the site of deposition or that due to their high insolubility and tendency to aggregate the pE-modified species are completely sequestered into the deposits soon after their generation, disappearing from the circulation. Recent data from our

laboratory using ABri and ADan derivatives, consistent with the findings in the AD field, highlight the relevance of the pE post-translational modification in the induction of structural changes, enhancement of intrinsic pro-amyloidogenic properties, and generation of ROS and mitochondria-mediated cell death mechanisms, all intertwined features relevant for the disease pathogenesis. Overall, it is clear that exacerbation of oligomerization/fibrillization concomitant with enhancement of the pathogenic characteristics of the molecule resulting from post-translational modifications is a common feature among different amyloidogenic proteins.

1.5 Mechanisms of disease associated with protein misfolding

A consistent feature that accompanies the accumulation of abnormally folded proteins in the CNS is the progressive damage and loss of vulnerable neurons. Although disease-specific differences exist among the different proteinopathies, many parallels are also found which may indicate similar mechanisms underlying the initiation of cell death. It is nowadays clear that more than a single factor and likely more than one cellular pathway are involved in the cellular dysfunction induced by protein aggregates and amyloid formation. Mounting evidence indicates that, in addition to a direct effect on cell viability related to the formation of oligomers/protofibrils and their ability to assemble into functional ion channel-like structures in lipidic environments, amyloid molecules are also able to induce apoptosis, to trigger oxidative stress, to generate an inflammatory response, and/or to activate the complement system. In turn, these pathways, separately or synergistically, are capable of producing different levels of cell damage and modulate cytotoxicity.

1.5.1 *Formation of ion channel-like structures*

The cytotoxic action of pathogenic amyloid assemblies may, at least in part, result from their interaction with cell membranes and the subsequent formation of ion-like channels [120]. Atomic force microscopy studies have revealed, in the case of Aβ, the formation of doughnut-shaped structures protruding out of the membrane surface with centralized pore-like depressions presumably representing individual channels

[121]. Electrophysiological data corroborated the formation of ion-permeable channels and demonstrated their dependence on the aggregation state of the Aβ peptides with a shift to larger single-channel conductances with increased peptide aggregation [122]. The formation of similar ion channels has also been observed for many other amyloid molecules including some related to systemic and localized noncerebral amyloidosis, such as β2-microglobulin, serum amyloid A, atrial natriuretic factor, calcitonin, lysozyme, transthyretin, and amylin. More relevant for the disorders of protein folding in the CNS, the ability to form ion channel-like structures has also been demonstrated, in addition to Aβ for other proteins associated with cerebral amyloidosis such as α-synuclein, and the more recently described ABri and ADan molecules linked to familial British and Danish dementias [121, 123, 124]. In spite of the common capacity to generate channels, structural, biochemical, and electrophysiological data demonstrated certain heterogeneity in the different multimeric channels assembled by the different amyloid species. This heterogeneity could either reflect conformational changes in the different amyloid structures, a simple difference in the number of subunits that form a single channel [121, 122, 125, 126], or a varying channel-forming activity depending on the nature of the lipid mixtures [124, 127]. Although the contribution of channel formation to disease pathogenesis remains to be further elucidated, many of the effects of amyloid *in vivo*, including Ca^{2+} dysregulation, membrane depolarization, mitochondrial dysfunction, inhibition of long-term potentiation, and cytotoxicity, may be attributed to channel formation in both plasma and intracellular membranes.

1.5.2 *Induction of apoptotic cell death mechanisms*

The exact mechanisms of cellular demise in neurodegenerative disorders remain to be fully defined. Nevertheless, in the case of AD as well as in other non-Aβ cerebral amyloidosis, evidence points to the oligomer-mediated activation of mitochondrial apoptotic pathways [97, 98, 128, 129] as key elements for the pathogenesis of the respective diseases. Recent data from our laboratory demonstrate that amyloid-mediated toxicity proceeds through these apoptotic mechanisms, with the induction of

comparable levels of DNA fragmentation by both Aβ and non-Aβ oligomeric assemblies, as it is the case of the ABri and ADan molecules [97, 98]. The apoptotic process was accompanied in all these cases by mitochondrial compromise involving alterations in the membrane permeability of the organelle and cytochrome c release to the cytoplasm, changes in mitochondrial membrane potential, and induction of oxidative stress mechanisms [96]. The presence of downstream caspase-9 activation, in addition to the abrogation of cell death in the presence of specific caspase-9 inhibitors, confirmed the involvement of intrinsic apoptotic pathways in all these cases. Whether in all cases the mitochondrial engagement results from a direct association of the oligomeric assemblies with the organelle leading to its dysfunction, as seems to be the case for Aβ [130], remains to be determined. Alternatively, it is conceivable that the initiation of intrinsic apoptotic pathways could originate in a direct interaction of the amyloid assemblies with plasma membranes and the formation of ion-channel-like structures, a common feature exhibited by many amyloid subunits, as described above. Notwithstanding, irrespective of the nature of the initiating events, the similarities in cell death pathways triggered by Aβ and non-Aβ cerebral amyloid subunits support the concept that different amyloids, despite entirely dissimilar origin and primary sequence, can cause similar pathological processes that ultimately lead to neurodegeneration and dementia.

1.5.3 *Mitochondrial dysfunction and oxidative stress*

A common feature of neurodegenerative diseases involving deposition of misfolded proteins is the extensive evidence of oxidative stress contributing to the dysfunction and/or death of neuronal cells. The brain requires an extraordinary amount of energy to maintain physiological functions and, since little energy is stored in this organ, mitochondria, mainly through the process of glucose oxidation [131, 132], continuously provide the required high production levels [133, 134]. These organelles not only exert central control of the cell bioenergetics but, as the major consumers of oxygen, they are also the most important generators of ROS [135]. Neuronal tissue is particularly sensitive to oxidative stress, and imbalance in pro-oxidant *versus* anti-oxidant homeostasis in CNS results in the

production of several potentially toxic ROS, including hydrogen peroxide, nitric oxide, superoxide, and the highly reactive hydroxyl radicals that participate in the initiation and/or propagation of radical chain reactions. In AD, PD, HD, and ALS, oxidative damage is found in every class of biological molecules within neurons, spanning from lipids to DNA and proteins [136–138]. This lipid peroxidation together with oxidation of proteins and DNA result in impaired cellular functions, formation of toxic species (not only peroxides, but also alcohols, aldehydes, ketones, and cholesterol oxides), altered enzymatic activity, and triggering of cascades of events leading to cell death, many of these common pathways for various neurodegenerative disorders [77, 87, 139–141]. Indeed, it is currently thought that the generation of ROS is intricately linked with the execution of intrinsic apoptotic mechanisms described above, either by providing the intracellular stress required for the initiation of the cascade or through direct modification of the protein components that facilitate formation of the apoptosome [142]. In this sense, recently published data highlight the relevance of oxidative stress in the induction of cell death mechanisms elicited by small oligomeric assemblies linked to the development of non-Aβ cerebral amyloidosis [98]. In this light, quenching ROS by co-treatment with the vitamin E analog Trolox efficiently inhibited activation of terminal caspase-3 and completely abolished the amyloid-induced toxicity and concomitant neuronal death [98].

1.5.4 *Inflammation-mediated pathways*

Compelling evidence has accumulated during the last decade pointing to a significant role of local inflammatory processes in the progression of neurodegenerative disorders including Alzheimer's, Parkinson's, and prion diseases [reviewed in Ref. 143]. These inflammation-related mechanisms are widely studied in AD [144]. In particular, complement activation and its pro-inflammatory consequences have been demonstrated to contribute extensively to disease pathogenesis [145], and inflammation-related cytokines are considered today a driving force in the neuropathological cascade associated with AD [for reviews, see Refs. 144 and 146]. Complement activation products co-localize with cerebral parenchymal and vascular deposits in AD and non-Aβ cerebral amyloidosis, thereby

indicating that the chronic inflammatory response, most likely initiated by the deposits, is a common phenotype to all these disorders [144, 147–149]. These deposit-associated components originate from direct activation of the complement system by Aβ and the non-Aβ amyloid peptides ABri and ADan, and once generated seem to participate, at least in the case of Aβ, in several key steps of amyloidogenesis including aggregation, microglial activation, and phagocytosis [147, 148, 150–153].

The presence of activated cytokine-expressing microglia co-localizing with amyloid deposits in affected brain areas — a clear indication of the existence of inflammation-related pathways — is also a common finding in AD and non-Aβ cerebral amyloidosis as well as in other diseases of protein folding in the CNS including PD [146, 154, 155]. Indeed, activated microglia has been shown associated with areas of neurodegeneration in both post-mortem human tissue and in mouse models of PD [156–158]. Further supporting a role for inflammation-mediated pathways in PD an increase in the pro-inflammatory cytokine profile was also demonstrated in CSF with elevated levels of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) [155]. Although results are, in some cases, controversial, there also appears to be a trend in CSF and plasma of AD patients with elevated levels of the pro-inflammatory mediators IL-1β, IL-6, and TNF-α. In contrast, reflecting the complexity of the disease mechanisms, a few anti-inflammatory cytokines (IL-1 receptor antagonist and IL-10) also seem to be elevated [159, 160]. These findings, together with the presence of increased levels of complement activation products, cytokines, chemokines, and free radicals, lead to the concept of neuro-inflammation as a self-propagating toxic cycle in which several factors — protein aggregates, abnormal cellular components, injured neurons, and abnormal synapses— activate microglia to release inflammatory mediators, which in turn exacerbate amyloid deposition and neuronal injury [161]. The idea, originally conceptualized for AD, is likely to be applicable to many, if not all, currently known diseases of protein folding.

1.6 Concluding remarks

The molecular pathogenesis of protein folding disorders is undoubtedly very complex and interlinks a diversity of mechanistic pathways, leading

to cell toxicity and death. Although AD is by far the most common and studied form of amyloid-related disorders in humans, recent findings on the molecular mechanisms of brain degeneration have demonstrated common features among this heterogeneous group of disorders. Histopathologic, genetic, biochemical, and physicochemical studies, together with the generation of transgenic animal models, strongly support the notion that this diverse group of pathological entities are caused by abnormal folding, aggregation/fibrillization, and subsequent tissue accumulation of particular proteins, specific for each disorder. The similarities in the physicochemical mechanisms ruling the aggregation/fibrillogenesis pathways and leading to similar end-point structures, as well as the existence of common factors able to modulate the fibrillogenesis process, together with the notion that comparable oligomeric amyloid assemblies, regardless of the primary structure of the amyloid subunit, have the capacity to elicit common pathological mechanisms that ultimately result in cell death, bridge together this wide range of dissimilar pathological entities, and suggest unifying mechanisms of disease pathogenesis.

Acknowledgments

This work was supported in part by the National Institutes of Health (Grant Nos. AG030539, AG044817, AG051266), the Alzheimer's Association, and the BrightFocus Foundation.

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Chapter 2

Oligomers at the Synapse: Synaptic Dysfunction and Neurodegeneration

Emily Vogler, Matthew Mahavongtrakul, and Jorge Busciglio

2.1 Introduction

Proteins start as a chain of amino acids synthesized through the translation of the genetic code found in DNA. This chain of amino acids then begins the process of folding to form hydrogen bonds between the donor and acceptor residues in the chain, with α-helices and β-sheets being the most commonly formed structures. Then the protein gains further stability through the formation of other covalent, ionic, and hydrophobic bonds. The folded protein may then form bonds with other proteins that result in the complete and functional protein structure.

Sometimes a protein will undergo a mutation, epigenetic modification, or other change in structure. In other cases, overexpression of the protein or defects in protein degradation interferes with the ability of the cell or tissue to maintain proteostasis. In these situations, the normal folding of the protein is frequently altered and it likely becomes a structure that cannot perform normal function and is also prone to abnormal aggregation. This can give a one-two punch to the affected tissue — loss of normal function and gain of toxic function — that ultimately results in cell death and, in the case of neuronal proteins affected, can lead to neurodegenerative disease.

In most neurodegenerative diseases, the aggregation of proteins into large insoluble aggregates was the first observation of disease pathology.

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As technology progresses, improving methods to identify and characterize toxic species in disease processes have made it apparent that small, soluble oligomeric aggregates are far more toxic than large aggregates that were initially observed. This chapter focuses on two aspects of misfolded proteins: the nature of toxic oligomers and the effects that oligomers have on synaptic function in neurodegenerative diseases (Figure 2.1).

2.2 Mechanisms of oligomer toxicity are related to protein conformation and misfolding

What is it about oligomeric forms that make them more toxic to cells than larger aggregates? Experiments with the 91 residue N-terminal domain of the *Escherichia coli* HypF-N protein, a stably folded α-helix/β-sheet protein that can be induced to form oligomers, protofibrils, and amyloid-like fibrils *in vitro*, give us insights into the structural basis for oligomeric toxicity. Varying incubation conditions result in HypF-N aggregating into stable oligomers with similar morphological and structural properties, but with some species being toxic, while others are non-toxic. The toxic species have a less tightly packed core with more hydrophobic residues exposed on the surface and higher structural flexibility than the non-toxic species, resulting in the ability of the toxic oligomers to penetrate the cell membrane and induce Ca^{2+} entry into the cell [1]. The toxic species also induces production of intracellular reactive oxygen species (ROS), increases membrane lipid peroxidation, triggers apoptotic pathways, and induces loss of cholinergic cells when injected into rat brains [2].

Another question is why are neurons more susceptible to damage than other cell types? Malchiodi-Albedi *et al*. [3] investigated this using oligomers of salmon calcitonin (sCTOs), a neurotoxic amyloid protein that interacts with lipid rafts in cell membranes to induce calcium-permeable pores, allowing the influx of extracellular calcium and resulting in loss of dendrites and a decrease in synaptic boutons. Significantly, they found this effect of sCTOs to be specific to mature neurons, but not immature neurons, astrocytes, or fibroblasts and that disrupting lipid rafts in mature neurons reduces sCTO toxicity. These results suggest that the much greater abundance of lipid rafts found in mature neurons compared to the other cell

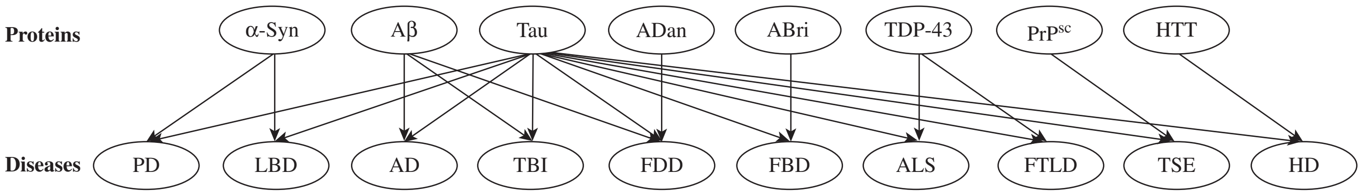

Figure 2.1 Summary of oligomeric proteins and associated diseases. α-syn, α-synuclein; Aβ, amyloid-beta; ADan, Danish amyloid; ABri, British amyloid; TDP-43, TAR DNA-binding protein 43; PrP^{Sc}, scrapie prion protein; *HTT*, mutant Huntingtin; PD, Parkinson's disease; LBD, Lewy body dementia; AD, Alzheimer's disease; TBI, traumatic brain injury; FDD, familial Danish dementia; ALS, amyotrophic lateral sclerosis; FTLD, frontotemporal lobar dementia; TSE, transmissible spongiform encephalopathies (Prion diseases); HD, Huntington disease.

types may provide a mechanism by which oligomers are preferentially destructive to neurons in neurodegenerative diseases.

The ability of toxic oligomers to disrupt lipid bilayers is implicated in many neurodegenerative disorders. Oligomers contributing to the pathologies of Alzheimer's disease (AD), Parkinson's disease (PD), Lewy body dementia (LBD), Creutzfeldt–Jakob disease (CJD), and Huntington's disease (HD) have been demonstrated to increase cell membrane conductance [4]. The amyloid beta oligomers (AβO) of AD can form pores allowing aberrant Ca^{2+} entry into cells [5], while alpha synuclein (α-syn) of PD can form various oligomers with a direct correlation between hydrophobic surface and cytotoxicity [6]. Also, the genetic mutations associated with PD to date are all in the N-terminal sequence involved with membrane binding, with the mutations either enhancing [7] or attenuating [8–10] binding properties or altering aggregation properties [11]. The expansion of the *HTT* protein in HD similarly disrupts the structure of lipid bilayers [12] and the nuclear membrane [13], while the prion pathogenesis of CJD is influenced by membrane binding and results in pore formation [14]. Both mutant forms of the BRI2 gene, ABri and ADan, associated with familial British dementia (FBD) and familial Danish dementia (FDD), respectively, aggregate into oligomers that induce the formation of pores in cell membranes that elicit single-channel ion currents [15]. These results indicate that the greater hydrophobicity and flexibility of an oligomer allows it to interact with lipid bilayers and disrupt cellular membranes, potentially interfering with synapse and vesicle membrane functions and altering protein–protein interactions critical for normal synaptic function.

2.3 Protein oligomers that interfere with synaptic function and the disorders they cause

2.3.1 *Alpha synuclein*

PD is one of the many neurodegenerative disorders in which early researchers observed clumps of aggregated protein and cellular debris in the brains of deceased patients. The aggregates were named Lewy bodies, after the physician who discovered them in 1912, and it was originally

thought that they were the culprits in the disease process, but Lewy body pathology does not correlate well with clinical symptoms of PD [16]. The identity of the primary protein that comprises Lewy bodies remained unknown until advances in genetics allowed identification of a missense mutation in a gene found in a family with a history of PD [17]. The protein coded by this gene was originally found to localize in synapses and so was named alpha-synuclein (α-syn). A primarily cytosolic monomer with highly flexible conformation, α-syn is also found bound to synaptic membranes where it folds into two α-helices. Though mutated monomeric forms are associated with toxicity through activation of TLR4 receptors on glial cells, leading to chronic neuroinflammation [18], the α-syn oligomers found in synapses early in the pathologies of PD and LBD have been demonstrated to be neurotoxic both *in vitro* and *in vivo* [19, 20].

The loss of dopaminergic neurons in the nigrostriatal pathway results in the movement disorders characteristic of PD, with α-syn playing a significant role in the deficiency of dopamine signaling that contributes to the loss. Along with regulating assembly of SNARE complexes in vesicle trafficking and release [21], α-syn also regulates several proteins required for normal dopaminergic function, including DAT (dopamine transporter) [22], VMAT2 (vesicular monoamine transporter 2) [23], and tyrosine hydroxylase [24]. Oligomeric α-syn has recently been demonstrated to bind the mitochondrial receptor TOM20 on dopaminergic neurons, interfering with transport of proteins into mitochondria and resulting in impaired cellular respiration and production of ROS [25]. It is proposed that the ability of α-syn to misfold into helical oligomers with structural similarity to the mitochondrial targeting signal (MTS) enables it to bind translocase of the outer membrane (TOM) receptors, as monomeric and fibrillary α-syn do not affect mitochondrial import. Consequently, the misfolding of α-syn into toxic oligomers, with both loss and gain of function, results in a knockout punch to synapses in dopaminergic pathways and significant damage to many other types of neurons.

The brain region in which α-syn accumulates gives rise to the clinical symptoms. Small aggregates of α-syn are found presynaptic in cortical regions in LBD, where they are associated with loss of pre- and postsynaptic proteins and dendritic spines [26], resulting in cognitive impairment. In PD, α-syn aggregates are first concentrated in the lower medulla

oblongata and anterior olfactory structures and progress upward to the cerebral cortex in the later stages of the disease, with motor impairment becoming symptomatic when α-syn pathology reaches the substantia nigra in the midbrain [22, 27]. The progression of α-syn pathology to higher brain regions may be facilitated by a prion-like mechanism where misfolded aggregates of α-syn seed the formation of misfolded proteins [28], which has been demonstrated to occur through direct neuronal connections [29, 30], and may lead to a variant of PD: Parkinson's disease with dementia (PDD), which has more cortical α-syn pathology than PD [31].

Identifying the events that lead to misfolding and aberrant accumulation of α-syn in the Lewy bodies found in the brain regions affected in PD, PDD, and LBD are critical in understanding the causes of these disorders.

2.3.2 *Tau*

When the German physician Alois Alzheimer inspected the brain tissue of a deceased patient with advanced dementia in 1906, he found tangles of fibrils inside the neurons, bundles of tangles on the surface of neurons, and thick tangles of fibrils where neurons were previously located [32]. The neurological disorder from which the patient had suffered was named after Dr. Alzheimer in 1910, but it was many years later that the components of the tangles were identified as the protein tau [33]. Currently, tau pathology is recognized as a common feature in multiple neurodegenerative conditions (Figure 2.1). Tau binds to and stabilizes microtubules in the axons of neurons in a phosphorylation-dependent manner. However, excessive phosphorylation of tau interferes with its ability to bind microtubules and also promotes tau aggregation into the paired-helical filaments (PHFs) that comprise the neurofibrillary tangles (NFTs) of AD [34] and relocation into cell bodies and dendrites [35].

Similar to α-syn, the primary toxic species of tau is oligomeric in form [36], with neurodegeneration beginning before the formation of PHFs [37] and electrophysiological alterations observed before morphological changes in mouse models of tauopathy [38]. Subcortical injection of tau oligomers, but not fibrils or monomers, into wild-type mice reduces synaptic vesicle-related proteins and impair mitochondrial function and memory consolidation [39]. Prefibrillar tau correlates with cognitive

dysfunction more so than fibrillar tau in mild cognitive impairment (MCI), which often precedes AD [40], and memory impairment is also correlated with accumulation of tau oligomers in transgenic mice expressing mutant tau [41]. The role of tau aggregation in long-term potentiation (LTP) and memory impairment is further supported by demonstration that switching off tau expression restores normal LTP and memory in transgenic mice expressing pro-aggregant mutant tau, while mice expressing anti-aggregant tau do not develop impaired LTP or cognition [42]. Tau oligomers also seed aggregation of tau in progressive supranuclear palsy (PSP), a parkinsonian neurodegenerative tauopathy [43], indicating that tau oligomers contribute to the spread of PSP pathology. In addition, tau oligomerization in the hippocampus has recently been shown to form as a result of traumatic brain injury (TBI), which accelerates onset of cognitive impairment [44].

The mechanisms of tau toxicity at the synapse are myriad. Injection of human tau into the squid giant axon results in tau hyperphosphorylation, synaptic vesicle aggregation in the presynaptic active zone, and neurotransmission impairment [45], demonstrating that abnormal tau phosphorylation disrupts synaptic function. Also, over-expression of tau inhibits axonal trafficking and interferes with mitochondrial activities at synapses, with mitochondria becoming concentrated in the cell body [46]. Post-mortem AD brains have reduced levels of the presynaptic protein synaptophysin in neurons bearing NFTs compared to tangle-free neurons [47]. Though tau is primarily axonal, hyperphosphorylated tau (p-tau) oligomers accumulate both pre- and postsynaptically in AD brains [48], where it can disrupt normal tau dendritic functions, including regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor clustering with PSD95 [49] and targeting the tyrosine kinase fyn to the postsynaptic density, where fyn influences *N*-methyl-D-aspartic acid (NMDA) receptor subunit interaction with PSD95 [50]. Expression of mutant tau interferes with activation of NMDA receptors and impairment of neurotrophic brain-derived neurotrophic factor (BDNF) signaling [51], as well as impaired glutamate metabolism and homeostasis [52]. Interaction between AβO and tau oligomers results in relocation of tau into synaptic spines [53] where it is associated with altered spine morphology [54, 55] and impaired LTP and memory [56].

Tau oligomers are also found in PD, LBD [57], HD [58], and very recently in chronic traumatic encephalopathy (CTE) [59] and in promoting neurodegeneration in glaucoma [60], highlighting the importance of understanding the mechanisms underlying the hyperphosphorylation and aggregation of tau into oligomers in neurodegenerative disease.

2.3.3 *Amyloid beta*

Dr. Alzheimer's investigation of the deceased dementia patient's brain also found small lesions with plaques in abundance throughout the cortex, which were later found to be made up of cellular debris and beta sheet fibrils identified as amyloid beta (Aβ) [61], a product of regulated intramembrane proteolysis of the amyloid precursor protein (APP). Cleavage of APP by the membrane-anchored protease β-secretase and subsequent cleavage by γ-secretase can produce Aβ peptides of 38, 40, or 42 amino acids with a soluble α-helix structure and C termini with varying hydrophobicity that result in differing aggregation properties [62]. The 42 amino acid Aβ monomer readily aggregates into soluble Aβ oligomers (AβO) and further aggregates and evolves into the insoluble β-pleated sheets that form the dense plaques found in AD brains.

One of the major conundrums of AD research has been the lack of correlation between Aβ plaque pathology and cognitive impairment, which would be expected as aggregation of Aβ is generally acknowledged as the primary cause of AD. However, the ability to differentiate between various forms of Aβ has led to the discovery that AβO are the more toxic species, killing cultured neurons at low concentrations, inhibiting LTP [63, 64], and impairing memory [65]. Accumulation of AβO early in the disease process of mouse models of AD, before the formation of plaques or cognitive deficits, results in altered synapse and mitochondria morphology suggestive of disruption of synaptic function and increased energy needs at the synapse [66]. In addition, TBI causes acute and rapid AβO accumulation, which may contribute to the development of AD later in life [148]. These observations are supported by the development of various models of transgenic AD mice to differentiate effects found in the presence and absence of soluble oligomers and insoluble plaques. One model accumulates AβO, but not Aβ plaques, developing deficits in LTP,

synaptic markers, and cognition along with tau hyperphosphorylation [67]. Another model in which the transcription of APP can be switched off after the development of Aβ plaque formation, resulting in plaque burden without the presence of AβO, demonstrates that removal of AβO after plaque formation restores cognition [68]. In humans, it has been found that non-demented individuals with AD pathology have significantly lower levels of AβO than demented individuals [69, 70]. These observations are further evidence that AβO are the prime movers in neurodegeneration and cognitive impairment.

Consistent with the hypothesis that the affinity of oligomers to bind membranes is a major mechanism in neurodegeneration, interstitial AβO rapidly bind GM1 ganglioside on cell membranes in a mouse model of AD [71], co-localize and preferentially bind lipid rafts in cell membranes, altering lipid raft size and morphology and inducing formation of cavities in membranes, thus reducing cell viability [72]. AβO also induce time-dependent neurotoxic alterations to expression of glutamate receptors, microglial activation, tau hyperphosphorylation, neuritic dystrophy, and reduction in synaptic proteins that are reversed by treatment with a drug that interferes with AβO binding to cellular membranes [73]. The effects of AβO on calcium trafficking span both AβO-induced pore formation and modulation of signaling channels. Total internal reflection fluorescence (TIRF) microscopy reveals that the increasing aggregation of AβO creates ever larger and highly active calcium-permeable pores in cell membranes [5], while application of AβO to HEK cells induces excitotoxic presynaptic calcium influx through P/Q- and N-type calcium channels that is reversed by calcium-channel inhibitors, preventing AβO-induced synaptic deficits [74].

AβO accumulate at synaptic sites in an activity-dependent fashion [75], where they disrupt synaptic function through various mechanisms. Hippocampal injections of AβO reduce vesicular acetylcholine transporter (vChaT) and glutamatergic synapses [76]. NMDA receptor function is disrupted by AβO through increased activation of NR2B NMDA receptor subunits resulting in LTP inhibition in cultured neurons and hippocampal slices [77, 78] and differentially altered trafficking and phosphorylation of NMDAR subunits in cultured hippocampal neurons [79]. Exposure to AβO reduces phosphorylation of AMPA receptor subunits blocking

extrasynaptic delivery of AMPA receptors and producing memory deficits in a transgenic mouse model of AD [80], while intracellular infusion of AβO into neurons enhances AMPAR-mediated synaptic transmission through insertion of calcium-permeable AMPARs [81]. AβO disrupt transport of synaptic proteins and mitochondria, interfering with dendritic spine development [82] and inhibit the motor protein Eg5, resulting in inhibition of LTP [83]. Synaptic functions are also disrupted by AβO through altered synaptic vesicle release by a reduced pool of recycling vesicles, slowed endocytosis but not exocytosis, impaired regeneration of fusion-competent vesicles [84], and disrupted synaptic vesicle endocytosis through depletion of the GTPase dynamin 1 [85].

Efforts to develop treatments for AD featuring antibodies designed to bind and clear Aβ have been hampered by many setbacks, such as lack of meeting target endpoints and deleterious side effects, which may be attributed to initiating treatment too far into the disease process and to the experimental therapeutics targeting Aβ plaques, not oligomers. Treatment of a mouse model of AD with an anti-Aβ monomer antibody and an aggregate-preferring antibody found that monomer-binding antibody bound and increased circulating Aβ in the bloodstream and aggregate-binding antibody-bound Aβ plaques increased circulating levels of Aβ, demonstrating the ability of the antibodies to bind Aβ and transport it into the bloodstream for clearance, but neither antibody affected AβO levels in the brain nor improved cognitive deficits [86]. These results highlight the critical importance of targeting AβO in the development of effective therapeutics for AD.

2.3.4 *BRI2*

In 1933, a family in Great Britain was found to have an inherited neurological disorder that was ultimately traced back to English forebears in the late 18th century. This disease was called FBD and is extremely rare, with some 350 individuals currently identified in the pedigree and 50 at risk of developing the disease. An even more rare, similarly heritable and neurodegenerative disease was identified in one Danish family in 1970, termed FDD, with 13 affected individuals. It was later found that both diseases were linked to mutations in BRI2, a gene

coding for the type II transmembrane protein Bri, which results in the loss of the normal stop codon. FBD has a point mutation in the stop codon resulting in an 11-amino acid extension with subsequent cleavage to produce the 34-residue ABri. FDD is associated with a decamer duplication preceding the stop codon that produces an overlong precursor protein with subsequent cleavage to produce the 34-residue peptide ADan, which is identical to the ABri N-terminal but with a distinct C-terminal of 10 mainly hydrophobic residues.

The pathology of both diseases feature diffuse, soluble amyloid deposits, NFTs of hyperphosphorylated tau, cerebral amyloid angiopathy (CAA), neurofibrillary degeneration, and ischemic white matter damage, but FBD also has plaque deposition in some brain areas. Symptoms are also similar, with muscular weakness in all four extremities, difficulty in articulation of speech, and loss of memory and dementia, and FDD also presents with cataracts, hearing loss, and loss of full control of bodily movements.

Bri, ABri, and ADan have two cysteine residues at the C-terminal that can form disulfide bonds, but the mutant C-terminal extensions result in ABri forming cross-linked oligomers when oxidized that are cytotoxic [87], resulting in apoptotic changes [88], and reduced ADan forming neurotoxic oligomers [89]. Pyroglutamate post-translational modification enhances oligomerization of both ABri and ADan, inducing oxidative stress and perturbations of mitochondrial membrane potential [90, 91]. Both ABri and ADan share a common feature of oligomers in that they induce the formation of pores in lipid bilayers, resulting in ionic transport across cell membranes, and although ADan forms larger pores than ABri, their conductances are similar [15]. The formation of ion channels may disrupt vesicle release and other synaptic processes.

Aggregation of oxidized ABri into oligomers generates hydrogen peroxide [92], consistent with findings that oxidized ABri inhibits LTP, disrupting synaptic plasticity in cultured hippocampal rat neurons [93]. A transgenic model of FDD expressing mutant human tau to study the interaction of ADan and tau found increased accumulation of tau and reduced levels of synaptophysin preceding plaque formation, suggesting that oligomeric ADan may mediate tau and synaptic pathology [94]. Although little is known about these two rare diseases, they also demonstrate the toxicity of oligomers to synaptic function.

2.3.5 *Huntingtin*

Nineteenth-century physicians noted a disorder characterized by abnormal, involuntary movements, termed chorea from the Greek word for dance, also marked by changes in personality and gradual impairment of cognition that ran in families down from the Middle Ages. And like so many other neurological diseases, it now bears the name of the physician who first thoroughly described the disorder, George Huntington, who also documented the autosomal dominant inheritance pattern of the disease in a paper published in 1872. The locus of causal gene was found in 1983 and advances in genetics allowed for the isolation of the gene in 1993, revealing a mutation resulting in a trinucleotide repeat coding for glutamine in a section of that gene, soon to be named *huntingtin* or *HTT*. The increasing number of repetitions in the chain of glutamines (polyQ repeats) correlates with increasing disease pathology, and individuals with fewer than 36 repeats will not develop the disease at all, while fewer than 26 repeats are not at risk to pass the disease on to their offspring.

The N-terminal segment of mutant *HTT* (*HTT*) influences mutant *HTT* protein aggregation, with increasing aggregation rates as polyQ repeats increase but maintaining a heterogeneous mixture of monomers, α-helix oligomers, and β-sheet amyloid fibrils that contain solvent-accessible α-helix segments [95]. *HTT* aggregates into intracellular deposits called inclusion bodies (IBs) in cortical and striatal neurons, though the presence of IBs does not correlate well with neuronal death. Transfection of mutant *HTT* into cultured striatal neurons shows that huntingtin aggregates in the nucleus and induces apoptosis; however, aggregate formation does not correlate well with huntingtin-induced apoptosis [96]. Long-term microscopic monitoring of striatal neurons transfected with mutant *HTT* of varying length of polyQ repeats demonstrate that neuronal death is dependent on the dose of *HTT* and the length of the repeats, and levels of diffuse *HTT* regulate cell survival, not the presence of IBs, suggesting that monomeric or less-aggregated *HTT* are the toxic species [97]. This hypothesis is supported by the isolation of prefibrillar *HTT* oligomers from brain tissue of HD-affected patients [98] and analysis of a transgenic mouse line known as the shortstop mouse, expressing a short fragment of

HTT with a polyQ expansion with nuclear inclusions but without other HD phenotype pathology, demonstrates that nuclear inclusions are not the toxic species [99].

Huntingtin is present both pre- and post-synaptic, interacting with NMDARs [100] and is associated with synaptic vesicle trafficking [101]. Conditional silencing of *HTT* in mouse cortex interferes with normal synapse development [102]. Maintaining normal levels of a homolog of the huntingtin protein in *Aplysia* is critical for normal LTP and both pre- and post-synaptic function [103]. Consequently, mutation can be expected to lead to neuronal damage through both loss and gain of *HTT* protein function.

Mutant *HTT* impairs normal synaptic functions through several mechanisms, including direct effects on neurotransmitter release, receptor response, and channel conductance. Pre-symptomatic transgenic mice expressing pathogenic polyQ repeats in mutant *HTT* have increased interaction of PSD95 with extrasynaptic NMDARs and subsequent activation of cell death pathways [104]. Hippocampal slices from these pre-symptomatic mice also have impaired vesicle release [105], while hippocampal slices from aged HD mice have profound deficits in LTP and lower amplitude and frequency of spontaneous excitatory postsynaptic currents (EPSCs) [106]. Co-cultures of cortical or thalamic neurons with striatal neurons from HD mice have reduced miniature excitatory postsynaptic current (mEPSC) frequency, readily releasable pool (RRP) of excitatory synaptic vesicles, and dendritic arborization in striatal spiny neurons (SPNs) [107], enhanced extrasynaptic NMDAR current, and associated cell death signaling [108, 109]. Mutant *HTT* targeted to pre-synaptic compartments inhibits phosphorylation of synapsin-1, a critical regulator of vesicle release [110]. Synaptic dysfunction is also induced by mutant *HTT* through interaction with the GTPase Drp1, fragmenting and dislocating mitochondria [111], and decreasing activity of the ubiquitin-proteasome system in mutant *HTT*-containing synapses [112]. Application *in vitro* of mutant *HTT* aggregated into diffusible oligomers and fibrils induce nuclear DNA damage, mitochondrial dysfunction, and cell death [113]. Mutant *HTT* binds the type 1 inositol (1,4,5)-trisphosphate receptor and increases its sensitivity to activation, which reduces endoplasmic

reticulum Ca^{2+} levels and leads to over-activation of neuronal store-operated Ca^{2+} entry and dendritic spine loss in a mouse model of HD [114].

Though preparation of isolated mutant *HTT* oligomers to identify oligomer-specific impairment of synaptic function remains difficult, there is ample evidence that suggests that these oligomers share toxicity common to other oligomeric proteins.

2.4 Other oligomeric proteins implicated in neurodegenerative disease

The cause of several brain-wasting diseases was found to be an abnormal isoform of the prion protein, PrP, rendering the misfolded protein infectious and neurotoxic. Prion is a product of the highly conserved *PrnP* gene, and consequently the misfolded form, PrP^{Sc}, causes disease in a spectrum of mammals, including CJD in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle. PrP^{Sc} forms amyloid plaques in affected brains and similar to other amyloids, oligomers are the more toxic form of the protein [115]. Toxic oligomeric mechanisms are proposed to inhibit the 26S proteasome [116] and disrupt cell membrane lipid rafts [117].

Neurological disorders can also feature degeneration of motor neurons, such as in amyotrophic lateral sclerosis (ALS). While familial ALS is primarily associated with mutation of the mitochondrial protein superoxide dismutase (SOD1), TAR DNA-binding protein 43 (TDP-43) is implicated in ALS pathology. TDP-43 has multiple functions in gene transcription and translation, and aggregates of the protein are hallmarks of frontotemporal lobar dementia (FTLD) and ALS, with more than 30 mutations of the gene found in individuals affected by these diseases. Mutation has been found to induce aggregation of TDP-43 [118, 119], with oligomerization interfering with its DNA binding capability [120].

These and other oligomeric proteins that are implicated in neurodegenerative diseases have not yet been shown to be directly synaptotoxic, but contribute to synaptic dysfunction through disruption of many cellular mechanisms, and therefore discovery of therapeutics that prevent misfolding and aberrant aggregation of these proteins is critical in developing effective treatments for many neurological disorders.

2.5 A case study of one mechanism by which oligomers disrupt synaptic function: AβO interference with zinc modulation of neurotransmission

Previous work from our laboratory has shown that synaptic zinc (Zn^{2+}) released during excitatory neurotransmission increases the formation and accumulation of AβO at synaptic sites and that the AβO–Zn^{2+} interaction accelerates oligomer formation [75]. Other research has demonstrated that Zn^{2+} has a high affinity for AβO [121–124], accumulates in Aβ plaques in AD brain [125], and disrupts synaptic function following sequestration by AβO [126], a significant finding as the reduction of synaptic Zn^{2+} has been demonstrated to result in excessive excitatory neuronal activity [127–130], cognitive impairment [131, 132], and altered neuronal signaling [133–135]. Dysregulation of synaptic zinc has been implicated in AD [136], and therapeutics targeting Zn^{2+} homeostasis have shown promise in treating both AD patients and mouse models of AD [137–141]. Although it is known that exogenous Zn^{2+} inhibits or activates many receptors and transporters, the effects of Zn^{2+} released at the synapse on modulation of downstream signaling molecules in these pathways, neuronal activity, and cognition are not well understood. To further investigate the dysregulation of zinc neurotransmission, we used zinc T3 transporter knockout (ZnT3KO) mice, which lack synaptic zinc and develop age-dependent cognitive impairment.

Zn^{2+} has differential modulation of NMDARs depending on subunit composition and zinc concentration [128, 142], and so we assayed the effect of genetic removal of synaptic Zn^{2+} on activation of proteins downstream in NMDAR signaling pathways. We found that while basal expression of AKT and Erk1/2 are reduced in ZnT3KO hippocampal slices, only the activity-dependent increase in phosphorylation of Erk1/2 (p-Erk1/2) was reduced in ZnT3KO mice, while p-AKT remained unaffected. We then explored the role of synaptic Zn^{2+} on NMDAR subunit activation of Erk1/2 using subunit-specific inhibitors of NR2A and NR2B and found that Zn^{2+} inhibits NR2B-mediated activation of Erk1/2. Increase in the expression of the neurotrophic protein BDNF is activity-dependent [143], linked to Erk1/2 signaling [144], and is differentially affected by NMDAR location, with synaptic NMDARs increasing expression and extrasynaptic

NMDARs inhibiting expression [145]. We explored transcription of *BDNF* mRNA, finding the activity-dependent increase to be reduced in ZnT3KO hippocampus.

The age-dependent reduction of synaptic proteins and pro-BDNF, along with impairment of cognition described in ZnT3KO mice [132] led us to look for other age-dependent alterations in ZnT3KO hippocampus. Previous reports found the genotype to have a lower threshold for induced seizures [146], consistent with findings that Zn^{2+} infusion into the hippocampus suppresses seizure activity [147], though there are no reports of observed seizures in ZnT3KO mice. We hypothesized that cumulative effects of aging may exacerbate seizure susceptibility in ZnT3KO mice and thus investigated alterations consistent with seizure activity in the hippocampus. We found age-dependent reductions of calbindin, increases in Neuropeptide Y, and aberrant mossy fiber sprouting. Consequently, we sought to determine whether the age-dependent increase in cognitive impairment characteristic of ZnT3KO mice may be due to increasing seizure activity as they age and so we used either an acute or chronic treatment with an anti-seizure drug, levetiracetam (LEV), to suppress seizures and investigate the effect of treatment on memory. We found that acute treatment with LEV did not rescue cognition, but chronic treatment prevented cognitive impairment.

These results suggest that interference with Zn^{2+} suppression of NR2B subunits can be a mechanism by which AβO induces excessive activation of extrasynaptic NMDARs, disrupting synaptic function and neurotrophic pathways including expression and homeostasis of BDNF. These results also suggest that synaptic Zn^{2+} suppresses excessive excitatory activity as removal of synaptic Zn^{2+} results in age-dependent increases in markers of seizure activity. The observation that long-term treatment with an anti-seizure drug prevents development of cognitive impairment in ZnT3KO further supports a role for synaptic Zn^{2+} in suppression of seizure activity. But while treatment with drugs targeting Zn^{2+} homeostasis has had success in treating mouse models of AD, they have yet to prevail in human clinical trials, and so preventing the formation or effective clearing of AβO should be a primary goal in AD research.

2.6 Conclusion

Misfolding of proteins into oligomers has many detrimental effects on cellular homeostasis, with both the loss of normal protein function and gain of toxic function by the misfolded protein. Either process will disrupt activity at the synapse and result in neurodegeneration and neurological disorders. Most current treatments aim to ameliorate symptoms of neurological diseases, with very few successful cures. The prevalence of neurological disease and the lack of effective therapeutics to prevent or cure many of them underscore the urgency for further understanding of what drives aberrant protein folding and to discover and identify strategies to prevent protein dysregulation and neutralize or clear misfolded proteins. Identifying commonalities in the formation of oligomers in neurological disorders may lead to a great leap forward in understanding the role of misfolded proteins in disease and developing successful treatments.

Acknowledgment

This work has been supported by a grant from the California Department of Public Health Alzheimer's Disease Program.

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Chapter 3

Prion-like Protein Seeding and the Pathobiology of Alzheimer's Disease

Lary C. Walker

3.1 Alzheimer's disease (AD) and the amyloid (Aβ) cascade hypothesis

The senile plaques and neurofibrillary tangles that were first clearly linked to dementia by Alois Alzheimer over a century ago [1] remain the defining histopathologic features of the disease that now bears his name (Figure 3.1A). Subsequent research has shown that the proteinaceous cores of the plaques primarily consist of extracellular, polymeric amyloid-β (Aβ) fibrils, and the tangles are formed intracellularly by the ectopic polymerization of tau protein [2]. The pathogenic relationship between these lesions in the Alzheimer brain is, to some extent, uncertain, but considerable evidence now supports the view that the aggregation of Aβ is a crucial early event, a concept embodied in what has been known as the "amyloid cascade hypothesis".

First formulated as such by John Hardy and colleagues in the early 1990s [3, 4], the amyloid cascade hypothesis remains the prevailing general theory for the pathogenesis of AD [5]. In the amyloid cascade, the aggregation of misfolded Aβ protein is thought to lead to the structural corruption of tau, and the proliferation of these heteromorphic proteins progressively impairs cellular homeostasis and brain function; this chain

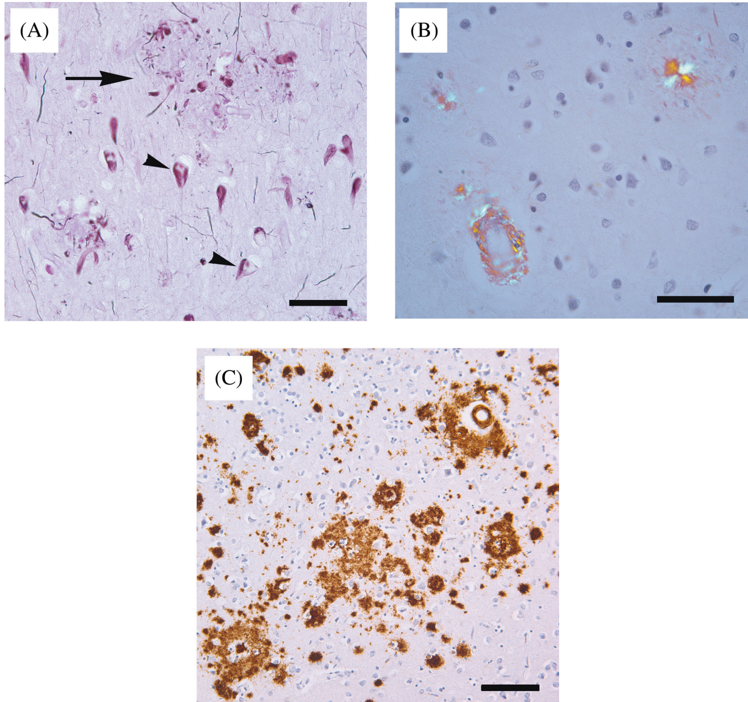

Figure 3.1 Neuropathological lesions in AD. (A) The canonical brain lesions in an AD patient, stained with the Naoumenko–Feigin silver method and periodic acid-Schiff reagent. A cluster of senile plaques with abnormal neurites is marked by the arrow, and two neurofibrillary tangles are denoted by arrowheads. (B) Congo red-stained amyloid plaque (upper right) and amyloidotic blood vessel (lower left) in the neocortex of an AD case. The tissue was imaged using crossed polarizing filters, with which Congo red-stained amyloid deposits characteristically show birefringence (orange-greenish); Nissl counterstain. (C) Aβ-immunostained section of the neocortex from an AD case. Note the variety of histologically detected deposits, including dense and diffuse parenchymal plaques and an immunoreactive blood vessel surrounded by parenchymal Aβ (upper right). Antibody 4G8 to Aβ, Nissl counterstain. Bars = 50 μm in (A) and (B), and 100 μm in (C).

of events ultimately results in the cognitive decline and dementia that define AD clinically [6].

The amyloid cascade hypothesis has adapted well to our increasingly nuanced understanding of AD pathobiology over the last quarter century, and its heuristic value is evident from the wealth of research it has helped to spawn. The impact of the concept has been diminished somewhat,

however, by the term *amyloid*, which has tended to draw attention away from the elements of protein aggregation that may be more closely involved in the neurodegenerative process. "Amyloid" is a generic term for deposits of insoluble, proteinaceous fibrils that exhibit distinctive staining properties with the dye Congo red (Figure 3.1B) and a characteristic X-ray diffraction pattern indicative of high β-sheet content [7]. Aβ is only one of at least 30 different proteins that can form amyloid in various organs of humans [7, 8]. Furthermore, burgeoning evidence indicates that Aβ-amyloid *per se* is a relatively benign expression of the disease process, which also involves the generation of cytotoxic oligomeric assemblies (see below). In this light, the "amyloid cascade" might more suitably be called the "Aβ cascade".

The principal support for the Aβ cascade derives from pathology, genetics, biomarkers, and experimental studies of the molecular mechanisms underlying the misfolding and aggregation of Aβ and tau [2, 6, 9–13]. Objections to the hypothesis often have emphasized the ambiguous relationship between the degree of dementia and Aβ plaque load, the failure of early therapeutic trials targeting Aβ, the inability to fully recapitulate AD in genetically modified mice, and the pathologic complexity of AD [14–16]. These criticisms have helped to focus and refine the hypothesis as well as to identify issues that need to be resolved. A strength of the Aβ cascade hypothesis, however, is that it has evolved to accommodate new information without changing its fundamental precept: that abnormal multimeric Aβ precipitates the series of events that ultimately lead to dementia in AD.

Many of these issues have been addressed in a recent review [6]. I will examine the major perceived shortcomings of the amyloid cascade hypothesis and then describe how the expanded prion paradigm furnishes a compelling mechanistic foundation for the Aβ cascade. Indeed, the prion paradigm has evolved into a unifying model for the molecular underpinnings of virtually all age-related neurodegenerative diseases [13, 17–23].

3.1.1 *Aβ plaque load and dementia*

One challenge to the amyloid cascade is the relatively weak correlation between the degree of dementia and the quantity of senile plaques in the

brain [15, 16]. This concern is based on the assumption that the plaques themselves are the primary cause of dementia. Amyloid plaques historically have garnered attention mainly because they are the most obvious histological sign that Aβ is accumulating in the brain [24]. While Aβ plaques unquestionably disrupt tissue integrity and contribute to disease severity [25–28], they are just one manifestation of abnormal Aβ; the most toxic Aβ assemblies likely take the form of small, soluble oligomers [24, 29–33] that exist in a shifting equilibrium with fibrillar Aβ in plaques [34–37] and can be particularly injurious to cells [38, 39]. In an analysis of Aβ plaques and oligomers in AD patients and non-demented controls, the quantity of oligomers correlated more strongly with plaque load in AD patients than in non-demented patients with a similar plaque burden [37]. Indeed, Aβ plaques may represent an attempt by the brain to neutralize aberrant Aβ by sequestering it in the form of less toxic, polymeric amyloid fibrils [6]. Accordingly, Aβ toxicity ensues when the balance shifts from the sequestration of Aβ in amyloid fibrils to the proliferation of diffusible oligomers [35].

Further evidence that amyloid itself is not obligatory for AD pathogenesis comes from a rare, autosomal dominant form of AD that is caused by a missense mutation (the "arctic" mutation) that alters an amino acid within the Aβ region of the Aβ precursor protein (APP E693G) [40, 41]. In these patients, the Aβ plaques consist of relatively diffuse deposits of Aβ protofibrils that lack the dense, congophilic core that defines classical amyloid. Even without significant amyloid, there is pronounced deposition of Aβ in the brain, along with the other essential features of AD: tauopathy and dementia [40, 41]. The ability to assemble into amyloid is a common feature of many pathogenic proteins [42], but the presence of *bona fide* amyloid is not obligatory in all brain disorders. This is particularly obvious in the prion diseases (below), in which amyloid is conspicuous in some but rare or absent in others, yet all prionopathies manifest severe neurodegeneration.

3.1.2 *Tauopathy and dementia*

Tauopathy occurs in many neurodegenerative diseases, both as the primary lesion in hereditary tauopathies, chronic traumatic encephalopathy, and

primary age-related tauopathy (PART), and as a secondary lesion in AD, British and Danish familial dementias, prion diseases, and others [43–52]. In AD, quantitative studies have demonstrated that both plaques and tangles correlate with the degree of dementia [53] but, paradoxically with respect to the Aβ cascade, tangles are more tightly linked to cognitive decline than are plaques [53–56]. In fact, experimental studies show that the full pathogenicity of Aβ in AD requires the expression of tau [57–59].

Although tauopathy is critically important for the cognitive impairment that defines AD, a key open question is how tau and Aβ are coupled in the Aβ cascade. Specifically, is tauopathy a non-specific response to the stress induced by Aβ aggregation [53], or do the proteins interact directly, for example, by cross-seeding? Laboratory studies have shown that Aβ aggregation induces a form of tauopathy in genetically modified mice [60–64]. The link appears to be unidirectional, however, in that tauopathy does not appear to actuate Aβ deposition [60]. In humans, the hereditary tauopathies are not obligatorily accompanied by significant Aβ deposition [53], but when Aβ deposits happen to coexist with tauopathy in these cases (usually in older patients), cognitive decline is exacerbated [65]. Elucidation of the Aβ–tau connection could yield novel therapeutic targets for AD at this crucial juncture of the Aβ cascade. The importance of tauopathy for the AD phenotype, however, does not override the role of Aβ aggregation as the prime mover.

3.1.3 *Clinical trials*

Another challenge to the Aβ cascade hypothesis has been the disappointing outcome of the first clinical trials in which Aβ was directly targeted in AD patients [66, 67]. These trials involved either removal of Aβ by active or passive immunotherapy, or the use of small molecules to lower the production of Aβ by blocking the action of the secretases (β- and γ-secretase) that release Aβ from APP. Despite evidence in several instances of target engagement in the brain, little or no clinical benefit has resulted from most trials to date [6]. Subsequent research, however, has confirmed what neuropathologists had suspected for years, i.e. that the pathogenic process in the brain begins well more than 10 years before the first clinical signs and that cerebral Aβ load has peaked by the time the

first clear cognitive deficits become apparent [9, 11, 12]. More recent clinical trials in which patients were treated in the earliest stages of clinical dementia have yielded hints of efficacy [6], and a recent phase 1b trial found that the monoclonal anti-Aβ antibody aducanumab dose-dependently clears plaques from the brain and may slow clinical decline [68].

The long, silent preclinical phase of AD — a characteristic of many chronic diseases [53] — indicates that the most effective treatment should begin as early as possible. For this reason, prevention trials have been initiated in which the Aβ cascade is targeted before the onset of symptoms [69, 70]. When placed in the context of recent biomarker and pathophysiologic research, the unsatisfactory outcomes of the first clinical trials targeting Aβ are likely to have resulted, at least in part, from the late timing of treatment [71]. To the extent that the appropriate target is engaged by an effective drug, and the trials are initiated early enough in the disease process, a negative outcome of ongoing prevention trials would necessitate a reconsideration of the Aβ cascade hypothesis. As therapeutic agents and strategies have continued to evolve [6, 68], wholesale rejection of the hypothesis seems increasingly unlikely.

3.1.4 *Animal models and AD*

The expression of AD-associated human transgenes has not yet yielded the complete AD phenotype in a rodent model [72, 73]. The introduction of the first successful transgenic mice expressing human APP in the mid-1990s unleashed a torrent of research on the pathogenesis of Aβ plaques [72, 74]. These mice were followed by models of tauopathy, presenilin expression, and by the co-expression of various transgenes (in most cases, the genetically modified mice have carried transgenes with mutations linked to hereditary human diseases) [73]. Genetically modified mice (and more recently rats [75]) have been a boon to investigations of the cellular and molecular mechanisms underlying the formation of plaques, cerebral amyloid angiopathy (CAA), and tangles, but discrepancies between the rodent phenotype and human AD have challenged the Aβ cascade; specifically, why do not rodents that generate copious, human-sequence Aβ plaques develop a more AD-like condition?

As far as we presently know, AD occurs only in humans. Non-human primates, our nearest biological relatives, express human-sequence Aβ, and in old age they develop abundant senile plaques and/or CAA [76, 77]. In addition to the most common human Aβ isoforms of 40 and 42 amino acids, other Aβ isoforms resulting from C- and N-terminal truncations or modifications are essentially identical in humans and monkeys [78]. When measured by enzyme-linked immunosorbent assays (ELISAs), the amount of Aβ in old monkeys sometimes exceeds that in humans with AD [78, 79]. Human-sequence Aβ occurs in other taxa such as canines, which also manifest Aβ proteopathy with age [80, 81]. No non-human species, however, has yet been found to develop the full pathologic phenotype of human AD, including, in particular, neurofibrillary tangles (along with Aβ plaques) [72, 76, 78].

The reasons for the apparent disconnect between Aβ deposition and tauopathy remain uncertain. However, there is evidence from the binding of the selective ligand Pittsburgh Compound B (PiB) that the multimeric Aβ in non-human species, including transgenic mice, non-human primates, and dogs, may have an altered three-dimensional architecture [78, 79, 82, 83]. Though still speculative, these findings suggest that the shape of the misfolded protein may be a critical determinant of its pathogenicity. If so, such proteopathic "strains" of aggregated Aβ would be predicted to differentially activate the Aβ cascade.

3.1.5 *Complexity*

Some have argued that the complexity of dementia, clinically and pathologically, mitigates against the relatively uncomplicated Aβ cascade model [15, 66]. Virtually all diseases are complicated by the organism's multifaceted response to a perturbation of cellular homeostasis. But as Rudolf Virchow often pointed out, the *cause* of the disease is not the *disease itself* [84]. Rather, disease is essentially the expression of the many ways in which the cells of the organism respond to the cause.

A thorough analysis of the brain of a patient who has died of AD will convince the observer that the disease itself — even in its purest form — indeed is complex. The Aβ plaques vary markedly in their morphology

(Figure 3.1C), cellular components, and distribution, both within an individual brain and among AD patients. What is more, Aβ in the walls of cerebral blood vessels (CAA) ranges from none to severe in different AD cases [85, 86]. Many other changes are prominent in the AD brain, including tauopathy, reactive gliosis, and loss of neurons and synapses. All of these changes are important components of the *disease itself*; as such, they are viable candidates for therapeutic intervention. They also can reveal putative risk factors for AD, such as inflammation, metabolic dysfunction, oxidative stress, DNA damage, or some facet(s) of senescence [15]. But these risk factors increase the probability of developing AD only inasmuch as they actuate the root *cause of the disease*, i.e. the misfolding, templated corruption, and aggregation of Aβ. Their occurrence in the absence of Aβ proteopathy in the brain is not, by definition, AD.

Another source of ambiguity about the role of Aβ aggregation in AD is the observation that dementia in the very old is associated with multiple pathologies, including vascular disease and assorted comorbid neurodegenerative conditions [66, 87–89]. AD is the most common *type* of dementia, but dementia can occur in more than 50 other conditions [90]. The presence of other causes of cognitive and behavioral dysfunction, many of which may be independent of AD, can unmask or accelerate dementia associated with the pathology of AD, and such comorbidity increases with advancing age [53, 91]. It is not uncommon, for example, to find in very old subjects TDP-43 proteopathy and/or synucleinopathy along with the canonical lesions of AD [92–94]. Furthermore, cortical microinfarcts significantly exacerbate cognitive decline in patients who also are incubating AD-like pathology [95]. While these co-existing lesions create the impression that what is diagnosed as AD is highly complex pathologically, in fact they mainly demonstrate that many things can go wrong in the senescent brain.

AD occurs in its purest form in younger patients with autosomal dominant mutations in the genes for APP or the Aβ cleaving subunits of the γ-secretases: presenilin-1 and presenilin-2. These hereditary forms of AD usually emerge before the age of 65, and they often lack the heterogeneous mixture of brain lesions in older patients, yet they exhibit the defining features of AD: Aβ deposition, tauopathy, and the progressive, intractable loss of cognitive capacity. AD is undoubtedly a complex disease, but the essential causative elements — as embodied in the Aβ cascade — are relatively straightforward and thus amenable to focused therapeutic

intervention. As discussed below, similarities in the pathophysiology of AD and prion disease, both of which involve seeded protein aggregation as an essential feature, support a seminal role of protein aggregation in AD. A similar causal relationship between proteopathy and neurodegeneration is likely in a host of other neurodegenerative maladies [13, 18, 20].

3.2 The prion paradigm

The essential molecular mechanism that drives the Aβ cascade bears important commonalities with the mechanism by which prions materialize and proliferate. Prions are assemblies of misfolded prion protein (PrP) that can infect other organisms by triggering a crystallization-like process of corruptive PrP templating in the host [18, 21, 96, 97]. Prion diseases can also arise endogenously, either idiopathically or as a result of mutations in the gene for PrP [18, 98–100]. Pathologically, the prionotic brain is marked by spongiform change (Figure 3.2), neuronal loss, astrocytosis, and the accumulation of misfolded PrP [45, 101, 102].

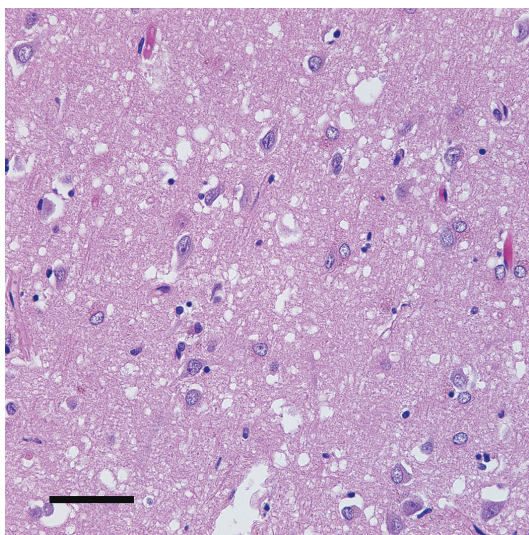

Figure 3.2 Neuropathological features in spongiform encephalopathies. Spongiform vacuolation (here seen as holes) in the neocortex of a CJD patient. Hematoxylin and eosin stain. Bar = 50 μm.

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In humans, prion diseases are rare, and most instances are either idiopathic (sporadic) or hereditary in origin. Creutzfeldt–Jakob disease (CJD), the most common human prionopathy, has a world-wide incidence of approximately 1–2 per million people [103] (http://www.cdc.gov/prions/cjd/about.html). Others include Gerstmann–Sträussler–Scheinker disease, kuru, fatal insomnias, variably protease-sensitive prionopathy, and variant CJD (which results from infection by the prions of bovine spongiform encephalopathy [BSE]). In non-human species, the prionopathies include scrapie in sheep, chronic wasting disease in cervids, BSE, and several others [97, 104–108].

Infectious prion diseases are more common in non-human species than in humans. Scrapie — the prototypical prion disease — has been known for centuries to be infectious in sheep [109, 110], though the infectious agent was not identified with reasonable certainty until the 1980s [97]. Chronic wasting disease is the only prion disease that is known to be spreading in a wild population of animals — mostly deer and elk in western North America.

Approximately 3,400 instances of human prion disease are known to have resulted from infection worldwide [21]: ~2,700 cases were the (now extinct) disease kuru among the Fore people of Papua New Guinea, and the rest include 231 cases of variant CJD (http://www.cjd.ed.ac.uk/documents/worldfigs.pdf) as well as ~469 iatrogenic cases caused by accidental exposure to prions in biologics such as cadaveric dura mater transplants, pituitary hormone preparations, and very rarely from sources such as corneal transplants and contaminated surgical instruments [111]. Cessation of cannibalism (transumption) among the Fore [112], and knowledge of the nature of prions and the potential infectivity of biologic material has essentially eliminated infectivity as a current cause of human prion disease [111]. As will be discussed below, there is now preliminary evidence that Aβ deposition has been induced in humans in rare instances in which Aβ seeds were inadvertently conveyed by contaminated biologics.

A key discovery that helped to explain the heterogeneous origins of prion diseases (infectious, idiopathic, or hereditary) was that PrP is normally produced by cells [113, 114]. This "cellular" type of prion protein (PrP^{C}) ordinarily assumes a non-pathogenic conformation with little β-sheet secondary structure. When PrP misfolds into a shape that is exceptionally

enriched in β-sheet [PrP-"scrapie" (PrP^{Sc})], the agent acquires the capacity to propagate by inducing other PrP molecules to misfold and aggregate into oligomers, protofibrils, and amyloid fibrils [18, 97]. For reasons that are not fully clear, aggregated, pure, recombinant PrP is ineffective at inducing disease [115], although aggregation of recombinant PrP in the presence of certain cofactors can enhance infectivity [116–118]. Whether prion diseases are human or non-human, hereditary, infectious or idiopathic, an essential feature of all prion diseases is that they progress by the seeded corruption and aggregation of PrP within the brain; this paradigm can furnish useful insights into many other neurodegenerative diseases.

3.3 The prion paradigm and AD

3.3.1 *Similarities between Aβ seeds and PrP-prions*

Aβ seeds resemble prions in virtually all of the characteristics by which prions are defined [119] (Table 3.1). *In vitro*, monomeric Aβ can be induced to aggregate by the preformed Aβ seeds in a manner resembling seeded

Table 3.1 Similarities between PrP-prions and Aβ seeds.[a]

Property	Prions	Aβ seeds
β-Sheet-enriched conformation	Yes	Yes
Potential to form amyloid	Yes	Yes
Seeds initiate pathology	Yes	Yes
Purified and synthetic proteins seed	Yes	Yes
Seeds instigate *de novo* deposition	Yes	Yes
Existence of distinct strains	Yes	Yes
Partial resistance to PK digestion	Yes	Yes
Resistance to high temperature	Yes	Yes
Resistance to formaldehyde fixation	Yes	Yes
Spread within and to the brain	Yes	Yes
Serial transmissibility in mice	Yes	Yes
Transmissibility to humans	Yes	Yes[b]

Notes: [a] Modified from [119].
[b] Transmissibility of Aβ lesions, but not AD.

crystallization [120]. Suggestive clinical and pathologic commonalities have long engendered speculation that, like the prion diseases, AD might be transmissible under certain circumstances [121, 122].

With the introduction of APP-transgenic mouse models in the 1990s [123–125], it became possible to definitively test the hypothesis that a prion-like mechanism drives the seeded induction of Aβ deposition in the living brain. Autopsy-derived, clarified brain extracts from AD patients injected into the brains of young, predepositing APP-transgenic mice stimulate the deposition of cerebral Aβ, usually after an incubation period of one or more months (depending on the nature of the extract and the host) [126–128]. The degree of Aβ seeding is proportional to the concentration of the brain extract [127, 129], and Aβ deposits are not induced by control brain extracts that are devoid of aggregated Aβ, or in non-transgenic host mice that do not express human-sequence Aβ [126, 127, 130]. Immunodepletion of Aβ from the injected extract abolishes the seeding effect [127, 131].

Aβ seeding thus requires both a donor brain extract containing aggregated Aβ and a host that is capable of depositing Aβ. The donor can be human, APP-transgenic mouse [127], or aged non-human primate [78]; thus, it is likely that any species can serve as an effective donor, as long as aggregated Aβ is present in the brain. In addition, both the seed and the host influence the morphotype of seeded Aβ deposits [127, 132]. Although aggregated synthetic Aβ (like recombinant PrP-prions [115]) is a relatively weak seed, increasing the dose of synthetic Aβ multimers and the incubation time results in seeded Aβ deposition *in vivo* [133].

Like PrP-prions, then, Aβ seeds are most potent when they are generated within the brain. Understanding why this is the case could enhance our understanding of the pathogenicity of multimeric Aβ in AD. Just as the seeding potential of synthetic PrP-prions is bolstered when certain cofactors are included in the medium during PrP aggregation [116–118], it is conceivable that similar cofactors can increase the *in vivo* potency of synthetic Aβ seeds. The pathobiology of Aβ, for instance, has been shown to be influenced by the lipid milieu [134].

Other features shared by Aβ seeds and prions include the formation of variant structural/functional strains [42, 127, 132, 135–137] and the variable size and proteinase K sensitivity of the seeds [138]. Aβ seeds can induce Aβ deposition *de novo* in animals that do not normally form

plaques or CAA within their lifetimes [130, 139], and seeds can be serially transmitted from the initially seeded mice to subsequent hosts [136]. In addition, as with prions, extremely small amounts of Aβ seeds can stimulate Aβ-proteopathy in susceptible hosts [129, 140]. Aβ seeds also are quite robust; they can withstand brief boiling [127], years in formaldehyde [141], and some can persist in the brains of APP-null mice for at least 6 months [142]. Furthermore, Aβ seeds resemble prions (and other proteopathic seeds) in that they can traffic systematically within the brain [143, 144] and from the periphery to the brain [145, 146]. The means by which Aβ seeds spread — by constrained diffusion or active cellular transport, for example — remains an important open question. *In vitro* studies have shown that Aβ seeds can be conveyed by neuron-to-neuron transfer [147, 148], and there is also evidence that macrophages can take up and translocate Aβ seeds [145, 149].

3.3.2 *Evidence for the prion-like seeding of Aβ in humans*

In the 1980s, a subset of individuals who had been treated years previously with hormones derived from human cadaveric pituitary glands were found to have contracted CJD due to contamination of the preparations with PrP-prions [150]. Brain samples from eight recipients who had died of CJD were examined for the copresence of AD-type lesions; four of them had significant Aβ deposition in the brain, and two others had sparse Aβ deposits [151, 152]. Subsequent studies have found that Aβ deposition also is present in the brains of CJD patients who had received PrP-prion-contaminated dura mater transplants [153, 154]. In many of these cases of apparent iatrogenic Aβ seeding, the patients were relatively young (the growth hormone recipients, e.g. ranged from 36 to 51 years of age at death); because Aβ deposition is quite rare at this age, it is likely that the lesions resulted in some way from the treatment. A recent analysis of patients treated with cadaveric human growth hormone, and who died of causes other than CJD, also found an increased incidence of cerebral Aβ proteopathy [155]. The most likely explanation is that some batches of growth hormone and dura mater were contaminated with Aβ seeds in tissues that originated from AD (or incipient AD) donors. This hypothesis is supported by the observation that some dura mater samples [156] and pituitary glands in AD patients [157] contain aggregated Aβ.

Interestingly, none of the [155] patients with Aβ deposits also had evidence of tauopathy [151, 156]. Direct (homotypic) seeding of tauopathy by aggregated tau has been demonstrated in rodent models [158–160] (see below), but whether the dura mater samples or pituitary extracts contained tau seeds at the time of administration is not known. In addition, it is not known whether the recipients would have developed tauopathy and the dementia of AD had they lived longer. Monitoring the surviving biomaterial recipients will determine whether they are at increased risk of AD or other neurodegenerative diseases with advancing age. A preliminary investigation of pituitary hormone recipients in the United States suggests that they are not more likely to develop AD (or Parkinson's disease) [157], although longer term follow-up is necessary to determine the risk with certainty. These reports represent the first evidence that the aberrant aggregation of a protein other than PrP can be initiated in the human brain by exogenous seeds. In light of experimental work on Aβ seeding *in vivo* (above), a direct, prion-like seeding mechanism appears likely.

3.3.3 *Similarities between tau seeds and PrP-prions*

Similar to Aβ seeding, tauopathy can be induced in the brains of tau-transgenic host mice by infusion of biological material containing aggregated tau [61, 158–164]. The resultant tauopathy then spreads systematically from the injection site to axonally connected areas [61, 165–167], consistent with the uptake, transport, and release of tau seeds by neurons [168–170]. Brain extracts from donors with different human tauopathies induce tau lesions in host mice that resemble the pathology in the corresponding human diseases [166, 171], suggesting that tau, like Aβ and PrP, can misfold into replicable strains [169]. Another similarity among these proteopathies is that tauopathy can be induced in the brain by tau seeds delivered to the peritoneal cavity [172]. Tau seeds also exist in a range of sizes [173] including, in addition to fibrils, small, soluble oligomers [163, 174] and fibril fragments [175]. Unlike Aβ seeding, tauopathy is readily inducible in wild-type mice, and (in tau-transgenic mice) by recombinant tau fibrils [160, 166, 174, 176], although recombinant tau is less effective than is tau derived from brain samples [177]. Studies *in vitro* have shown that aggregates of tau are taken up by macropinocytosis and released

[177, 178] and that neuronal activity increases the release of tau *in vitro* as well as the amount of tauopathy *in vivo* [179].

While these findings highlight the prion-like properties of tau seeds, tauopathy in humans, like Aβ-proteopathy, has been shown to arise only from the *endogenous* generation of seeds. In a model of this endogenous process, genetically modified mice in which a pathogenic human tau transgene is restricted mainly to projection neurons of the entorhinal cortex [180, 181] were shown to develop tauopathy first in the entorhinal cortex and subsequently in axonally connected areas [180, 181]. Although weaker expression of transgenic tau in other brain areas could contribute to the pattern of lesion progression [182], when considered in light of evidence for the orderly proliferation of tau lesions along neuronal pathways in AD [183–185], the mouse experiments implicate neuronal transport mechanisms in the proliferation of neurofibrillary-type pathology. Furthermore, recent *in vivo* imaging studies indicate a general involvement of the connectome in AD and other neurodegenerative disorders [186–189].

The evidence thus supports the hypothesis that the aggregation of Aβ and tau occurs by a prion-like mechanism. To date, we cannot conclude that AD *per se* is transmissible from person to person, and there is no evidence for infection under everyday circumstances. Rather, it is most likely that AD begins within the brain with the endogenous misfolding, corruptive templating, and self-assembly of Aβ [13]. The cascade then progresses to include tauopathy and the other features that characterize the AD phenotype. The exact mechanism by which tau and Aβ are linked in the Aβ cascade remains an important unanswered question.

3.4 Wide range of prion-like mechanisms

It would be surprising to discover a disease mechanism that does not bear similarities to normal functions in nature. The proliferation of cells, for example, is an essential process in development, tissue renewal, and repair, but when the usual restraints on cell division become compromised, cancer can ensue. Similarly, the induced folding and polymerization of proteins is important for the formation of subcellular components such as the cytoskeleton [190], and prion-like processes of induced protein folding

have been implicated in the storage of biologically active peptides [191], in regulating gene function [192–194] and in stabilizing the molecular substrate for memory [195]. In yeast, prion-like proteins ("yeast prions") convey heritable information from mother to daughter cells [196–198].

Neurodegenerative diseases represent a corruption of the protein polymerization mechanism in which misfolded proteins escape normal proteostatic controls and proliferate in the form of oligomers, inclusions, and/or extracellular masses. This process of seeded protein aggregation, which in disease states can be likened to a molecular malignancy [199], now appears to be fundamental to virtually all age-related neurodegenerative diseases [18, 19, 21–23, 158, 200–225]. In addition, several systemic amyloidoses have been shown to arise and propagate by a seeding mechanism [210, 214, 226, 227]. Because every disease manifests itself in a unique way, each is likely to have a disease-specific cascade that is rich with therapeutic targets. The prion paradigm provides a unifying framework within which the fundamental molecular abnormality — seeded protein aggregation — can be rationally targeted therapeutically. As a shared mechanism, proteopathic seeding also presents untapped opportunities for fruitful cross-talk among researchers investigating seemingly distinct diseases, nearly all of which currently lack effective treatments.

Acknowledgments

This work was supported by NIH grants R21NS077049, AG025688, AG040589, RR00165, OD11132, and the MetLife Foundation. Mathias Jucker and the members of his laboratory in Tübingen have been the source of many fruitful discussions, which were facilitated by an award from the Alexander von Humboldt Foundation. I also gratefully acknowledge the insightful input of David Lynn, Yury Chernoff, Anil Mehta, Noel Li, Harry LeVine, and Silke Vogelgesang, as well as the technical assistance of Jeromy Dooyema. There are no conflicts of interest to declare.

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Chapter 4

The Tau Misfolding Pathway to Dementia

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4.1 Introduction to tauopathies

Neurodegenerative diseases linked to dementia are becoming more commonplace as society ages. In many cases, inclusion bodies in the brain can be detected that are caused by the buildup of aggregated proteins including α-synuclein, Aβ, and tau. These inclusions can be formed in different areas of the brain with varying phenotypic effects.

Tau pathology associated with dementia is present in many different disease types which are more universally known as tauopathies. This family of disease shares the accumulation of abnormally hyperphosphorylated tau which forms intracellular deposits that might be morphologically different depending on the disease, but always linked to dementia. Tauopathies include, among others, Alzheimer's disease (AD), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), cortical basal degeneration (CBD), Pick's disease, progressive supranuclear palsy (PSP), dementia pugilistica, amyotrophic lateral sclerosis, and tangle-only dementia [1, 2] [as reviewed in Ref. 3]. The presence of tau inclusions in the brain of sports players and soldiers has been in the news recently and has helped to identify chronic traumatic encephalopathy as a new tauopathy [4]. Brain imaging tools are being developed to study the increase in abnormal tau inclusions in the people that have experienced repetitive mild traumatic brain injury linking the injury to deposit patterns found in

this disorder [5]. Abnormalities in the tau protein is a primary event which lead to neurodegeneration and dementia [6].

Recent work in AD patients using brain imaging correlates the levels of tau inclusions, and not levels of plaque formation, with declining cognitive abilities in patients with AD [7]. As early as 1998, mutations in *MAPT*, the gene for tau, were discovered in patients with FTDP-17 providing unequivocal evidence that changes in tau are enough to induce dementia [8–10]. These findings further allowed researchers to begin studying the effects of single mutants on tau phosphorylation, microtubule stability, and neurofibrillary tangle (NFT) formation [8–10]. Through this work, it was found that all of the mutations in the *MAPT* gene are dominant and probably result in a gain of toxic function [11].

Tau pathology in AD appears to be progressive through the propagation of hyperphosphorylated tau through anatomical connections in the brain [12–14]. This chapter will focus on the biochemical characterization of the post-translational modifications that contribute to tau's conformational change and the gain of tau toxic function induced by hyperphosphorylation. At the end of the chapter, we will present a model of the mechanism of action and the potential for therapeutic design based on the new and exciting research in the field.

4.2 Microtubule-associated protein (MAP) tau: Isoforms and normal physiology

The *MAPT* gene is located on chromosome 17q21.1 as a single copy gene and is transcribed into pro-mRNA which undergoes alternative splicing to generate mRNAs that are translated into six different isoforms found in the brain [as reviewed in Ref. 15]. The isoforms of tau differ by the number of C-terminal repeat sequences (3R or 4R) in the microtubule binding domain (MTBD) and N-terminal inserts (0N, 1N, or 2N) (Figure 4.1). The six isoforms were cloned and expressed heterologously [16], and when the recombinant proteins were analyzed in microtubule polymerization reactions, the 4R proteins had a faster rate of polymerization than 3R proteins regardless of the N-terminal repeat composition [16].

In normal adult brains, the 4R and 3R proteins are expressed in a nearly 1:1 ratio, with the N domains being expressed at various levels, i.e. 0N at

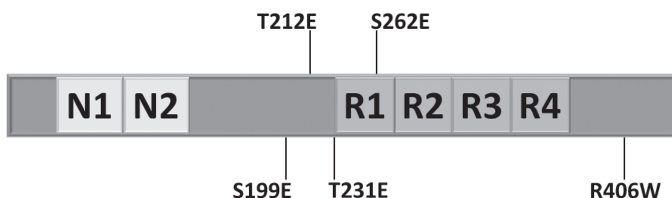

Figure 4.1 Cartoon of Pathological Human tau (PH-Tau). This is the 2N4R form of tau. The mutations indicated are the pseudophosphorylated sites and the FTDP-17 mutation R406W that convert tau into the human pathological form of tau, PH-Tau.

40%, 1N at 50%, and 2N at 10% [17]. In AD brains, all six isoforms appear to be expressed as in normal brains both at the mRNA and protein levels [17, 18]. Similar observations have been made with other tauopathies including Downs Syndrome, amyotrophic lateral sclerosis, Niemann–Pick disease Type C, and some FTDP-17 mutations [17]. Analysis of Pick's disease brain samples indicate that expression of 3R proteins is higher than that of 4R proteins [17]. A deeper look at the mRNA levels indicated that transcription of 3R and 4R occurs at similar levels and that in soluble tau fractions the protein levels appear to be relatively similar, however the sarkosyl-insoluble fractions have much higher levels of 3R proteins compared to 4R [18]. Conversely, tauopathies that appear to express 4R proteins at higher levels than 3R proteins include CBD, PSP, and other FTDP-17 mutations as observed at both the mRNA and protein levels [17, 18].

4.3 Post-translational modifications of tau and the implications in creating a toxic molecule

Tau is an intrinsically disordered protein whose conformation and function are dependent on the post-translational modifications which help to mediate function through protein–protein interactions. This has been found to be a common theme in proteins involved in neurodegenerative diseases besides tauopathies including α-synuclein in Parkinson's disease (PD) and huntingtin in Huntington's disease (HD) [as reviewed in Ref. 19]. The major modifications will be discussed below. Tau has also been shown to be methylated [20], nitrated [21, 22], polyaminated [23–25], glycated [26], and O-glycosylated [27, 28], however these are beyond the scope of this chapter.

4.3.1 *Tau phosphorylation*

Tau is a phosphoprotein that contains a low level of phosphorylation under normal physiological conditions. Phosphorylation occurs at Ser, Thr, and Tyr residues on a protein. The tau protein contains many potential phosphorylation sites as it contains 80 Ser or Thr residues and five Tyr residues. Of these 85 residues, under physiological and/or pathological conditions, approximately 71 of them can be phosphorylated [29, 30]. Many of these sites flank the microtubule-binding domains in the proline-rich region, which is located between residues 181–235 and 396–422. Some of these sites are phosphorylated under normal physiological conditions where tau will contain ~2–3 moles of phosphate per mole of protein [31]. Under certain conditions, increased phosphorylation of tau appears to be necessary for function as tau is hyperphosphorylated in the fetal brain where higher-than-normal levels of tubulin are present during development of the neuronal networks [32–34].

In tauopathies, particularly AD, the level of phosphorylation can increase up to ~12 moles of phosphate per mole of protein as studied in our laboratory [31, 35]. When tau is hyperphosphorylated, the interaction with tubulin is lost, polymerization into microtubules is inhibited, and even more, AD phosphorylated tau (P-tau) appears to gain the toxic function of disrupting preformed microtubules [reviewed in Ref. 36]. The higher levels of phosphate also result in the oligomerization and aggregation of tau leading to the formation of paired helical filaments (PHFs), characteristic of pretangles in the neurons of Alzheimer's patients [37]. If hyperphosphorylated tau becomes dephosphorylated, the interactions with tubulin are restored and an increase in tubulin binding and microtubule growth is observed [38, 39]. This post-translational modification of tau is mainly regulated through kinases and phosphatases.

Four groups of kinases have been found to phosphorylate proteins: proline-dependent protein kinases (PDPKs), non-PDPKs, tyrosine protein kinases, and protein kinases that phosphorylate tau on serine or threonine residues followed or not by a proline [29]. Tau is mainly phosphorylated by PDPKs, including mitogen-activated protein kinases and cyclin-dependent protein kinase 5 (Cdk5), at about 50% of these sites (Ser/Pro, Thr/Pro) [40]. The active kinases in the non-PDPK group include: tau-tubulin kinases 1 and 2, casein kinases 1 and 2, dual-specificity tyrosine-phosphorylated and

regulated kinase 1A, phosphorylase kinase, Rho kinase, protein kinase A, protein kinase B/Akt, protein kinase C, and protein kinase novel [29]. Furthermore, the motifs SXXXS or SXXXD/E and RXRXXS/T are recognized by the protein kinases that phosphorylate tau on serine or threonine residues including glycogen synthase kinase (GSK), 3α and 3β, and AGC kinases (such as mitogen- and stress-activated protein kinase 1) [29].

GSK-3α and GSK-3β play a role in the generation of both NFTs and amyloid β plaques. In their interactions with tau, these kinases can modify several sites on tau which results in the formation of NFTs (as reviewed in Refs. 3, 36). Previous work has shown that GSK-3, *in vitro*, can phosphorylate up to 40 different Ser/Thr residues on tau which can only be compared to casein kinase 1 in the number of potential phosphorylation sites [39]. Furthermore, microtubule assembly promoted by tau is reduced upon phosphorylation by this protein both *in vitro* and in cells [41, 42]. Another kinase, Cdk5, along with its regulator protein p35, is important in brain development and is highly expressed in neurons. When p35 is truncated to p25, the Cdk5/p25 complex can phosphorylate tau in a pattern similar to phosphorylation observed during mitosis as well as mitotic-like tau phosphorylation observed in AD brain [30, 43, 44]. Serine residues in the KXGS motif can be phosphorylated by MAP-microtubule affinity regulating kinase (MARK). These motifs are mostly found in the microtubule binding repeats of tau. Homologs have been found in species ranging from yeast to fruit flies and in all cases are involved in cell-cycle control, cellular polarization, neuronal migration, differentiation, and cell signaling [45]. Since MARK phosphorylates Ser residues in the MTBDs, tau loses affinity for binding to microtubules resulting in the formation of tau aggregates [46]. In flies, when dMARK is overexpressed, tau becomes phosphorylated at Ser262/356 and tau toxicity increased [47, 48]. Taken together, this work shows that the kinases described above can significantly affect the phosphorylation of tau, thereby modulating its function in neuronal cells [49–52].

Protein phosphatases (PP) can be found associated, either directly or indirectly, with microtubules to counteract the actions of the kinases and can control the phosphorylation state of tau [53]. PP-1, PP-2A, and PP-2B are phosphatases that have been shown *in vitro* to dephosphorylate tau [54]. Another phosphatase, PP5, has been shown to dephosphorylate

tau, at similar sites as PP-2A, in PC12 cells when overexpressed [55]. The phosphatase that regulates GSK-3's kinase activity by binding to tau is PP-2A and is, therefore, considered a major tau phosphatase [56–61]. This protein accounts for 70% of tau phosphatase activity in the human brain [55]. Reduction of PP-2A has been observed in brains of patients with AD [55]. In mice, the loss of phosphatase and tensin homolog (PTEN) in the cerebellar neurons resulted in neurodegeneration which was associated with activation of Cdk5 and pERK1/2 and hyperphosphorylation of tau and neurofilaments implicating the PTEN/pAkt pathway in the mediation of neurodegeneration [62].

The tau protein contains five Tyr residues at positions 18, 29,197, 310, and 394 (based on 2N4R tau). Of these residues, only Tyr394 has been shown to be normally phosphorylated, whereas Tyr18, 197, and 394 have been observed to be phosphorylated in the brains of patients with AD. PHF samples taken from post-mortem brains had tau protein that was phosphorylated at residues 18 and 394 [63]. Tyrosine kinases Abl and Arg have been shown to phosphorylate Tyr394 by two independent mechanisms [63]. Interestingly, Arg has been shown to play a role in the oxidative stress response and during neuronal development, two key time points for tau function. Another Tyrosine kinase, Fyn, was expressed at higher levels in a transgenic mouse model of AD and Tyr18-phosphorylated tau was found in NFTs [56, 64, 65]. From this data, it appears that Fyn, c-Abl, and Arg are critical kinases in the neurodegenerative process.

4.3.2 *Acetylation*

Acetylation at lysine residues can be a regulatory post-translational modification of protein function. *In vitro*, acetylation of a recombinant tau protein using acetyltransferase p300 resulted in the acetylation of 23 Lys residues as determined by mass spectrometry analysis [66]. Using a polyclonal antibody generated against a tau peptide acetylated at positions 163, 174, and 180, the first evidence of acetylated tau (ac-tau) was observed in PS19 tau transgenic mice [66]. This same study showed that patients in Braak stages I–IV had higher levels of acetylated tau than those in later stages. In AD and other tauopathies, tau that has been acetylated

at residues Lys174, Lys274, and Lys280 has been observed in tau inclusions [67–70]. Furthermore, the acetylation at Lys280 (ac-Lys280) appears to impair the tau-mediated microtubule stabilization, thereby enhancing tau aggregation [67]. Interestingly, it appears that ac-Lys280 is not present in Pick's disease, and ac-Lys274 is not present in argyrophilic grain disease indicating different acetylation patterns among the tauopathies [67, 69]. The role of ac-tau remains unclear at this point, but the levels of ac-tau appear to correlate to the amount of phosphorylation and ubiquitination of tau.

4.3.3 *Ubiquitination and protein degradation*

Ubiquitin was observed in AD brains in most of the pathologies including NFTs, dystophic neurites, and neuropil threads, but unlike phosphorylated tau, ubiquitin was not found in pretangle neurons indicating a role downstream of phosphorylation [71, 72]. PHF-tau appears to have increased levels of ubiquitination when compared to AD tau and AD P-tau [31]. Three sites of ubiquitination were observed in PHF-tau extracted from human brain by mass spectroscopy located in the MTBD at residues Lys254, Lys311, and Lys353 [73]. Polyubiquitination at these residues appears to be through linkages at Lys6, Lys11, and Lys48 of the ubiquitin moiety, with the majority of linkages being at Lys48. This indicates a pathway toward proteasomal degradation. Other groups have shown that polyubiquitinated tau has linkages at both Lys48 and Lys63 which could indicate that differential processing of the protein as Lys63 has been shown to be involved with cellular signaling, DNA repair, and/or autophagic degradation [74, 75]. The E3 ligase carboxyl terminus of the Hsc-70-interacting protein (CHIP) has been shown to be the enzyme responsible for tau ubiquitination in conjunction with complex comprised of heat shock protein 70 (Hsp70) and other heat shock proteins [74, 76, 77]. The ubiquitination reaction requires the full-length CHIP protein and the MTBD of tau [77]. CHIP was found to co-localize with neuronal inclusions from multiple tauopathies including AD, Pick's disease, PSP, and CBD [74]. Though there is debate about the tau target protein structure (i.e. 3R *versus* 4R, wild-type tau *versus* phosphorylated tau), there appears to be no debate about CHIP as the ubiquitin ligase.

Modification of tau by a small ubiquitin-like modifier appears to be in direct competition with ubiquitin modification at Lys340 [78]. This modifying protein regulates many cellular processes and in some cases, modulates this regulation by blocking ubiquitination [79].

The inclusions in AD are comprised of insoluble protein aggregates that appear to be linked to deficiencies in the proteasomal and/or autophagic pathways. These proteins, including tau, are tagged with modifications (mainly ubiquitin) that would implicate these pathways as a normal means of protein clearance that if inhibited in some way could result in the buildup of these aggregates. The proteins, when not too large, can be degraded through the proteasome, but as the aggregates increase in size, autophagy becomes the major source of protein degradation [75]. The degradation pathways are decided through chaperone proteins including Hsp90, Hsp70, FKBP51, p62, and CHIP [75, 80, 81]. PHF tau found in NFTs has been shown to impair proteasomal activity through direct association [82, 83]. These results strongly implicate the role of proteasomal degradation in neurodegenerative disorders including tauopathies.

4.3.4 *Proteolysis of tau*

Fragmentation of tau is observed in brains of patients suffering from tauopathies including AD and Pick's disease as well as in cellular and mouse models. These fragments appear to be generated by a variety of proteases including aminopeptidases, thrombins, caspases, and calpains [as reviewed in Ref. 84]. The majority of focus has been on calpains and caspases, which can lead to potential aggregate-prone molecules. Cleavage of tau by caspase-3 at Asp421 has been characterized by many groups in AD and Pick's disease [85–89]. The cleavage of tau at Asp421 has been shown to promote the aggregation propensity of the tau molecule [86]. The use of antibodies specific for phosphorylation, conformation, and Asp421 cleavage fragments has allowed the timeline of tau processing to be determined in these tauopathies [87–90]. In general, it appears that phosphorylation of tau is the early event which leads to a change in conformation of tau which is recognized by the Alz50 antibody where the N-terminus of the protein interacts with the MTBD. This conformational change leads to the cleavage at Asp421 by caspase-3 which does not seem

to be affected by phosphorylation at Ser422. Changes in the conformation of tau are then observed by the antibody Tau-66 which recognizes when the proline-rich region of tau interacts with the MTBD followed by cleavage at Glu391, recognized by the antibody MN423, by an unknown protease. This pathway appears to be similar in both AD and Pick's disease indicating that this processing may be similar in other tauopathies.

4.4 Tau: Normal biological function and pathological gain of function

4.4.1 *Microtubules and tau in AD*

A decrease in microtubules is observed in neurons of patients with AD. Further investigation indicated that the concentration of tau is several times increased in neurons of AD brains [31]. There are three different pools of tau in the brains of AD patients: AD tau is most similar to normal tau and is not hyperphosphorylated; AD P-tau is a soluble hyperphosphorylated tau; and PHF-tau is insoluble and hyperphosphorylated. AD tau is decreased by about 60% compared to tau found in normal brain. AD P-tau, as well as normally phosphorylated tau, can be isolated from AD brain in solution [31]. To determine the microtubule-promoting activity of tau from AD brains, we studied their biological activity during *in vitro* assembly of microtubules, with or without previous treatment with alkaline phosphatase [38]. We found that AD tau has normal microtubule-promoting activity; conversely, AD P-tau did not promote microtubule assembly but the activity was recovered upon dephosphorylation. AD P-tau preincubated with normal tau prior to the addition to tubulin inhibited the normal tau-microtubule-promoting activity and destroyed microtubules already present. This was probably due to interactions between tau and AD P-tau, thereby sequestering it from the tubulin.

4.4.2 *AD P-tau has a prion-like behavior*

Using a solid-phase binding assay, we verified that AD P-tau was able to bind normal tau [91]. To better quantitate this binding, we determined the binding in solution. Surprisingly, in solution, the AD P-tau binding to

normal tau was non-saturable, and visualization by electron microscopy showed us that the products were bundles of filaments [91]. These results suggested that hyperphosphorylation of tau could change the conformation of the protein in such a way that the change could be transferred to the normal protein, thereby seeding tau self-assembly. The conformational change transfer by AD P-tau to normal tau is a property of a prion protein. This prion-like activity of AD P-tau was further determined to disrupt the microtubules formed by normal tau or by the other neuronal MAPs, MAP1b, and MAP2 [91, 92]. Amorphous aggregates are formed when AD P-tau binds to MAP1b and MAP2 [92].

4.4.3 *Tau self-assembly and "AD P-tau-like" protein behavior is induced by hyperphosphorylation*

In AD, hyperphosphorylation of tau appears to precede the appearance of the tangles [93]. Tau is a phosphoprotein that normally contains ~3 moles of phosphate per mole of protein, however its hyperphosphorylated form may contain ~7–10 moles of phosphate per mole of protein [31] which results from the appearance of new phosphorylated sites. Degenerating neurons appear to have tau that has self-assembled into tangles of PHFs and straight filaments (SFs). AD P-tau was able to self-assemble into tangles of PHFs mixed with SFs at varying pHs [37]. The PHFs generated had similar dimensions to those of AD PHFs: a wide part of ~20 nm, which narrowed to ~10 nm at every ~80 nm. Within the bundles of PHFs, some 4-nm protofilaments and SFs of ~15 nm, similar to the SFs in AD, were also observed. Dephosphorylation of the AD P-tau resulted in a protein that was unable to self-assemble [38] suggesting that hyperphosphorylation of tau is a requirement for its self-assembly into tangles of PHFs/SFs.

To confirm the role of hyperphosphorylation in the conversion of normal tau into a toxic molecule that has aggregation propensities, the six isoforms of recombinant tau (r-tau) were individually treated with protein kinases present in normal brain extract and followed its ability to bind normal tau and to inhibit its microtubule-promoting activity [37, 94]. Rat brain extract-treated r-tau became hyperphosphorylated with the increase to ~12 moles of phosphate per mole of the protein (phosphorylated tau, P-tau) which is similar to AD P-tau. P-tau also bound to normal tau and

was able to self-assemble into tangles of PHFs/SFs in a phosphorylation-dependent manner and inhibited the microtubule assembly activity [37]. These results suggested that hyperphosphorylation could convert tau into an AD P-tau-like state.

Several reports have shown that FTDP-17 mutations decrease tau's ability to promote tubulin assembly into microtubules [95] or increase the ability of tau to self-assemble [96]. We proposed that these mutations may change the conformation of tau making it a better substrate for phosphorylation [97]. Phosphorylation assays using r-tau with FTDP-17 mutations R406W, P301L, V337M, or G272V resulted in faster rate and greater phosphorylation extent (~16–18 moles *versus* ~12 moles of phosphate per mole protein) than normal tau *in vitro* [98]. This increase in phosphorylation probably correlates to an increased number of sites that become modified based on the higher phosphorylation stoichiometry. We also found that fewer moles of phosphate per mole of protein were required for filament formation in the mutant proteins.

4.4.4 *Pseudophosphorylation of tau as a means to study toxic gain of function*

The term hyperphosphorylation has been the topic of much discussion over the last few years. There seems to be confusion as to whether the toxic effect is because of a general increase in the amount of phosphate per molecule or because of increased phosphorylation at specific sites within a molecule. Pseudophosphorylation is a method used to mimic the negative charge of the phosphate group and the length of the carbon side chain by replacing the codons for Ser or Thr residues with that for Glu in the *MAPT* gene. This is a widely accepted approach to mimic phosphorylation [99–104]. A mouse model was developed to study hyperphosphorylated tau using a tau protein with 10 pseudophosphorylation sites [105]. This mouse did not appear to have any of the hallmark traits of dementia-related neurodegeneration, indicating that it is more likely phosphorylation at specific sites than overall phosphate per molecule. This understanding lead to a closer examination of the protein conformation. The structure of tau, and other intrinsically disordered proteins, may be determined by long-range interactions which can be modulated by phosphorylation and other

post-translational modifications [106]. Intermolecular association has been linked to interactions through the MTBD while self-assembly appears to be inhibited by the flanking regions of this domain [98, 99, 107] (Figure 4.2). As a disordered protein, tau has little defined secondary

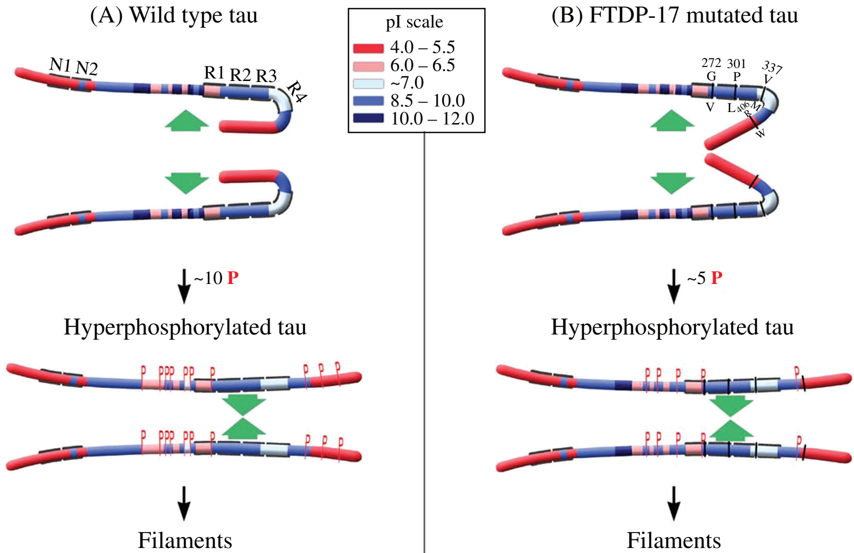

Figure 4.2 A hypothetical scheme of the phosphorylation-induced self-assembly of wild-type and FTDP-17 mutated tau proteins. Tau self-assembles mainly through the MTBD/repeat R3 in 3R tau proteins and through R3 and R2 in 4R tau proteins (R2 and R3 have β-structure). Regions of tau molecule, both N-terminal and C-terminal, to the repeats are inhibitory. Hyperphosphorylation of tau neutralizes these basic inhibitory domains, enabling tau–tau interaction. In the case of the C-terminal region beyond Pro397 (398–441), a highly acidic segment masks the repeats. Phosphorylation (*red P*s) of tau at Ser396 and/or 404 opens this segment, allowing tau–tau interaction through the repeats. FTDP-17 mutations make tau a more favorable substrate for phosphorylation than the wild-type tau. The mutated tau proteins achieve the conformation required to self-assemble at a lower level of incorporated phosphate. Although the FTDP-17 mutant tau proteins have conformations that are more prone to polymerize, in the absence of hyperphosphorylation, the highly basic segments and the C-terminus interfere with polymerization. Phosphorylation sites are indicated by *red P*s at Ser/Thr positions in tau (left panel): 199, 202, 205, 212, 231, 235, 262, 396, 404, and 422; and in FTDP-17 mutant tau (right panel): 199, 212, 231, 262, and 396, respectively. Reprinted with permission from [36].

structure. There are regions of tau that have strong basic charge (p*I* > 9) that are separated from other segments of tau by Pro residues which can induce a bend in the amino acid chain. These very basic regions that are N-terminal to the MTBDs can mask the intermolecular attraction of the MTBD. Three residues in this region, Thr212, Thr231, and Ser262, appear to be 50% phosphorylated when tau begins to polymerize [98], thus decreasing their theoretical p*I* and increasing the probability of tau self-assembly. On the C-terminal side of the MTBD, there is a basic region up to Pro397 that is followed by an acidic segment. Phosphorylation at Ser396 and/or Ser404 may open up this segment and increase intermolecular interactions and increasing tau self-assembly. Using this information, phosphomimetics were studied in the presence and absence of mutations related to FTDP-17, since it was shown to increase the phosphorylation effect. To determine which residues to change to Glu, r-tauR406W was hyperphosphorylated *in vitro* and the levels of phosphorylation at the time tau self-assembled were determined to be about 5 moles of phosphate incorporated per mole of protein by about 2 h of incubation. Upon analysis, nine sites were found to be phosphorylated about 50% at the time of self-assembly: Ser199, Ser202, Ser205, Thr212, Thr231, Ser235, Ser262, Ser396, and Ser404. From these results, we generated vectors in which the tau gene was mutated at each site to Ala (non-phosphorylatable) or Glu (pseudophosphorylated) in the normal tau or R406W background.

Upon transfection into PC12 cells, the vectors containing Ala mutations acted similarly to non-mutated r-tau at each of the sites tested. Mutations to Glu, in most cases, resulted in tau dissociation form tubulin, but complete microtubule disruption was not observed [102]. This indicated to us that a single phosphorylation event was not enough to convert tau into an AD P-tau-like toxic molecule.

After multiple combinations containing two or three pseudophosphorylation sites, it was determined that the strongest effect was observed with the triple-mutant r-tauT212E/S235E/S262E which bound weakly to microtubules in CHO cells and decreased tubulin staining. This pseudophosphorylated tau appeared to be aggregated in both the cytoplasm and nuclear space and was able to sequester normal tau in a manner similar to that of tau isolated from AD brain [102]. When compared to wild-type tau, we found that Ser199 in the pseudophosphorylated tau was very

highly phosphorylated. This suggests that phosphorylation at these four sites is able to convert tau into a toxic species which was enhanced by the FTDP-17 mutation R406W. We decided to use the phosphomimetic of tau at these four sites with the R406W mutation and we named it Pathological Human tau (PH-Tau).

4.4.5 *Toxic gain of function observed in a tauopathy models*

We generated tau-transgenic flies to study PH-Tau effects *in vivo*. We found in *Drosophila* that PH-Tau expressed in a pan-neuronal fashion has a marked effect on the olfactory learning [3]. We have recently developed and characterized a new mouse model in which PH-Tau is expressed in neuronal cells under the control of the calcium-calmodulin kinase II promoter [108]. This model expressed the protein at two different levels: PH-Tau$_{low}$ (4% of normal tau when the promoter is repressed) and PH-Tau$_{high}$ (14% of normal tau when the promoter is induced). Substantial differences in cognitive abilities, synaptic morphology, and neuronal loss were observed between PH-Tau$_{low}$ and PH-Tau$_{high}$ [108]. Low levels of PH-Tau resulted in cognitive deficits and reduced CA1 synapse number, while high levels of PH-Tau caused neuronal death primarily in CA3 as well as astrocytosis in certain brain regions with no apparent effect on CA1 synapses. Interestingly, PH-Tau had distinct biochemical properties when expressed at low and high levels that could account for the different phenotypes. At low PH-Tau, we observed a high-molecular-weight tau species (~100 kDa) that was significantly reduced when high levels of PH-Tau were induced. Preliminary work in this mouse model indicates disruptions in mitochondrial morphology in the CA1 and CA3 regions of the hippocampus of mice expressing PH-Tau. These changes may be due to mitochondrial dysfunction that has been shown to play an increasing role in AD (see below) [109–116].

4.5 Effects of tau propagation on cellular function and AD pathology

4.5.1 *Tau and mitochondria*

The involvement of mitochondria, particularly mitochondrial dysfunction, in the development of neurodegenerative diseases is increasing

[109–115, 117–119]. Physiological processes, including oxidative phosphorylation (OXPHOS) and Ca^{2+} regulation, are disrupted when mitochondria do not function properly [111]. In AD, it has been shown that both Aβ and various forms of tau are linked to mitochondria and result in deficiencies in OXPHOS and/or increased apoptotic activity [as reviewed in Ref. 118]. Cellular localization and respiratory function of mitochondria are dependent on the balance of mitochondrial dynamics [120] as well as cellular localization which is dependent on axonal transport which may be disrupted by modifications of tau.

Neuronal cells have synapses far away from the nucleus and, therefore, correct cellular distribution of mitochondria is especially significant [121]. In neuronal cultures that overexpress tau, thereby increasing microtubule stability, mitochondrial transport along dendrites was diminished and mitochondria number decreased [122]. As described above, post-translational modifications resulting from increases in oxidative species due to mitochondrial dysfunction may modulate the ability of tau to aggregate into fibrils and NFTs [123]. When brains from an AD mouse model, 3XTg.AD, and AD patients were analyzed, hyperphosphorylated tau coimmunoprecipitated with Drp1, a protein involved in mitochondrial fission [117]. In a different tauopathy mouse model, rTg415 that expresses Tau P301L, hippocampal neurons were studied and the length of the mitochondria was found to be significantly increased, either as a function of increased fusion or decreased fission [124]. This has also been observed in brains of *Drosophila* that express TauR406W and either the overexpression of Drp1 or the inhibition of MARF, an Mfn homolog, resulted in normal sized mitochondria [124]. The elongated mitochondria resulted in increased superoxide production and cell-cycle activation each of which was rescued when the mitochondria size was normalized.

The role of tau in mitochondrial function has been entering the forefront of tau pathological studies. The phosphorylation state of tau has been implicated in the degeneration of neuronal cells [102] and linked to mitochondrial dysfunction [125]. As described above, tau gets ubiquitinated by the E3 ligase CHIP. This may be involved in the cellular distribution of mitochondria in mouse neurons [126, 127]. A mouse model that results in the formation of plaques and NFTs was studied and it was observed that both the plaques and the tangles affected the OXPHOS of

the mitochondria [128]. More recently, reduction of soluble tau expression resulted in proper mitochondrial distribution in the neurons of rTg4510 mice [127]. These data help to support the mitochondrial cascade hypothesis which connects mitochondrial dynamics and hyperphosphorylation of tau and aggregation of Aβ [as reviewed in Ref. 129]. When studying the correlation of mitochondrial health and tau, ATP production was decreased chemically and it was observed that, except in the MTBD, the phosphorylation state of tau was decreased [125].

4.5.2 *Tau in the nucleus*

Although the attention to tau has mainly been focused on its ability to stabilize microtubules, now it is evident that tau is a multifunctional protein and besides the stabilization of microtubules there are many more functions of tau that are not clear yet. Many of the studies showed localization of tau in the nucleus. Nuclear tau was identified in both AD brain and normal (control) brain, however it is hard to say whether nuclear tau is generated from a distinct isoform or whether one or more of the six known isoforms can localize in the nucleus. The studies also indicate that nuclear tau can exist in both phosphorylated and dephosphorylated states, which varies depending on tau localization in the nucleus. This localization of tau in the nucleus is very important as the nucleus is involved in many cellular processes. We have shown that PH-Tau translocated into the nucleus when expressed in cells [102] suggesting that the conformational change that makes tau toxic can also alter its subcellular distribution.

In 1975, Bryan *et al.* [130] showed that RNA could inhibit microtubule assembly *in vitro*, through the reduction of the activity of tau. Furthermore, brain-depolymerized MAPs bind the DNA with high affinity and DNA can inhibit microtubule assembly in a concentration-dependent manner [131] indicating that MAPs have more affinity to the DNA than to the microtubules [131, 132]. Further studies indicated that when tau gets phosphorylated, it dissociates from the DNA. A more recent study to further characterize the nature of this interaction revealed strongly reduced or loss of capability of tau phosphorylated by GSK-3 for binding and protecting the DNA against thermal denaturation [133]. Much work has been done to show that tau binds DNA in the nucleus and protects it from

damage by oxidative stress and heat shock [134–136]. This protection appears to be due to the binding of dephosphorylated tau [135] to the minor groove of the double-stranded DNA [134]. Taken together, these studies indicate that tau hyperphosphorylation might affect its ability to both stabilize microtubules and protect DNA.

Several reports indicate that tau may be involved in the organization of nucleolar organizer region (NOR) as well as protection of the DNA [137, 138]. The nucleolar tau for the most of the cases is dephosphorylated [139, 140]. It appears that a fraction of nuclear tau co-localizes with the pericentromeric heterochromatin (PCH) in a manner that indicates tau plays a role in the organization of the NOR and may mediate transcription of ribosomal RNA genes [137]. In knockout tau neurons, the expression of non-protein coding RNAs was affected by disrupted clustering of tau, and other proteins, to the PCH region [138]. Interestingly, in AD brains, a significant decrease has been observed in nuclear tau which is associated with changes in ribosome biogenesis regulating proteins, such as upstream binding factor, nucleolin, and nucleophosmin [141]. Therefore, changes in conformation and/or phosphorylation state of tau can affect nuclear localization, DNA binding, and heterochromatin organization and can thereby affect the expression of many proteins during pathogenesis.

4.6 Conclusions

Taking into consideration what we have learnt so far, modifications of tau can modulate different events at the cellular levels with important consequences for its physiology. Though it appears to be very clear that tau hyperphosphorylation is an early event in the process of neurodegeneration, many different modifications of tau modulate its phosphorylation and conformation. Therefore, they could also be influencing the pathway to the pathological state. We have observed tau translocation into the cell nucleus [102] and different effects on neuronal physiology based on the level of PH-Tau expression [108]. We could picture different scenarios where the levels of hyperphosphorylated tau start appearing in the cell because of kinase overactivity, phosphatase deficiency, failure in the clearance system, or a combination of them. At the beginning of the diseases, the conformationally modified tau might move in the cell, translocating in

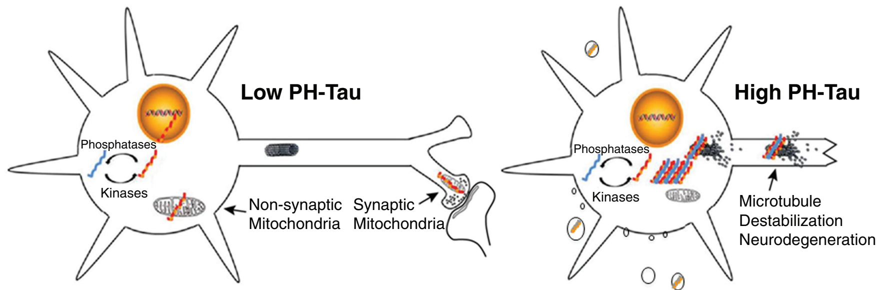

Figure 4.3 Proposed mechanisms of neurodegeneration. (Left) Low level of PH-Tau expression results in translocation to the nucleus, synaptic dysfunction, and mitochondrial disruption. The presence of tau in the nucleus might be involved in alterations of protein expression. (Right) High levels of PH-Tau expression results in protein aggregation, microtubule disruption, and loss of synapses. As a result of cell death or cell-altered metabolism, tau can be released from the cells, and it is possible that the released conformationally altered tau molecule can propagate the disease to neighboring cells.

the nucleus, locating in synapses, and interfering with mitochondria homeostasis (Figure 4.3, left). As a consequence, cognitive impairment without significant structural changes might be observed [108].

The presence of hyperphosphorylated tau in the nucleus can alter the interaction with DNA [142] and may influence protein expression, in turn affecting cellular function. Our preliminary studies suggest that tau can be involved in the regulation of mRNA stability hence altering the protein expression patterns. As the pathological tau increases in the neurons, it leads to toxic effect on the cytoskeleton and retrograde neurodegeneration (Figure 4.3, right). It is known that hyperphosphorylated tau, especially when it has other mutations, causes not only a destabilization of the microtubules, but also the actin microfilaments [143]. Zeiosis of the cell membrane is expected after the disruption of the microfilaments, as they are a major factor in membrane stability. As the membrane pinches off during exocytosis, there is the release of hyperphosphorylated tau-containing membrane vesicles throughout the surrounding cellular environment which has been shown in cell culture models. We propose that these vesicles drift towards and interact with surrounding cells and the contents are taken up by endocytosis. It has been shown that hyperphosphorylated tau sequesters

healthy tau protein and causes healthy tau to become pathological [91]. Despite these different mechanisms, it appears that reducing the levels of hyperphosphorylated tau remains a key target for tauopathies, in combination with therapies to prevent cytoskeleton disruption [40]. These novel steps in the process of neurodegeneration need to be unraveled to point to new targets. Transmission of toxicity through tau might be addressed with immunotherapy.

Taken together, these findings reviewed here show that hyperphosphorylated tau acts as a prion-like protein, spreading pathology to surrounding cells. As the pathological protein moves from cell to cell, it sequesters healthy tau, which causes a disruption of all cytoskeleton components, destabilization of the organelles, disruption of protein synthesis and eventually zeiosis, continuing disease transmission.

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Chapter 5

The Biology and Pathobiology of α-Synuclein

Joel C. Watts, Anurag Tandon, and Paul E. Fraser

5.1 Introduction

α-Synuclein (α-syn) is highly expressed in nervous tissue and linked to the pathophysiology of synucleinopathies such as Parkinson's disease (PD) [for reviews, see Refs. 1 and 2]. It is a member of a family of proteins that include the homologous β- and γ-synucleins which are also present in the central and peripheral nervous systems. The initial link of α-syn to disease was established by its isolation from insoluble aggregates from Alzheimer disease (AD) tissue. This led to the term non-amyloid component (NAC) which corresponds to the central domain of α-syn [3]. However, the identification of a missense α-syn A53T mutation indicated a primary association with autosomal dominant inheritance of PD. This connection to PD was further supported by the discovery of additional mutants (E46K, H50Q, G51D, and A53E) as well as α-syn locus triplication leading to disease-causing increases in protein expression [4]. A number of other PD genetic factors have been identified such as the ubiquitin ligase Parkin, PTEN-induced kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2), and protein deglycase DJ-1 [5]. Binding of α-syn to Parkin and their convergence with PINK1 to impact mitochondrial dynamics suggest interconnected pathways of these genetic components in the etiology of PD [6].

After AD, PD is the most common neurodegenerative disease and is characterized by extensive loss of dopaminergic neurons in the substantia

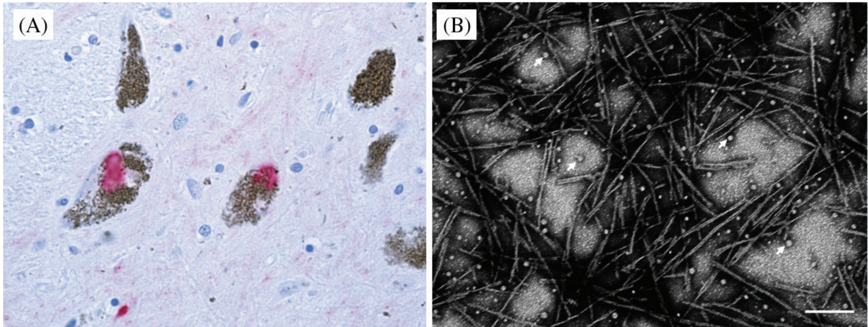

Figure 5.1 PD pathology and α-syn fibril formation. (A) Lewy body pathology (pink) within the substantia nigra indicating the accumulation of α-syn fibrillar deposits in the neuronal cell body. The pathology image is courtesy of Dr. Naomi Visanji (University Health Network, Toronto). (B) Negative-stain transmission electron microscopy (TEM) of polymerized recombinant α-syn indicating the typical fibrillar structures and small oligomeric aggregates (arrows). Scale bar in (B): 250 nm.

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nigra pars compacta leading to the clinical manifestations of this movement disorder [7]. The main pathological features are intraneuronal Lewy bodies (Figure 5.1A) and Lewy neurites which are aggregated protein deposits primarily composed of α-syn [8, 9]. Similar insoluble α-syn aggregates are found in astrocytes in cases of multiple system atrophy (MSA). There has been considerable interest in determining the factors involved in α-syn aggregation and its relationship to PD neurodegeneration. A major focus of these investigations has been the examination of α-syn native structure and the conformational changes involved in misfolding and oligomer formation. More recently, it has been shown that α-syn aggregates can undergo cell-to-cell transfer to propagate Lewy body pathology in a prion-like manner [10, 11]. This raises the possibility that α-syn aggregates spread from initial foci to other vulnerable neuronal types and even to peripheral enteric neurons which has broad implications for therapeutic interventions.

Early investigations into α-syn revealed its localization to presynaptic compartments suggesting a potential involvement in neurotransmission-related pathways [12]. Its membrane binding properties indicate a potential role in synaptic vesicle trafficking, recycling, and/or fusion events related to exocytosis and neurotransmitter release. The initial discovery of

α-syn from purified synaptic vesicles is consistent with a role in synaptic activity [13]. However, the exact physiological function of α-syn remains under scrutiny. This overview will examine, in more detail, the features of α-syn structure, function, and its contributions to pathological disease pathways.

5.2 Structure, misfolding, and aggregation

α-Syn is classified as a natively unfolded protein due to the largely random conformation of the isolated protein in solution [14]. A number of proteins fall within this classification including the microtubule-associated tau protein which is the primary component of intracellular neurofibrillary tangles found in AD. The sequence of α-syn (140 amino acids) is composed of three major domains: N-terminal region, NAC, and the C-terminal tail (Figure 5.2). These individual domains contribute to the unique structural features of α-syn and its physiological properties.

The N-terminal domain, spanning residues 1–60, contains four repeats of ~11 amino acids which have a conserved KTKEGV consensus sequence. These repeats share similarity with the lipid-binding domains of apolipoproteins and represent the principal α-syn sequence involved in membrane interactions [15, 16]. These repeating motifs are predicted to fold into two amphipathic α-helical conformations that span residues 3–37 and 45–92 [17]. The helical conformation of α-syn is stabilized upon binding to membrane bilayers and displays a preferred binding to

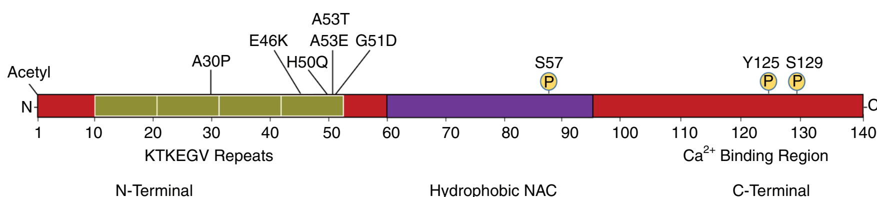

Figure 5.2 A schematic representation of α-syn indicating its three predominant domains (N-terminal, the hydrophobic NAC, and C-terminal). The location of PD-associated mutations within the N-terminal KTKEGV repeats (green rectangles), which constitute the main lipid binding domain, are shown above the protein. Post-translational modifications such as serine (S129, S87) and tyrosine (Y125) phosphorylation and N-terminal acetylation are also indicated.

acidic phospholipids similar to those found in synaptic vesicles [18, 19]. All of the known PD-related α-syn mutations are localized to the helical repeats within the N-terminus indicating that this domain represents a critical functional element.

The central region, residues 61–95, has been termed the NAC due to the original co-isolation of this sequence from protein deposits from AD tissue. This largely hydrophobic domain is the primary driving force for the nucleation of α-syn aggregation and ultimately the assembly into amyloid-like fibrils [3, 20]. A high-resolution structure of an 11-residue peptide from the fibril forming core of the NAC domain has been recently solved using micro-electron diffraction which revealed a well-defined β-sheet organization similar to other amyloidogenic peptides and proteins [21]. These fibrillar aggregates were also found to induce cell toxicity *in vitro*. The C-terminal domain contains a number of proline residues and is enriched in acidic residues that confer a high degree of backbone flexibility and may be an attenuating factor in α-syn aggregation [22]. This has been supported by the observation that deletion of the C-terminal domain results in an increased propensity for α-syn fibril formation [23]. The acidic groups are located within two 16-residue tandem repeats and it has been proposed that this structure is essential for calcium binding [24]. The calcium-binding properties of α-syn are consistent with its proposed involvement in synaptic exocytosis and neurotransmitter release which is a highly calcium-dependent event. Wild-type and mutant α-syn have also been shown to interact with calmodulin in a calcium-dependent fashion [25]. It has been suggested that calcium-mediated control of α-syn function may be facilitated by its association with calmodulin within cells. The C-terminal domain also forms a number of long-range interactions with regions of α-syn such as the observed electrostatic binding to positively charged residues within the N-terminal sequence [26, 27]. Alteration of N-terminal electrostatic characteristics through the introduction of the PD-related E46K mutant results in an increased aggregation propensity suggesting that the dynamic interactions between domains have significant implications for pathological fibril formation [28].

Numerous studies have concluded that purified α-syn forms an intrinsically disordered or natively unfolded structure in solution [29, 30]. This translates into an essentially random structure for the majority of the

protein sequence but the N-terminus does display some tendencies toward an α-helical conformation [31]. The unfolded nature of α-syn has been under debate following the proposed native tetrameric structure of protein isolated from cells under nondenaturing conditions [32]. These findings created a controversy in the field as subsequent studies from several laboratories that isolated α-syn from a variety of sources (brain tissue, erythrocytes, mammalian, and bacterial cells) supported the disordered monomeric structural model [33]. These observations were countered by a series of investigations using cross-linking approaches which indicated that cellular α-syn existed primarily in an oligomeric tertiary structure [34]. More recently, it has been demonstrated that amino acid substitutions within the KTKEGV consensus repeat in the N-terminal domain as well as PD-associated mutants were capable of shifting the equilibrium from tetrameric to monomeric α-syn structures [35, 36]. Additional investigations have proposed the existence of α-helical octamers or possibly higher-order multimers to represent functional α-syn species [37, 38]. These structures may be formed by membrane association and have been linked to α-syn-related SNARE complex assembly. While the debate on the native structure continues, it is not inconceivable that α-syn is capable of assembling into a wide range of conformations depending on the environment as well as associations with potential binding partners and membrane bilayers.

Recombinant α-syn readily adopts fibrillar and oligomeric structures *in vitro* (Figure 5.1B). Oligomeric aggregates of α-syn and many other amyloidogenic proteins have been examined but the exact structural features and makeup of these structures does remain somewhat elusive. Depending on the preparation protocol and particular purification processes used, α-syn has been found to assemble into aggregates such as the ordered tetramers to higher-order polymers containing multiple α-syn polypeptides [39–41]. The pathophysiological relevance of oligomers has been a matter of considerable interest. It has been proposed that oligomers represent the most cytotoxic species of α-syn and represent a critical element in PD-related neuronal dysfunction. This has been supported by the examination of α-syn mutants, E57K and E35K, which stabilize oligomeric aggregates and prevent subsequent fibril formation [42, 43]. The oligomers formed by these mutants have been shown to be highly toxic to

neurons. A comparable relationship of oligomer assemblies and enhanced toxicity has been described for the β-amyloid (Aβ) peptide associated with AD [44]. In this case, Aβ aggregates forming the so-called Aβ-derived diffusible ligands (ADDLs) are a key pathogenic factor in synaptotoxicity and neuronal loss in AD.

The various oligomeric structures are likely to be transient in nature and undergo interconversion between monomeric and aggregated forms. They may also represent intermediates in the fibril formation pathway which is a multistage nucleation-dependent process. The fibrillogenesis pathway is initially characterized by a lag phase where the individual α-syn monomers assemble into aggregated nuclei. This is followed by an elongation phase where coalescence of the aggregates leads to an exponential growth into protofibrils and, ultimately, mature fibrils. The oligomers may correspond to the on-pathway nucleation species as well as off-pathway aggregates that do not culminate in amyloid-like fibrillar assemblies. However, it has been clearly demonstrated that the addition of preformed oligomers or nucleating seeds results in a dramatic reduction of the lag phase and results in more rapid fibril formation. This process is also relevant to the prion-like properties of α-syn as discussed below.

The clustering of PD mutations within the N-terminal domain has a number of consequences for α-syn folding and function. Their localization within the lipid-binding region has a pronounced effect on membrane interactions, with the A53T and H50Q mutations displaying a greater affinity for bilayers [45]. In contrast, the A30P, G51D, and A53E missense mutations result in a decreased propensity for membrane binding [16, 46, 47]. However, the PD-linked mutants do not by themselves have any dramatic effect on the generally unfolded structure of α-syn with the exception of the A30P mutant which exhibits a slightly lower degree of α-helical conformation [31]. The mutations do have effects on the aggregation rates of α-syn with the E46K, H50Q, and A53T mutants having a greater propensity to form fibrils. The mutations also have effects on the morphologies of the fibrils formed such as diameters and overall lengths. However, further investigations continue to explore the mechanisms of action as to how these mutations impact the pathological pathways of PD.

α-Syn undergoes a number of post-translational modifications such as phosphorylation, acetylation, and nitration that have an impact on its

structure and cellular function. Although trace levels of phosphorylated α-syn are detectable in normal brains, virtually all α-syn accumulated within Lewy bodies in PD brains is phosphorylated on serine 129 (S129) [48]. Phosphorylation at serine-87 (S87) has also been demonstrated, and this results in decreased aggregation rates, although the physiological significance of this modification has been debated [49]. Tyrosine phosphorylation of α-syn at residue 125 (Y125) has also been observed in *Drosophila* models as well as in human brain tissue [50]. Y125-phosphorylated α-syn was found to be decreased in PD patients and also appeared to decrease oligomer formation. α-Syn can be phosphorylated *in vitro* by polo-like kinase 2 (PLK2) which targets S129 to generate modified protein for biophysical and biological characterization [51]. Analysis of the purified α-syn indicated that misfolding and fibril formation of phosphorylated isoforms were detected earlier and membrane binding of α-syn monomers was differentially affected by phosphorylation depending on the PD-linked mutation. Binding of wild-type α-syn to presynaptic membranes was not affected by phosphorylation, whereas A30P α-syn binding was greatly increased and A53T was slightly lowered, implicating distal effects of the C-terminal domain on the membrane binding domains. Endocytic vesicle-mediated internalization of preformed fibrils into non-neuronal cells and dopaminergic neurons matched the efficacy of α-syn membrane binding. In addition, the disruption of internalized vesicle membranes was enhanced by phosphorylated α-syn which suggests a potential means for misfolded extracellular or lumenal α-syn to access cytosolic compartments.

Acetylation of the α-syn N-terminal domain has been observed for the endogenous protein isolated under mild conditions. This modification tends to enhance the α-helical conformation of the N-terminal domain and also increase α-syn affinity for membrane bilayers [52]. Analysis of isolated Lewy bodies revealed that α-syn is nitrated at all tyrosine residues [53]. The examination of chemically modified α-syn indicated that nitrated dimers could accelerate fibril formation and decrease membrane binding [54]. S129-phosphorylated α-syn has been shown to exist in both mono- and di-ubiquitinated forms that reflect a potential proteasome-mediated degradation [55]. These ubiquitinated species are also found in Lewy body deposits and are particularly enriched in the periphery of these

inclusions as shown by immunohistochemistry. Finally, α-syn can be enzymatically conjugated to small ubiquitin modifiers (SUMO) on lysine residues located within the C-terminal domain [56]. SUMOylation can also alter protein aggregation and degradation of related proteins such as tau in AD. However, the impact SUMO modification has on α-syn pathology remains under investigation.

5.3 Membrane binding and cellular function

Both α- and β-syn are broadly expressed in brain, with a preferential localization at nerve terminals. In contrast, γ-syn is expressed only sparsely in the central nervous system (CNS) but more prominently in peripheral nerves. Synuclein gene deletions in rodents suggest that synucleins serve an inessential regulatory function, and despite their broadly overlapping expression in brain, loss of one or both α- and γ-syn does not affect presynaptic morphology or function [57–63]. Moreover, even triple synuclein knockout animals display only modest age-related presynaptic deficits [38, 58, 64, 65].

The considerable sequence homology among α, β, and γ synucleins and the overlap in their localization nevertheless implicate some functional redundancy. For example, the amino-terminal repeat sequences (i.e. KTKEGV or similar), which are essential for an amphipathic helical conformation and membrane association, are present in all three synuclein isoforms [66]. Studies in intact neurons and isolated nerve terminals suggest that membrane-bound α-syn is in dynamic equilibrium with a freely diffusible cytosolic pool [67, 68]. Cytosolic α-syn has generally been assumed to be intrinsically unstructured suggesting that α-syn dissociation from membranes requires unfolding of the α-helix which can be reformed upon subsequent attachment to lipids. In this model, where α-syn folding and unfolding is linked to its cycle of membrane binding and dissociation, the conformational changes are unlikely to be energetically favorable in both directions, implying that energy input is necessary for repetitive membrane cycling. One possibility is that α-syn membrane binding is linked to the GTP/GDP cycle of the small GTPase Rab3a and the ATPase function of its GDI/Hsp90 chaperone complex [69].

Because of the main primary sequence differences between the synucleins reside within the C-terminal domain, this region likely governs isoform-specific interactions. For example, neurodegeneration associated with murine CSPα deficiency is rescued by the overexpression of α-syn but not of γ-syn, whose shorter C-terminal sequence lacks the synaptobrevin binding site [70]. The apparent phenotypes of different α-syn mouse models have not resolved a clear indication of α-syn function. Nevertheless, its affinity for high-curvature lipids and high concentration at nerve terminals clearly indicates a role at presynapses, and interactions with various synaptic vesicle proteins such as synaptobrevin II, Rab3a, and synapsin III complement its proposed chaperone function in SNARE complex assembly [37, 38, 69, 71]. Consistent with a role in dopaminergic neurotransmission, multiple reports have linked α-syn to vesicular and plasma membrane monoamine transporters and to tyrosine hydroxylase activity [72, 73].

5.4 α-Syn proteostasis: proteasome, autophagy, and lysosomal pathways

Ubiquitin-proteasome and lysosomal pathways have been implicated in α-syn protein degradation, and studies using inhibitors of these pathways suggest that the lysosome is the preferential route for α-syn oligomers and aggregates [74–78]. Significant cross-talk between these pathways is evident, however, as experimental inhibition upregulates the protein degradation by the other [79]. The delivery of α-syn to the lysosome is mediated by both chaperone-mediated autophagy (CMA), which depends on recognition of CMA-targeting motifs by Hsc70 and co-chaperone complexes and Lamp2, and macroautophagy, which is regulated by multiple autophagy-related gene (Atg) products which mediate a multistep process to envelope cytosolic components and organelles into double-membrane vesicles that merge with lysosomes [80]. Lysosomal inhibition results in the accumulation of high-molecular-weight α-syn species, and the relative contributions of CMA or macroautophagy can vary depending on the cell type and metabolic activity [81]. In addition, accumulation of α-syn oligomers can themselves impede both pathways exacerbating and causing further defects in proteostasis [77, 82].

Several genes associated with the autophagy-lysosomal pathway have also been linked to PD. Mutations in the vacuolar protein sorting-associated protein 35 (Vps35), a subunit of the retromer complex that regulates retrograde trafficking of protein cargo from the endosome to the trans-Golgi network, cause autosomal dominant PD [83]. The resulting defect interferes with Atg9 trafficking and autophagosome formation that impairs α-syn degradation and causes the formation of α-syn aggregates [84, 85]. The PARK9 locus encoding the lysosomal P5-type ATPase, ATP13A2, is linked to autosomal recessive forms of an unusual early-onset Parkinsonism. Loss of function mutations in this gene impair lysosomal function and cause α-syn accumulation. Despite this link, Atp13a2-deficient mice develop age-related motor dysfunction, neuropathological changes, and lysosomal dysfunction, but with little or no corresponding α-syn accumulation [86, 87]. Loss of function mutations in GBA1, which encodes a glucocerebrosidase, cause a lysosomal storage disorder called Gaucher's disease and increase the risk of PD [88]. GBA mutations induce α-syn accumulation and aggregation in cell and animal models [89, 90].

Therapeutic titration of these protein degradation pathways could offer potential clinical benefits by augmenting the removal of pathogenic α-syn and to overcome the age-related decline in the efficiency of the autophagy-lysosomal protein degradation machinery. For example, several approaches that have been tested in cell and animal models to increase the degradative capacity or restore autophagy-lysosomal pathways (i.e. viral-mediated Beclin1, LAMP2, glucocerebrosidase, or ATG7 expression, rapamycin treatment, or induction of lysosomal biogenesis) reduced α-syn pathology and neuronal deficits [81, 90–93]. Although fundamental questions regarding the specificity of the treatment to particular cells and affected pathways remain, these approaches to modify α-syn proteostasis could be used to complement other α-syn-specific therapeutic approaches such as passive immunization or anti-aggregant therapy that are presently in clinical trials.

5.5 Prion-like properties of α-syn

Evidence is accumulating that the pathological α-syn aggregates in PD and related disorders may be non-cell autonomous and instead may be

capable of spreading from cell-to-cell, resulting in a cascade of protein misfolding that is reminiscent of what occurs in prion diseases such as Creutzfeldt–Jakob disease (CJD) [94, 95]. In the prion disorders, the normally α-helical cellular prion protein (PrP^{C}) adopts a misfolded, β-sheet-rich conformation (PrP^{Sc}) that is able to bind to and convert PrP^{C} into additional copies of the disease-associated PrP^{Sc} conformer [96]. The ability of PrP^{Sc} to self-propagate (i.e. catalyze its own formation) underlies the infectious nature of the prion diseases and explains how the pathological protein aggregates are able to spread within the brain.

Studies from Braak *et al*. [97] revealed that α-syn pathology follows a stereotypic progression in the CNS; deposition is first observed in the brainstem followed by the substantia nigra and amygdala, and then in cortical regions at later disease stages. One possible interpretation is that α-syn aggregates, like prions, are spreading from cell-to-cell during PD. Some of the earliest symptoms of PD relate to dysfunction of the enteric nervous system, and α-syn pathology within the gut has been observed in PD patients [98]. A recent study in rats has demonstrated that injection of α-syn into the intestine results in spreading of the protein to the dorsal motor nucleus of the vagus in the brain [99], potentially indicating that PD may originate in the gut and then spread *via* a prion-like mechanism to the brain. In 2008, it was revealed that Lewy bodies were found in fetal dopaminergic tissue that had been grafted into the striatum of PD patients ~11–16 years earlier [100, 101]. Since Lewy pathology would not be expected to occur in tissue of this age, this data has been interpreted as evidence for host-to-graft propagation of misfolded α-syn species.

Recent studies have provided direct evidence that both soluble and aggregated forms of α-syn can be transferred from one cell to another. α-Syn inclusions were found in mouse cortical neural stem cells that were grafted into transgenic (Tg) mice that overexpress α-syn [102], and α-syn species secreted from cells were taken up by neighboring cells in culture [103]. Fibrils generated from recombinant α-syn or brain-derived α-syn aggregates seed the intracellular aggregation of α-syn when applied to cultured cells, including primary neurons [104–106]. Transfer of α-syn between interconnected brain regions has also been observed in mice [107, 108]. A key unresolved issue is deciphering the mechanism by

which α-syn aggregates can spread from cell to cell. Unlike PrP^{Sc}, which exists on the cell surface and can directly contact neighboring neurons, α-syn aggregates are found in the cytoplasm. Thus, there must be a mechanism that permits α-syn aggregates to exit the cell, be taken up by another cell, and then template the aggregation of α-syn in the recipient cell. Candidates for this process include exocytosis/endocytosis [109] and macropinocytosis [110]. Interestingly, several recent reports have identified putative cellular receptors for α-syn fibrils, which may mediate their internalization. These include heparan sulfate proteoglycans [110], the α3 subunit of the neuronal Na^+/K^+-ATPase [111], and the lymphocyte activation gene 3 (LAG3) protein [112]. Confirmation of these proteins as *bona fide* α-syn receptors could lead to new a strategy for blocking cellular α-syn uptake and thus halting the spread of protein misfolding. The existence of extracellular α-syn species also provides hope that antibodies may help mitigate the spread of α-syn pathology in the brain. Indeed, treatment of mice injected with α-syn fibrils with anti-α-syn monoclonal antibodies reduced the induction of α-syn pathology in the brain [113].

Intracerebral injection of synucleinopathy-laden brain extracts from aged, symptomatic M83 Tg mice, which overexpress A53T-mutant human α-syn, into young M83 mice induced cerebral α-syn deposition and accelerated the onset of clinical disease in the recipient animals [114–116]. Inoculation of preformed recombinant α-syn fibrils is also sufficient to initiate disease in M83 mice [115]. This prion-like acceleration of disease also occurs following intramuscular or intraperitoneal injection of pathological α-syn aggregates [117, 118]. Whereas brain extracts from PD patients do not induce disease when inoculated into M83 mice, extracts from MSA patients are highly effective at initiating a progressive synucleinopathy with comparatively short incubation periods [116, 119]. Thus, not all aggregated α-syn species exhibit equivalent prion-like properties when injected into Tg mice. Inoculation of Tg mice expressing wild-type human α-syn with aggregated α-syn induces moderate amounts of α-syn deposition in the brain, but does not generate clinical illness [120]. Similarly, widespread α-syn pathology is induced upon inoculation of nontransgenic mice or rats with preformed fibrils [121, 122] or Lewy body extracts from either PD or demential with Lewy bodies (DLB) patients [123, 124], but no overt clinical signs of a neurological illness are observed.

It is becoming increasing clear that the self-propagating α-syn aggregates observed in the synucleinopathies share many similarities with PrP^{Sc} in the prion diseases (Table 5.1). Both PrP^{Sc} and α-syn aggregates can induce a progressive and ultimately lethal neurodegenerative disorder when injected intracerebrally into susceptible Tg mice [115, 116]. Moreover, like PrP^{Sc}, α-syn aggregates can exist as distinct conformational variants (i.e. strains) with unique pathological properties [39, 125, 126]. However, unlike PrP^{Sc}, it has not yet been demonstrated that α-syn aggregates are capable of transmitting a fatal disease to nontransgenic mice, and there is no evidence of human-to-human transmission of a synucleinopathy [127], which occasionally occurs with prion diseases [128].

There has been considerable debate over what to call the self-propagating α-syn aggregates that are present in the synucleinopathies.

Table 5.1 Prion-like attributes of α-syn aggregates.

Characteristic	PrP	α-Syn	Key Reference(s) for α-Syn
Induction of protein aggregation in Tg mice expressing mutant precursor protein	Yes	Yes	[114–116]
Induction of protein aggregation in Tg mice expressing wild-type precursor protein	Yes	Yes	[120]
Induction of protein aggregation in non-Tg mice	Yes	Yes	[121]
Induction of protein aggregation in cultured cells	Yes	Yes	[104–106]
Induction of a lethal disease in Tg mice expressing mutant precursor protein	Yes	Yes	[114–116]
Induction of a lethal disease in Tg mice expressing wild-type precursor protein	Yes	No	[120]
Induction of a lethal disease in non-Tg mice	Yes	No	[121]
Progressive spreading of protein aggregation	Yes	Yes	[97, 115]
Neuroinvasion following peripheral inoculation	Yes	Yes	[117, 118]
Horizontal or iatrogenic transmission in humans	Yes	No	[127]
Zoonotic transmission	Yes	No	
Serially transmissible	Yes	Yes	[119]
Existence of distinct strains	Yes	Yes	[39, 125]

Some investigators have proposed that they should be referred to as "prions", since they exhibit transmission properties similar to PrP^{Sc} [129]. Others have argued that the term "prion" inappropriately implies that these agents can be transmitted from human to human or animal to human [130]. Other terms that have been used to distinguish self-propagating protein aggregates from authentic prions include prion-like, prionoid, and proteinaceous nucleating particles [131, 132]. Regardless of the nomenclature, there is a broad consensus that α-syn aggregates can become self-propagating during disease and that the cell-to-cell propagation of misfolded α-syn may be responsible for the progressive spreading of protein aggregation observed in the synucleinopathies.

5.6 *In vivo* modeling of synucleinopathies

A number of conventional transgenic mouse models have been generated using human α-syn expression driven by different promoters. α-Syn-overexpressing mice develop features of synucleinopathies (i.e. deposition of aggregated α-syn in the brain) that are accompanied by loss of striatal dopamine and impairments in motor function (for review, see Ref. 133). High levels of α-syn expression also lead to mitochondrial impairments, oxidative stress, and neuronal loss. However, the observed neuronal loss is not overly dramatic, and features of the PD-like phenotype can be observed to varying degrees in different mouse lines. The α-syn pathology can also frequently occur in brain regions, such as the spinal cord, which are not typically associated with the human disease [134, 135]. The occurrence of PD-related pathology in animals expressing human wild-type or PD α-syn mutants (e.g. A53T, A30P) correlates with transgene distribution as well as expression levels and is consistent with a proposed toxic gain of function for α-syn aggregates. In addition, unlike nonmammalian models, the majority of α-syn transgenics express the homologous endogenous murine protein which may also complicate the outcomes and pathologies in these models.

Great strides have also been made in the development of nonmammalian models of PD which have included the *Caenorhabditis elegans* nematode and *Drosophila melanogaster*. Several *C. elegans* models have been generated through the use of promoters specific for dopaminergic neurons as well as muscle [136, 137]. When expressed in body wall muscle cells,

fluorescent-tagged α-syn assembles into readily observable and quantifiable inclusions making these models useful vehicles for assessing different therapeutic approaches and modulators of α-syn activity [137, 138]. Compared to the relatively small number of dopaminergic cells in *C. elegans*, *Drosophila* has a broad network of several hundred dopaminergic neurons which contributes to the more complex behaviors of these insects. α-Syn can be controlled by selective promoters that allow for expression within specific classes of neurons, pan-neuronal expression or in nonneuronal tissues such as the eye which is a common target for *Drosophila* transgenesis [139, 140]. Similar to mouse models, high levels of neuronal expression are associated with neuronal loss and the formation of cytoplasmic inclusions of human α-syn. These models have been useful for identifying modifiers of PD-related neurodegeneration and also for testing various hypotheses related to the pathogenesis of the synucleinopathies [141–144].

The vertebrate and invertebrate modeling of PD and synucleinopathies, in particular, continue to evolve and hold considerable potential for advancing our understanding of the molecular etiology of PD as well as the development of effective therapies.

5.7 Concluding remarks

α-Syn is an interesting and complex molecule from the perspective of its structure, cellular function, and pathobiology of PD and related disorders. Since its discovery in 1988, numerous studies have provided a wealth of information that has significantly advanced our understanding of α-syn. However, many questions still need to be answered as part of ongoing and future investigations. Assembly into oligomers and the fibrils found in Lewy bodies as well as the prion-like seeds involved in cell-to-cell spreading represent key targets in the clinical treatment of α-syn-mediated neurodegeneration.

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Chapter 6

Impact of Loss of Proteostasis on Central Nervous System Disorders

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6.1 Introduction

As organisms encounter many different kinds of stress, several mechanisms that modulate cellular homeodynamics have been developed in order to ensure the correct structure and function of biomolecules in cells. The vast majority of cellular functions are performed by proteins that are organized in large assemblies and largely work as complicated energy-demanding protein machines. Proteome stability is maintained by an elegant compartment-specific system called the proteostasis network (PN). PN under stress conditions is activated to rescue, when feasible, or degrade unfolded, misfolded, and/or damaged polypeptides, and this process is also known as a triage decision (fold, hold, or degrade) [1]. Proteome quality control (PQC) consists of two main arms, namely *arm I* (proteotoxic stress-related cellular responses) which is represented by

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several modules namely the unfolded protein response (UPR) of the endoplasmic reticulum (ER) and the armada of intra- and extracellular chaperones, and *arm II* which includes the main proteolytic systems in the cell, i.e. the ubiquitin proteasome system (UPS) and the autophagy lysosome pathway (ALP).

Loss of proteostasis is associated with many age-related diseases such as neurodegeneration, cancer, and metabolic disorders [2]. In particular, the common trait of neurodegenerative diseases is neuronal death largely due to accumulation of aggregate deposits consisting of abnormal polypeptides. Dysfunction of the PN modules causes (among others) protein misfolding and aggregation, resulting in the disruption of neuronal homeostasis and finally in neurodegenerative diseases, including Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) disease; amyotrophic lateral sclerosis (ALS), as well as other neurological disorders, that are mentioned and discussed below [3]. Deciphering the loss-of proteostasis-related molecular events that underlie the pathology of these devastating diseases will help to better understand the underlying mechanisms and also to develop new therapeutic strategies. Herein, we provide an overview of the role of proteostasis on neurodegeneration.

6.2 Ubiquitin-proteasome system

The UPS is composed of the ubiquitin-conjugating enzymes and the 26S proteasome [4]. UPS plays a central role in cellular homeodynamics, as it degrades both short-lived poly-ubiquitinated normal proteins and non-functional or misfolded polypeptides [4], whereas oxidized proteins are, most likely, degraded by the proteasome in a ubiquitin-independent way [5, 6].

Ubiquitin (Ub) is a well-conserved (mainly in eukaryotes) 76 amino acid protein [7, 8]. It can form different chains that are linked *via* its seven Lys residues, namely Lys (K) 6, 11, 27, 29, 33, 48, and 63, and also *via* the N-terminal methionine (Met) within Ub, which can be fused to the C-terminal di-glycine motif of another Ub. Ub chains are attached to proteins by an adenosine triphosphate (ATP)-dependent manner which is mediated by a series of enzymes [9]. More specifically, Ub is activated at

the expense of ATP and transferred to the active site of a Cys residue of a Ub-activating enzyme (E1). The activated Ub is then further transferred to the active site of a family of Ub-conjugating enzymes named E2s. Finally, the E3 Ub-ligases ligate the carboxyl group of the C-terminal Gly residue of Ub to the e-amino group of an internal Lys in the target protein; degradation of the target protein by 26S proteasome requires (in most cases) poly-ubiquitination at Lys 48 [4, 10].

The 26S proteasome is a complicated protein machine of ~2.5 MDa that consists of the 20S core particle (CP) and of one or two 19S regulatory particles (RPs) that bind to one or both ends of the 20S CP [11]. The 20S CP in eukaryotes consists of 28 α- and β-type subunits divided into four rings [12, 13]. The internal rings are constituted of β-type subunits (β1–7) and the outer rings from α-type subunits (α1–7). The 20S core has a hollow cylindrical α–β–β–α organization and carries the catalytic center with the three peptidase activities [14]. More specifically, the proteasomal proteolytic activities are located in the inner rings where the β1, β2, and β5 subunits display caspase-, trypsin-, and chymotrypsin-like peptidase activity, respectively [15, 16]. The 19S RP (or PA700) contains 20 subunits which are evolutionarily conserved and organized into two subcomplexes, namely the base and the lid [17–23]. The base consists of six AAA-type ATPases (Rpt1–6) and three non-ATPase subunits, namely Rpn1, Rpn2, and Rpn13 [19, 20, 24, 25]. Each ATPase is involved in distinct functions during protein degradation [26, 27], while the two non-ATPase subunits, Rpn1 and Rpn2, have an Arm/HEAT motif forming α-helical solenoids [28, 29]; notably, the Rpn2 subunit can bind directly to the *a*-ring of the 20S CP [30]. The lid contains nine non-ATPase subunits (Rpn3, Rpns5–9, Rpn11, Rpn12, and Rpn15) with unique sequences, structures, and functions [19, 20, 24, 25, 31]. The base and the lid are connected *via* the Rpn10 linker subunit forming thus the 19S RP [19]. The Rpn3, Rpn5–7, Rpn9, and Rpn12 subunits display a C-terminal winged-helix fold that is flanked by a helical segment and it is known as the proteasome-CSN-initiation factor 3 motif, whereas the Rpn8 and Rpn11 subunits have an Mpr1-Pad1 N-terminal (MPN) domain. The Rpn13 and Rpn10 subunits function as poly-ubiquitin receptors [23, 32]. The MPN domain of the Rpn11 subunit exerts a deubiquitinating enzyme

(DUB) activity [23, 33, 34]; deubiquitination of the substrate is also exerted by the enzymes Uch37 and Usp14 [23]. The assembly of 19S RP is less understood as compared to the 20S CP assembly; nevertheless, both require chaperones, self-assembly, and proteolytic maturation of subunits [35–39]. Formation of the 26S proteasome (stabilization of 19S and 20S binding) is not so well understood; however, the Ecm29 factor (a HEAT-repeat protein), along with the Rpn6, Rpt6, Rpn1, and Rpn2 subunits are found to participate in the stabilization of 19S RP and 20S CP [30, 40–42].

UPS is implicated in crucial functions of the nervous system such as the synaptic function and synaptic plasticity, the interneuronal communication, and the main brain functions of memory and learning [43–45]. The dysfunction of UPS has been associated to abnormality of synaptic functions in neurodegenerative diseases including AD, HD, PD, and ALS [43, 44]. The main common characteristic of these disorders is the abnormal deposition of insoluble, mainly ubiquitinated, protein aggregates or inclusion bodies within the neurons [46–48]. The presence of ubiquitinated proteins in almost all the neurodegenerative disorders highlights, albeit indirectly, the impact of UPS (and ALP; see below) in neurodegenerative pathology [49–53].

AD is the most frequently occurring progressive form of dementia and it is characterized by the presence of extracellular amyloid plaques which are (mainly) composed of insoluble fibrillar deposits of amyloid beta (Aβ) aggregates, as well as of intracellular accumulation of Aβ oligomers. AD-related Aβ peptides are generated by the amyloidogenic pathway from the cleavage of amyloid precursor protein (APP) by either α-secretase, the β-site APP-cleaving enzyme (BACE1), or γ-secretase [54]. On the contrary, the peptides that are generated by the cleavage of APP by the non-amyloidogenic pathway are not associated to AD [55]. The function of Aβ peptides under physiological conditions is not well understood; yet, they have been implicated in antioxidant responses [56, 57], metabolic pathways regulation [58–60], and it also seems that Aβ peptides regulate the transcription of AD-associated genes [61, 62]. Aβ oligomers appear to have a more toxic effect compared to Aβ peptides [63, 64]. Specifically, Aβ oligomers have been associated with increased

levels of Ub due to ER stress and reduction of proteasome activity *in vitro* and in mouse brain lysates [65–67]. Moreover, Aβ oligomers activate the Flavin adenine dinucleotide (FAD)-dependent oxidoreductase domain containing 2 (FOXRED2) which promotes ER-mediated neuronal cell death *via* inhibition of proteasome activity [68]. In addition, the Aβ40 and Aβ42 (consisting of 40 or 42 amino acids, respectively) oligomers, which are the most representative Aβ peptides, were found to inhibit proteasome activity in cell-free assays in a dose-dependent manner [69]. Reduced proteasome activity has been described in different brain areas of AD patients; this loss-of-proteasome functionality, however, was not associated with decreased proteasome expression [70]. This intriguing finding could be probably due to the activation of nuclear factor erythroid 2-related factor-2 (Nrf2), as an attempt to restore the loss of proteostasis [71]. Interestingly, enhanced proteasome degradation alleviated the Aβ toxicity in *Caenorhabditis elegans* [72, 73], while it was recently shown that the γ-secretase activating protein (GSAP) is ubiquitinated and then selectively degraded *via* the UPS, providing further evidence for the role of UPS in the regulation of Aβ precursor protein and Aβ formation [74].

Furthermore, along with the regular Ub, the Ub+1, a mutant form of Ub, has been found in brains of AD patients [75, 76]. Interestingly, it was shown that 20S proteasome degraded Aβ40 and Aβ42 peptides *in vitro* [69] and the E3 Ub-protein ligase Parkin accelerated the proteasome-mediated degradation of Aβ peptides [77]. Parkin overexpression ameliorated the phenotype in AD mice, as it improved locomotor and memory performance of AD mice [78]. However, no Aβ deposition was found in the brains of Parkin-mutant patients [77]. The ER-associated degradation (ERAD) Ub ligase Hrd1 has also been correlated to AD [79, 80]. Specifically, Hrd1 promoted APP ubiquitination and degradation, resulting in decreased generation of Aβ [79]. Moreover, the brain-enriched RING finger E3 ligase was found to be increased in AD brains and neuronal cells exposed to injurious insults [81]. Furthermore, the protein expression of Ub C-terminal hydrolase L1 (Uchl1), which belongs to the Uch family of DUBs, was found to be reduced in sporadic AD brains [82] and its overexpression rescued learning and memory deficits in AD model mice [83].

Another hallmark of AD is the neurofibrillary tangles (NFTs) which are primarily composed of hyper-phosphorylated tau protein (p-tau); p-tau can interact with monomeric and oligomeric Aβ increasing the neurotoxicity [84]. The E3 ligase Carboxyl terminus of Hsp70-Interacting Protein (Chip) mediates the ubiquitination of tau (primarily in its phosphorylated form) and its degradation by the UPS in order to prevent its accumulation and formation of NFTs [85, 86].

PD is mainly associated with the abnormal increase in phosphorylated forms of α-synuclein. The aggregates of α-synuclein form the accumulations known as Lewy bodies (also composed of Ub); Lewy bodies induce the progressive degeneration of dopaminergic neurons [87]. It is also known that α-synuclein is degraded by proteasome and that α-synuclein aggregates and the induced mutant α-synuclein impair normal proteasomal functionality [88–90]. Moreover, patients with sporadic or familial forms of PD displayed altered proteasomal function [90]. Correspondingly, proteasome inhibition in mice overexpressing α-synuclein resulted in severe neurodegeneration [91]. Recently, it was shown that the neurons derived from PD-induced pluripotent stem cells (iPSCs) (PD-iPSCs were generated by a sporadic early-onset PD patient) exhibited abnormal α-synuclein accumulation and downregulation of the proteasome and of the antioxidant pathways [92]. Notably, another study showed that proteasome activity was not decreased in brain regions with Lewy body pathology [93]. Furthermore, mutations of Parkin which ubiquitinates the α-synuclein and its post-translational modifications were associated with PD [94–98]. In the *Drosophila* model, Parkin loss of function exhibited muscle and neuron degenerations which are highly reminiscent of PD [99]. Familial forms of PD are also characterized by genetic mutations in Uchl1 [100–102]. Cerebrospinal fluid levels of Uchl1 in PD patients were significantly decreased compared to healthy controls [103], while except for the UPS-related proteins, the tau protein is also thought to contribute to PD, but the mechanism is still unclear. Finally, it has been proposed that α-synuclein fibrils interact with tau, inhibit its function to stabilize microtubules, and promote tau aggregation leading to dysfunction of neuronal cells [104].

HD is characterized by the accumulation of insoluble aggregates or inclusions of polyglutamine (polyQ) expanded mutant huntingtin (*HTT*)

protein in the brain [105, 106], as well as by Ub accumulation [10]. Although UPS activity in the whole-cell homogenates of HD mice brains was not reduced [107–109], impaired UPS activity in the synapses of HD mice was detected [109]. In addition, the natural compound quercetin, probably through proteasome activation, protected against mutant *HTT*-mediated cell death at low doses [110]; in support, proteasome activation ameliorated the toxic effects of HD in *C. elegans* [72]. The Ub-related enzymes have also been involved in HD pathology [53, 111–115]. Specifically, it was shown that the upregulation of the DUB Ub-specific protease-14 (Usp14) [112] or the overexpression of K48-specific E3 ligase, UBE3a [111], reduced cellular *HTT* aggregates enhancing their degradation *via* the UPS. In addition, ubiquilin-1 overexpression delayed the *HTT* aggregates accumulation in the R6/2 mouse model of HD [113]. The Ub-activating enzyme E1 (UBE1) has been involved in the differential accumulation of mutant *HTT* in the brain and in peripheral tissues [114]. Interestingly, the overexpression of E3 Ub ligase WWP1 or of the E2 Ub-conjugating enzyme UBE2N has been correlated to mutant *HTT* aggregation [53, 115], making them attractive targets for HD therapy. Recently, increased tau oligomers levels (as compared to healthy subjects) were detected in HD brains [116, 117] and a possible interaction between tau and *HTT* was suggested [117]; however, these data are contradictory to studies from Fernandez-Nogales *et al*. [118] who reported no interaction between *HTT* and tau in HD.

ALS is a fatal neurological disorder that is characterized by the loss of motor neurons in the brain and spinal cord. The motor neuron degeneration mainly results by the formation of Ub-positive nuclear inclusions composed of superoxide dismutase 1 (Sod1) and transactive response DNA binding protein 43 kDa (TDP-43) or FUS [119, 120]. Similar to the aforementioned CNS disorders, several components of UPS are reportedly impaired in ALS [120–122]. Knockout of the regulatory proteasome subunit Rpt3 promoted the accumulation of FUS and TDP-43 in motor neurons [51]. Interestingly, strong immunoreaction of proteasome in ALS neurons was found, providing further evidence for UPS involvement in ALS pathogenesis [123]. In addition, ALS-related mutations in the Ub-like protein ubiquilin-2 decreased the rate of proteasome degradation

resulting in the accumulation of poly-ubiquitinated proteins, including the ALS-associated protein TDP-43 [124, 125].

In summary, in most of the aforementioned studies, UPS functionality declines in neuron diseases. Thus, it is anticipated that either genetic or pharmacologic mild activation of UPS activity that could maintain a non-pathological proteome in these post-mitotic cells will likely result in slowing down, or even abolishing, the development of CNS disorders during aging.

6.3 Autophagy-lysosome pathway

Autophagy (or autophagocytosis) is a highly conserved catabolic pathway that degrades substrates in lysosome, and along with UPS (which is also a highly catabolic process), comprise the most important key players in PQC. In contrast to the UPS that targets mostly short-lived proteins, autophagy degrades small cytoplasmic portions, long-lived proteins, as well as entire cellular organelles, such as ER, mitochondria, peroxisomes, nuclei, ribosomes, and even cellular invaders such as bacteria or viruses (xenophagy) [126–129]. The autophagic turnover of intracellular constituents is crucial for cellular proteostasis, nutrient sufficiency, and various aspects of development in eukaryotes. There are three different types of autophagy that have been so far described, all of which deliver a variety of cytoplasmic components to the lysosome for degradation: (a) microautophagy, (b) macroautophagy, and (c) chaperone-mediated autophagy (CMA). Microautophagy is characterized by the direct lysosomal engulfment of substrates, which occurs by invagination of lysosomal membrane; subsequently, the cargo is degraded *via* acidic lysosomal proteases. On the other hand, macroautophagy (the main autophagic pathway) involves the formation of a double membrane, known as autophagosome that engulfs cytoplasmic proteins and organelles; the autophagosomes are then targeted to lysosomes where they fuse [130]. Studies in yeast have identified more than 30 autophagy-related proteins (Atgs), many of which are evolutionarily conserved in mammals [131, 132]. Briefly, the three main steps in autophagosome formation are the *initiation* phase, the *nucleation*, and the *expansion* of the isolation membrane. The process initiates at the

phagophore assembly site (PAS) where proteins of the UNC51-like kinase (Ulk) complex [Ulk1 or Ulk2, Atg13, FAK family kinase interacting protein of 200 kDa (FIP200), and Atg101] participate to autophagosome formation [133]. During the nucleation stage, the Ulk complex recruits class III PI3K complex [consisting of BECLIN 1 (Becn1, Atg6 in yeast), vacuolar protein sorting 15 (VPS15), VPS34, and Atg14] [134] leading to a mass production of phosphatidyl inositol 3-phosphate. Finally, at the expansion stage, the Atg12–Atg5, Atg16 (Atg16L is its functional counterpart in mammals) complex is activated and it facilitates the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3 or LC3, mammalian homolog of yeast Atg8) with phosphatidyl ethanolamine. Autophagy could be triggered by either mTOR-dependent or -independent pathways. The third type of autophagy, CMA, involves the recognition of a pentapeptide motif (KFERQ) by the cytosolic chaperone heat shock cognate 70 kDa protein (Hsc70). Hsc70 guides the target proteins to lysosome for degradation *via* a complex containing the lysosomal-associated membrane protein 2A (Lamp2A) receptor and Hsp90 or lys-Hsc70 (a luminal form of Hsc70) [135, 136]. When macroautophagy is inhibited, either genetically or pharmacologically, CMA is upregulated [137]. Generally, autophagosomal impairment has been implicated in several diseases, such as cancer, bacterial, or viral infections, while recently several studies have focused on the role of autophagy in healthy neurons, as well as in neurodegenerative disorders. Evidence suggests that under normal conditions, neuronal cells maintain low levels of autophagosomes or decreased rate of autophagosome biosynthesis, even during short period of limited availability of nutrients. Notably, during aging, protein degradation, including autophagy, is reduced even more [138–140]. Dysfunctional autophagy has been described in the pathology of numerous CNS diseases, including not only chronic disorders (such as proteopathies), but also acute injuries. Under these pathological conditions, autophagosomal degradation could be either deteriorated or overactivated leading to organelle disorganization and accumulation of autophagosomes [141–143]. Autophagy seems to have a very important role in the degradative pathway of aggregates and specifically in the degradation of poly-ubiquitinated (polyU) proteins. In particular, studies have shown that the SQSTM1

protein (Sequestosome 1 or Ub-binding protein p62) co-localizes with LC3 and becomes sequestered in autophagosomes. This underlines the significance of autophagy as a main degradation pathway for polyU aggregates [144]. A number of additional findings, in the field of neuro-degenerative pathology, implicate autophagy in AD progression [145]. Specifically, Becn1 mRNA was found to be decreased in brain tissue of AD patients, and Becn1 protein expression was reduced (as compared with age-matched controls) in the brain cortex of AD patients [146]. Nonetheless, EM images from AD neurons and AD brains showed an increased number of autophagosomes and MAP1LC3-I/MAP1LC3-II levels [147]. Probably, low levels of Becn1 cause a deregulation of endo-somal–lysosomal degradation over time and result in a progressive increase in intracellular vesicular compartments.

Several pharmacological studies, using autophagy inducers or inhib-itors, have been performed to better understand the involvement of autophagy in the degradation of protein aggregates. Interestingly, in various experimental models (e.g. fly, mouse, or human cell lines) of HD, the specific mTOR inhibitor rapamycin (also an inducer of autophagy) leads to reduction of polyQ/polyA (polyglutamine/polyala-nine expansions) aggregates [148]. Thus, autophagy is activated, in order to degrade and reduce protein aggregates, suggesting a protective role of this proteolytic pathway [149]. Similar results were obtained in cell lines for α-synuclein [150], as well as for wild-type tau in the fly model [151]. These studies suggest that autophagy could be used as a potent anti-neurodegenerative target by facilitating the clearance of aggregated proteins [152, 153]. Although the pharmacological studies have helped to broaden the knowledge about the role of autophagy in neurodegeneration, most of them are focused on upstream pathways that regulate autophagy, such as the mTOR/AKT pathway, and not directly to the process itself.

In order to specifically target autophagy and in parallel minimize side effects, genetic methods have been particularly useful. More specifically, siRNA knockdown of LC3 in cell-culture HD models or RNAi-mediated deletion of Atg proteins such as bec-1, Atgr-7, and Ce-Atg18 in *C. elegans* increased the accumulation of polyQ aggregates, confirming the data of pharmacological studies [154]. Another key molecule in autophagy

process is the cytoplasmic histone deacetylase 6 (Hdac6), which is related to the microtubule- and dynein-dependent intracellular movement of polyU protein aggregates [155]. It has been demonstrated that RNAi-mediated Hdac6 knockdown aggravates autophagosomes–lysosomes communication [156]. In contrast, Hdac6 overexpression can suppress neurodegeneration caused by proteasome dysfunction or by the polyQ toxicity in transgenic flies *via* autophagy induction, suggesting a crosstalk between UPS and autophagy [157].

CMA seems to be also implicated in CNS disorders and specifically in PD. Human PD brains display high levels of Hsp90 that correlate with the levels of insoluble α-synuclein [158]. In addition, immunohistochemistry and EM data demonstrated that Hsp90 and α-synuclein co-localize in Lewy bodies, Lewy neurites, and glia cell inclusions in PD patients, as well as α-synuclein transgenic mice [159].

Although it seems clear that both neurons and axons depend on proper autophagy functionality for maintaining cellular proteostasis, additional studies should be conducted in order to clarify the exact role of autophagy in neuronal cells.

6.4 Molecular chaperones

An essential PN component is the heterogeneous group of molecular chaperones that are key players in proteostasis. Chaperones are also called heat shock proteins (Hsps) because of their upregulation under conditions of oxidative stress and raised temperature (the prototype of stress) that typically result in significant proteome instability [160]. Molecular chaperones are responsible for the proper folding of newly synthesized nascent proteins, but they also intervene in order to rescue unfolded, misfolded, and/or damaged proteins, a triage process known as *fold*, *hold*, or *degrade*. Chaperones collaborate with many different proteins to achieve their function and they are highly specific even though they possess a rather wide spectrum of potential substrates. To achieve their function, chaperones undergo many structural conformational changes and since they are composed of highly coordinated moving parts with different energy demands, they represent true protein machines [161]. Due to their involvement in diverse cellular pathways such as protein trafficking, protein degradation,

and assembly of macromolecular complexes, chaperone malfunction leads to abnormal polypeptide accumulation in the form of aggregate [162] and significant proteome instability. As already mentioned, extensive research is currently being performed concerning disease-related polypeptide aggregation; that is because deposition of protein aggregates in the central nervous system results in dysfunctional neuronal cells and leads to the manifestation of diseases such as AD and HD. Therefore, examination of the role of chaperones in modulating or preventing neurodegeneration offers a window for new therapeutic opportunities.

Molecular chaperones are classified according to their molecular weight in Hsp90, Hsp70, Hsp60, and the small Hsps (sHsps); there are also chaperones functioning at the extracellular milieu like the molecular chaperone Apolipoprotein J/Clusterin (CLU). sHsps are ATP-independent, while the functionality of the high-molecular-weight Hsps is highly dependent on ATP. In most cases, the function of chaperones is facilitated by co-chaperones like the BAG-domain containing family (Bag1–6), the TPR-domain containing family (Chip, Hip, Hop), and the DnaJ-domain containing co-chaperone Hsp40 [163].

The sHsps have a size range of 10–40 kDa and they have in common a conserved 100 amino acid α-crystallin domain that can form oligomers [164]. sHsps have been implicated in many cellular functions and are able to bind to unfolded or misfolded proteins and prevent their aggregation [165]. Several studies have associated sHsps with neurodegeneration where it is hypothesized that sHsps confer a protective role against diseases, such as HD. Specifically, HspB6, HspB7, HspB8, and HspB9, members of the sHsps family, were found to decrease polyQ aggregation *in vitro* [166]. HspB8 was also found to clear polyQ aggregates by forming a complex with Hsp70 and its co-chaperone Bag3 [167]. Reportedly, the complex of Hsp70–HspB8–Bag3, in co-operation with the selective autophagy receptor p62, was found to activate autophagy-mediated degradation of polyQ and also to reduce neurotoxicity [167].

Members of the sHsp can influence the development of AD by affecting Aβ aggregation. Specifically, HspB5 was found to bind to Aβ fibrils and inhibit their elongation and growth [168]. Studies in cell and animal models have associated another member of the sHsp family, namely the HspB1 protein, to ALS. Overexpression of HspB1 along

with Hsp70 in neuronal cells expressing mutant versions of Sod1-protected cells from mutated Sod1-induced cell death [169], while HspB1 overexpression was found to protect motor neurons *in vivo* from cell death induced by nerve crush [170]. Mutations in Sod1 are also, likely, implicated in the onset of ALS, since in a transgenic mouse model overexpressing a mutant version of Sod1 ($Sod1^{G93A}$) and HspB1 a significant amelioration of muscle force, as well as a rise in motor neuron survival of the spinal cord was reported [171]; however, the observed positive effects were short-lived.

A wide class of molecular chaperones that depicts about 1–2% of total cellular protein content in normal cells is the Hsp90 family [161]. Proteins of the Hsp90 family are involved in many different cellular processes such as cell-cycle progression, signal transduction, protein degradation, and apoptosis and as a result they interact with many different "client" proteins [172]. Hsp90 is a homodimer with each monomer comprising an N-terminal ATP-binding domain (N-domain) that interacts with co-chaperones, a central domain that binds client proteins and is responsible for ATP hydrolysis, and a C-terminal dimerization domain (C-domain) [3]. Hsp90 undergoes a series of conformational changes through ATP-induced transition between an open and a closed conformation, in order to bind target proteins and release them properly folded. Binding of ATP promotes lid closure, a structure over the nucleotide binding pocket that boosts N-domain dimerization and forms a closed conformation. Closed dimer conformation prompts ATP hydrolysis, with subsequent N-domain dissociation, substrate release, and return to the open conformation [173]. There are convincing evidence relating Hsp90 to AD pathology, since tau protein is a target of Hsp90, and inhibition of Hsp90 is followed by tau destabilization, thus tau aggregation and toxicity can be largely controlled through Hsp90 [85, 174]. For this reason, many small-molecule inhibitors of Hsp90 have been developed in order to degrade tau and release Aβ burden. However, these inhibitors exhibited many off-target effects and therefore new approaches are being explored for inhibition of the Hsp90/tau complex or of Hsp90-specific co-chaperones [175]. Another prominent inhibitor of Hsp90 is 17-AAG, which promotes the expression of different chaperones such as Hsp70, Hsp40, and Hsp60; the upregulation

of these chaperones protected neurons against Aβ toxicity both *in vitro* and *in vivo* [176].

Another abundant Hsp family in the cell is the Hsp70 group of proteins. Hsp70 chaperones are involved in many different cellular processes in collaboration with specialized co-chaperones, namely the Hsp40 family [177]. Hsp70 proteins are comprised of two domains, an amino-terminal ATPase domain and a carboxy-terminal substrate-binding domain constituted of a hydrophobic binding pocket and a helical lid segment [178]; the lid segment remains open even after ATP binding and closes after ATP hydrolysis. Energy from ATP hydrolysis is sufficient for lid closure and stability of the bound client protein to reach its corrected folded state [179]. Hsp70, in order to disaggregate large aggregates, co-operates with several proteins namely Chip or Hsp100, ATPases, "unfoldases" and "disaggregatases" that deliver substrates for degradation to specific proteases like the proteasome [180–182].

Extensive research has been performed in relation to Hsp70 and α-synuclein aggregation. Most importantly, Hsp70 was found to regulate α-synuclein aggregation both *in vitro* and *in vivo*. Overexpression of Hsp70 in cells reduced the levels and toxicity of insoluble α-synuclein [183]. Moreover, in an α-synuclein transgenic mouse model, Hsp70 expression was found to lower insoluble α-synuclein aggregates [183], probably through inhibition of fibril formation.

In AD, Hsp70 expression, along with Hsp40 and Hsp90 co-expression, delayed Aβ aggregation; thus, a combination of chaperones upregulation might be more appropriate approach to prevent Aβ aggregation [184]. The Hsp70 and Hsp40 (DnaJ) families are also implicated in HD, where they were found to reduce polyQ aggregation in both cells and *Drosophila* model [185, 186]. Members of the Hsp40 family were also found to suppress polyQ aggregation especially the neuronal DnaJ protein HSJ1 (DnaJB2) [187]. In support, HSJ1a overexpression exhibited a neuroprotective role in an R6/2 mouse model of HD [188] and it suppressed aggregation of mutant *HTT* in brain.

Another group of Hsps is the Hsp60 family, also called chaperonins. Chaperonins are protein machines that ensure the correct protein folding and are divided into two subgroups. Group I, which is present in chloroplasts, bacteria, and mitochondria, and group II, which is found in the

eukaryotic cytosol and in archaea [189]. Group I includes the bacterial chaperonin 60 (GroEL) and the co-chaperonin 10 (GroES) [190]. GroEL is shaped in a barrel-like, "lid" structure, where non-native proteins are captured. After ATP binding, GroES is recruited, closes the "lid", and creates an isolated chamber where target proteins cannot further misfold and can either retain this conformation or fold properly [191]. In group II, the chaperonin-containing Tcp1 (CCT; also known as TriC), which binds to cytoskeletal proteins, and the archaeal thermosome are included [192]. Hsp60 seems to act in synergy with the other chaperones to reduce toxicity in neurodegenerative disorders, as already reported for AD [176]. Upregulation of the Hsp90, Hsp70, and Hsp60 chaperones by a hydroxylamine derivative called Arimoclomol (that prolongs activation of HSF1) leads to delayed progression of ALS disease in SodD1^{G93A} mice, along with an improvement in muscle function and in motor neuron survival in the late stages of the disease [193].

Molecular chaperones are not only found intracellularly and, in fact, many operate in the extracellular space, which is a rather challenging environment as it lacks ATP and it is constantly affected by environmental stressors. Haptoglobin [194], α(2)-macroglobulin [α(2)M] [195], and CLU [196] are glycoproteins that exhibit ATP-independent chaperone properties and are characterized as extracellular molecular chaperones. These chaperones seem to ensure extracellular proteome stability by targeting unfolded or misfolded proteins in the biological fluids for lysosomal proteolysis *via* endocytosis [197]. CLU is a heterodimeric secreted glycoprotein that is implicated in many different processes such as aging, cancer, metabolic diseases, and neurodegeneration [198]. CLU functions in an sHsp-like way and it is upregulated after several types of stress [198]; these findings are in accordance with the many stress-related regulatory elements found in the CLU gene promoter [199]. Moreover, it is characterized as an amyloid-associated protein due to its co-localization with fibrillary deposits [200]. Specifically, regarding AD, plasma CLU levels are linked with brain atrophy and rapid clinical progression [201]. Genome-wide studies also exhibited a link between CLU levels and the severity and risk of AD [201]. CLU inhibits the Aβ aggregation and therefore prevents further formation of protein aggregates, but it also causes the induction of Aβ soluble forms that are toxic to neurons [202]. Furthermore,

in neuronal cells, it is involved in the Dik-Wnt-JNK pathway and forms complexes with Aβ fibrils which are neurotoxic [203]. Nevertheless, further studies are needed to better clarify the role of CLU in AD pathogenesis.

Overall, it is clear that chaperones are important key players in neuronal proteostasis and they represent a reasonable therapeutic approach.

6.5 Unfolded protein response

Cells express a pool of thousands of different proteins that need to be tightly controlled for proper cellular structure, organization, and function. As mentioned, PN is an assembly of distinct dynamic molecular pathways and functional modules that control proteome dynamics during protein synthesis, folding, trafficking, and degradation [204]. The ER is a major site for synthesis of proteins, steroids, cholesterol, other lipids, and the main storage site for cellular calcium [205, 206]. Therefore, perturbations in ER homeostasis can lead to the accumulation of misfolded and/or damaged proteins. Homeostasis of protein folding and quality control in the ER is maintained by the sensor system known as the UPR [206]. In mammalian cells, UPR activates three major signaling pathways initiated by prototypical ER-localized stress sensors: the activating transcription factor 6 (ATF6), the pancreatic ER kinase (PKR)-like ER kinase (PERK) and the inositol-requiring enzyme-1 (IRE1), which collaborate to maintain proteome balance. In chronic ER stress, the UPR triggers apoptotic cell death, thus eliminating damaged cells [206].

Under normal conditions, all three effectors bind to the ER chaperone 78 kDa glucose-regulated protein/binding immunoglobulin protein (Grp78/BIP) on their luminal domains [207]. The mechanism of stress sensing by these proteins is poorly understood, but one widely accepted model involves the dissociation of the chaperone Grp78/Bip after the accumulation of unfolded proteins.

Once activated, the UPR regulates translation and gene transcription in order to increase the folding capacity and eventually to contend with stress conditions. ATF6 is a transcription factor that is initially synthesized as an ER-resident transmembrane protein. During ER stress, ATF6 dissociates from Bip, traffics to the Golgi, and undergoes proteolytic

processing to release the cytosolic domain; this event produces the active ATF6f form. Cytosolic ATF6f is then imported into the nucleus to induce the expression of protein quality control genes, either independently or in collaboration with XBP1s [208, 209]. The second pathway of the UPR is guided by PERK. Upon ER stress, PERK is activated by autophosphorylation and it then activates the ubiquitous translation initiation factor eIF2α; it also indirectly inactivates eIF2. The eIF2α phosphorylation allows the specific translation of activating transcription factor 4 (ATF4), which upregulates many important genes involved in the redox control, metabolism, and folding, and it also transiently inhibits protein synthesis [210, 211]. The UPR also activates the third UPR sensor, namely IRE1, which *via* splicing of the inactive X box-binding protein 1 (XBP1) mRNA, produces the active spliced XBP1 (sp-XBP1) form [212]. The IRE1 pathway is one of the most well-studied branches of the UPR. Unfolded proteins induce activation of the IRE1 ribonuclease activity upon oligomerization on the ER membrane. The active form of XBP1 controls the expression of genes with X-box elements in their promoters; these genes mainly encode for ER chaperones and proteins involved in the ERAD [212–215]. There is a crosstalk between the three pathways and modulation of one will affect the signaling of the other two. Moreover, ER contains a series of chaperones, including Hsp70 BiP, Hsp90-like Grp94, calnexin, calreticulin, thiol-disulfide oxidoreductases, and protein disulfide isomerases (PDIs), which are all involved in the appropriate folding of ER proteins [208, 216]. Activation of the UPR signaling pathways helps fighting cellular stress by suppressing the translation of new proteins (thereby reducing the load of unfolded/misfolded proteins), as well as by inducing the expression of ER chaperones and activating the proteasome to degrade misfolded/unfolded proteins via ERAD [217]. The failure of the UPR-mediated adaptive events results in apoptosis [218]. As mentioned, accumulation of protein aggregates is a common feature of neurodegenerative disorders; this finding highlights the role of UPR in neuron pathological states. As a result, many studies have shown that UPR is activated in most neurodegenerative pathologies.

Glia cells, oligodendrocytes of the CNS, and Schwann cells from the peripheral nervous system, produce the myelin sheath which is vital for the function of the nervous system. Myelin sheath wraps axons and participates

in the myelinating process. Several studies show that ER and UPR play an important role in disorders of myelinating glia, including multiple sclerosis, Pelizaeus–Merzbacher disease, and Charcot–Marie–Tooth disease [219], suggesting that glia cells are highly susceptible to ER stress [220].

In PD, aggregates of wild-type or mutated α-synuclein activate UPR probably due to the inhibition of the ER-Golgi transport [221], while other studies indicate that activation of CBP1 and ATF6 may have a protective effect [222–226].

In AD, the early stages of the disease are accompanied by the activation of UPR, through BiP induction, PERK phosphorylation, increased levels of XBP1 splicing forms, and accumulation of Grp78 [227, 228]. UPR activation is thought to have a protective role in the early stages, but a continuous persisting activity may contribute to neuronal death, probably by activating autophagy [227, 229]. In addition, post-mortem tissue from the brain of AD individuals has provided evidence for ER dysfunction [230]. Moreover, studies suggest that UPR may play a direct role in AD progression through tau phosphorylation [231, 232].

Post-mortem brains from patients with ALS show increased concentrations of the ER stress markers [233], suggesting a connection between UPR and ALS pathology. Notably, deletion of neuronal XBP1 delays the onset of disease in a mouse model of familial ALS and depletion of XBP1 in cultured neurons reduces Sod1 aggregates and its toxic effects through enhanced autophagy [234]. Several evidence associate induction of UPR with HD, however contradicting data have been observed about the role of the XBP1 and the pathology that still needs to be clarified [235, 236].

In summary, plenty of studies performed in genetic and pharmacological models have contributed to increase the knowledge about the role of the UPR in the CNS. However, the proposed role of the UPR is often controversial and difficult to be translated to human brain. For instance, it is not clear which is the equilibrium line between the beneficial effect of UPR activation and its cytotoxic effect on neuronal cells.

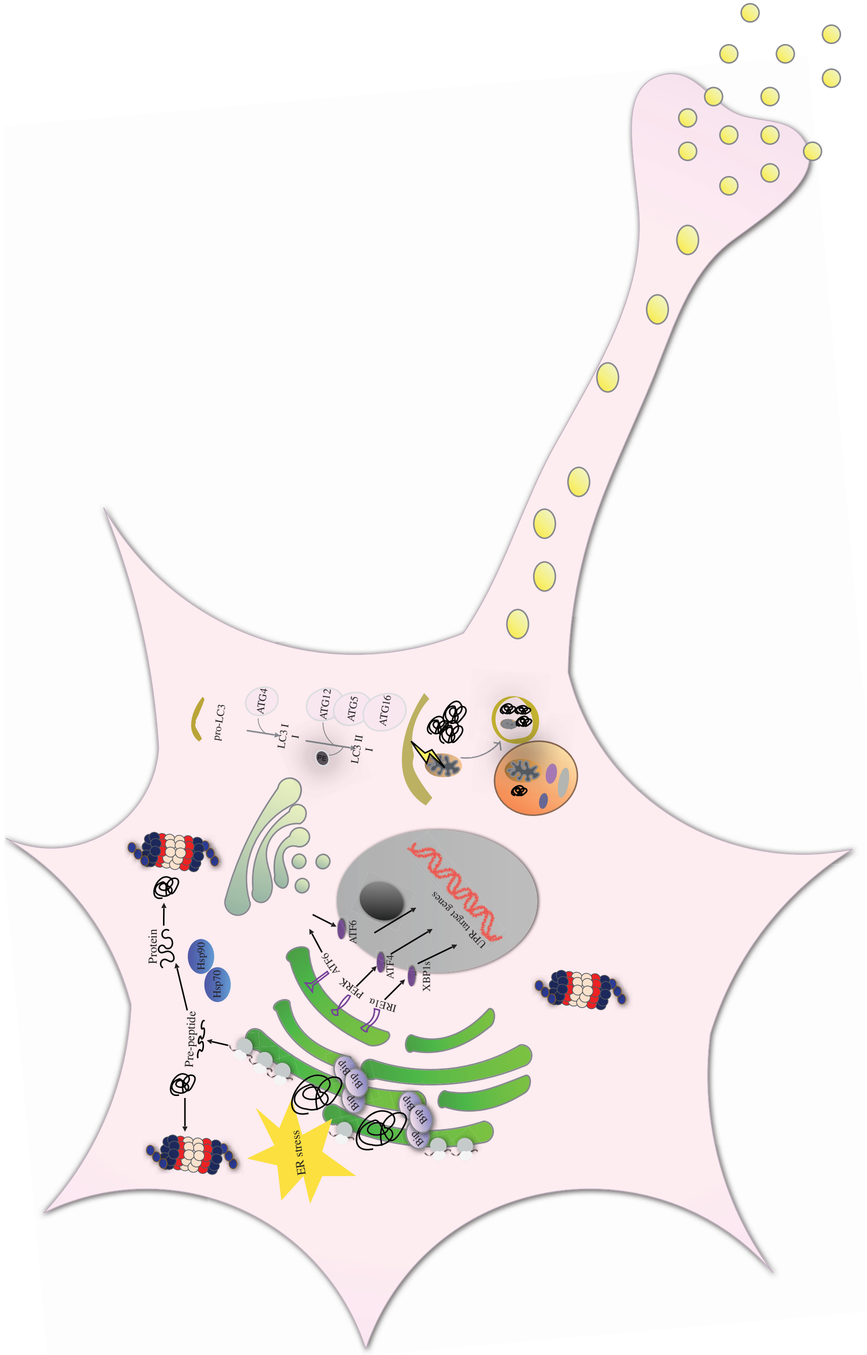
6.6 Concluding remarks and perspectives

It is becoming clear that PN functionality plays a crucial role in maintaining the optimal neuronal state (Figure 6.1) and its deregulation is closely

associated to the neurodegenerative disorders. Several studies have shown that activation of protein repair or degradation systems reduces the rate of age-related protein aggregate formation and/or accumulation, thus resulting in decreased age-related neurotoxicity. UPS or ALP activation alone or in parallel with the overexpression of the molecular chaperones Hsp70, Hsp40, and some sHsps reportedly diminished the polyQ and polyU aggregates and the toxicity they induce [72, 110, 149, 167, 185, 186]. Therefore, increased functionality of protein clearance mechanisms contributes to the amelioration of age-related diseases. Interestingly, UPR activation seemed to have a protective role in the early stages of neuropathologies, while long-term UPR activation contributed to neuronal death [227, 229]. Hence, the protein repair and degradation pathways provide alternative and in some cases different strategies and targets for treatment of neuropathologies. It is crucial to understand which approach and what time point (i.e. state of disease; early–late) would be appropriate for therapeutic interventions.

Tau is another protein that seems to act at different molecular events and is involved in almost all common neuropathologies [74, 85, 86]. Several data indicate that PN modules affect the activity and degradation of tau protein. UPS was found to ubiquitinate and degrade tau along with the GSAP enzyme, which participates in the formation of Aβ pathological peptides. Moreover, chaperone Hsp90 regulates tau stabilization and it was shown that Hsp90 inhibition led to tau clearance [85, 174]. ER stress reduced the degradation rate of tau due to decreased tau-Chip binding [232], and therefore UPR or Chip activation likely could offer protection against tau accumulation and related pathologies. Consequently, tau could be considered as another potential target for neurodegeneration.

Finally, a variety of studies have highlighted the dysfunction of PN during aging [4, 71, 216, 237, 238] and as the neurodegenerative symptoms occur mainly in older people, it is unclear whether aggregates accumulation is causative to PN dysfunction or *vice versa*. Moreover, it would be interesting to find out whether deregulation of a single component of PN, distinctly from the other modules, could prompt the neuronal pathogenesis. Genetic or pharmacological models along with live imaging and proteomics have helped to better understand the mechanism involved in the pathogenesis of neurodegenerative diseases. However, it is worth mentioning that most of the studies are focused principally on the pathological form of the protein that



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leads to neurodegeneration and not on its wild-type form. This approach is (in most cases) not fully representative of the actual molecular processes that relate to the development of these diseases which are (as mentioned) induced by changes/mutations in the physiological benign form of the protein.

Considering that several proteins and multiple pathways are involved in neurodegenerative disorders, a combination of different drugs that act at different molecular levels and stages of the disease (i.e. see the different effects of UPR activation in early or late stages) should probably be considered during the development of new therapeutic approaches or in clinical treatment.

Taking into account the aforementioned observations and realizing that neurodegeneration is mostly (if not entirely) an age-related condition, further studies on aging etiology and its causative relation with degeneration of post-mitotic tissues will surely contribute to the better understanding of the molecular events that underlie neuronal pathology and also in designing new therapeutic strategies.

Figure 6.1 Schematic depiction of the main PN components' functional implication in the maintenance of neuron homeodynamics. The maintenance of the optimal neuronal state is a multistep regulatory process, in which the PN plays a crucial role. PN consists (mainly) of molecular chaperones, the UPR of the ER, the UPS, and the ALP. Cytosolic chaperones, such as Hsp70 and Hsp90, are involved in the folding of the newly synthesized peptides and in the recognition and refolding of misfolded or unfolded proteins. The unfolded or improperly assembled ER-synthesized proteins (ER stress) activate the UPR; this network consists of three types of ER stress sensors, namely the IRE1a, PERK, and ATF6 transcription factors, that upregulate the expression of several proteins in order to restore ER proteostasis. Cytosolic or ER misfolded proteins that cannot be repaired are ubiquitinated by E3 ligases and degraded mainly by the UPS. When the repairing system of cellular chaperones or the degrading network of proteases is overwhelmed, an extensive deterioration of cellular organelles occurs, which then prompts the activation of autophagic responses. Autophagy starts with the formation of the autophagosome where the Atg16L complex is composed from the autophagy-related proteins Atg12–Atg5. The Atg16L complex conjugates phosphatidyl ethanolamine (PE) to the microtubule-associated protein 1 Light Chain 3 (LC3) to generate the LC3 II receptor. Finally, the membrane engulfs the cargo, closes its ends, and fuses with lysosomes in order to degrade its content. Protein aggregates and/or dysfunction of the PN can (among others) alter the number and transport of synaptic vesicles and of organelles (e.g. mitochondria), along the axons leading to initiation and/or progression of neuron degeneration.

Acknowledgments

We apologize to those authors whose work was not cited due to space limitations. Research studies at the group of IPT are financially supported by the following EU grants: TASCMAR (EU-H2020, GA 634674), MICROSMETICS (FP7-PEOPLE-IAPP 2013, GA 612276), and MEDIHEALTH (H2020-MSCA-RISE-2015, GA 691158).

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Chapter 7

Protein Misfolding and Mitochondrial Dysfunction in Amyotrophic Lateral Sclerosis

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7.1 Introduction

7.1.1 *Amyotrophic lateral sclerosis: Clinical and genetic features*

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with rapid fatal outcome, within only 1–5 years of diagnosis. The incidence of ALS is approximately 2 in 100,000 per year [1]. In ALS, cortical and spinal motor neurons degenerate, resulting in muscle weakness and atrophy and ultimately in severe paralysis, which affects virtually all muscle, including limbs and bulbar districts. Death often occurs because of respiratory insufficiency. Presently, there are no treatments for ALS patients, besides Riluzole, which prolongs life only for a few months.

Etiologically, ALS can be divided into familial (fALS) and sporadic (sALS) forms. fALS accounts for approximately 10% of the cases and is caused by a multitude of genetic mutations. To date, over 30 different gene mutations (an updated list of ALS genes is available at http://alsod.iop.kcl.ac.uk/) have been associated with fALS that occur with frequency in the population varying from relatively common (i.e. 40% of all familial cases) to extremely rare (only a few cases ever identified). Unfortunately, the etiology of sALS, which accounts for 90% of ALS cases, still remains largely unknown.

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Historically, the first fALS mutations in [Cu–Zn] superoxide dismutase (SOD1) were discovered in 1993 [2]. Since then, hundreds of studies on mutant SOD1 have shed some light on the mechanisms of fALS, also thanks to the development of powerful transgenic models that recapitulate the disease in mice [3]. However, SOD1 mutations only represent approximately 10% of all fALS and it has been challenging to determine whether the discoveries in the pathogenesis of SOD1 fALS apply to other forms of the disease as well. In the last few years, largely because of the widespread availability of whole-genome sequencing techniques on large cohorts of patients, a plethora of genes, with remarkably variable ontology, have been associated with ALS [4, 5]. Often, the same mutations can cause variable clinical phenotypes, even in different members of the same family. Clinical manifestations can vary from frontotemporal lobe degeneration (FTLD) to ALS or a combination of both. One of the most poignant examples of such variability in clinical manifestations is the hexanucleotide expansion in the first intron of C9Orf72 [6, 7], a gene with still unknown function, which is responsible for the most common form of FTLD and fALS, but also a subset of sALS [8].

7.1.2 *Pathways leading to ALS*

Several diverse molecular pathways are affected in different forms of fALS. For example, mutations found in TAR-DNA binding protein 43 (TDP-43 [9]), matrin3 (MATR3 [10]), and fused in sarcoma (FUS [11, 12]) point to RNA metabolism and the dynamics of stress granules [13–15]. Mutations in ubiquilin 2 (Ubqln2 [16]), vesicle-associated membrane protein-associated protein B (VAPB [17]), p62/sequestosome (SQSTM1 [18]), and valosin containing protein (VCP [19]) indicate protein quality control and autophagy as alternative molecular pathogenic pathways in ALS. Mutations in vesicle-associated protein B (VAPB [17]) and the mitochondrial protein CHCHD10 [20–22] suggest that alterations in ER and mitochondrial homeostasis are also involved in ALS. Remarkably, despite the apparent differences in the genetic causes of fALS, these diverse molecular and cellular mechanisms of disease wind up converging into common pathogenic pathways that cause motor neuron degeneration.

An important feature of ALS, which has been amply demonstrated in animal and cell-culture models of ALS, is the non-cell autonomous nature. It was shown that various types of glial cells harboring pathogenic mutations in SOD1 and other ALS-related genes affect the viability of motor neurons. For example, genetic ablation of mutant SOD1 selectively from astrocytes [23], microglia [24], or oligodendrocyte precursors [25] significantly improves the disease phenotype in transgenic SOD1 mice. Through co-culture experiments, it was demonstrated that mutant glial cells exert a direct active toxicity on motor neurons by secreting toxic molecules [26, 27], whose nature has not yet been fully understood. Nevertheless, it is likely that the same molecular mechanisms that play a role in cell autonomous motor neuron toxicity, such as RNA metabolism, proteostasis, and mitochondrial function, can also cause toxicity from glial cells. For example, several ALS mutant proteins, including SOD1 [28] and TDP-43 [29], activate pro-inflammatory programs in microglia largely through NF-κB signaling, resulting in neuro-inflammatory responses that contribute to motor neuron demise.

Overall, the breadth and diverse pathogenic pathways, which have been implicated in ALS, are perhaps unmatched by any of the other major neurodegenerative diseases. Proposed cellular targets include RNA metabolism, axonal transport, energy metabolism, ER stress, intracellular calcium imbalance, cell death signaling, proteins, and organellar quality control. Any of these targets could potentially be affected by the accumulation of misfolded proteins.

7.1.3 *ALS, a disease of protein misfolding and aggregation*

A significant subset of fALS and sALS is characterized pathologically by the presence of intracellular protein aggregates and inclusions. In some instances, the mutant proteins that cause the disease represent a major component of the aggregates, as in the case of mutant SOD1 [30] or TDP-43 [31]. Otherwise, mutations in seemingly unrelated proteins could induce aggregation in different proteins, such as, for example, C9Orf72-induced TDP-43 inclusions [32]. Indeed, cytoplasmic aggregation of TDP-43, particularly the phosphorylated form of the protein, is one of the pathological hallmarks of ALS, including sALS [33]; however, TDP-43

aggregates are not specific for ALS/FTLD, since they are found in various other neurodegenerative diseases [34, 35].

The pathogenic role of protein misfolding and aggregation in ALS could involve many potential pathways. Aggregation can cause protein mislocalization resulting in lack of function in the physiological compartments, such as the nuclear depletion of TDP-43 caused by its cytosolic sequestration. Misfolded proteins can also form aberrant and persistent interactions with proteins with which they do not ordinarily partner, such as in the case of mutant SOD1 in mitochondria [36–38], or cause excessive stabilization of physiological interactions, such as for mutant FUS in stress granules [39–41]. Aggregates of mutant proteins and aberrant protein–protein interactions can interfere with cellular processes, such as axonal transport [42, 43], autophagy [44], proteasomal degradation [45, 46], nuclear-cytoplasmic transport [47, 48], and mitochondrial functions.

7.1.4 *Mitochondrial dysfunction in ALS*

Numerous lines of evidence link ALS to mitochondria, since mitochondrial abnormalities are evident in both fALS and sALS. Mitochondrial involvement in ALS has been investigated quite extensively and includes various functional, structural, and dynamics components. Mitochondrial morphological abnormalities [49, 50], reduced calcium capacity [51], transport impairment [52–54], bioenergetic dysfunction [55, 56], and aberrant reactive oxygen species (ROS) production [57] have been proposed as pathological contributors to ALS. Mitochondrial abnormalities are also common to other forms of fALS [53] and sALS [58, 59]. Mitochondrial dysfunction in mouse models of ALS appears prior to symptom onset, suggesting a pathogenic role, but because of the widespread intracellular distribution of many of the mutant proteins, it is often difficult to determine whether mitochondria are primary or secondary targets. However, the discovery of fALS mutations in a gene encoding for a mitochondrial protein, CHCHD10 [20, 21], suggests that motor neuron toxicity can arise directly in mitochondria.

This chapter will examine the effects of ALS-linked mutant proteins interacting with mitochondria and affecting their structural and functional integrity.

7.2 Misfolded proteins that associate with mitochondria in ALS

7.2.1 *SOD1, the first ALS protein found in mitochondria*

7.2.1.1 *SOD1 function and dysfunction*

SOD1 was the first gene found to be causative of fALS [2]. The encoded protein (superoxide dismutase 1) is a superoxide scavenger that catalyzes the dismutation of superoxides into hydrogen peroxide and molecular oxygen. The active enzyme requires both copper and zinc ions, an intramolecular disulfide bond between cysteine residues 57 and 146, and homodimerization. Over 100 mutations have been identified affecting the 153 amino acid of SOD1, with no specific domains as hotspots for mutation occurrence. Many of the mutations are missense, resulting in a single amino acid substitution, but some mutations cause truncation of the protein. Since not all missense mutations result in loss of the dismutase function and inheritance is autosomal dominant, a toxic gain of function of the mutant protein is thought to be the cause of the disease.

A common feature of SOD1 mutant proteins is structural destabilization [60], resulting in the formation of detergent-insoluble aggregated species [61]. Misfolding of SOD1 can result in exposure of internal cysteines, which become available to establish aberrant intermolecular disulfide bonds. Therefore, altered SOD1 conformation can create a linkage platform for SOD1 oligomers [62, 63]. Misfolded mutant SOD1 can also form very large intracellular inclusions, defined as “skein-like” inclusions, which are found in ALS post-mortem spinal cord motor neuron cell bodies [63]. Furthermore, aberrant oxidation and misfolding of wild-type SOD1 has been suggested to play a pathogenic role in sALS [64]. The removal of misfolded SOD1 by expression of a chaperone, TorsinA, in a *Caenorhabditis elegans* model of SOD1-fALS, which lead to degradation of aggregated mutant SOD1 and to a motor phenotype improvement [65], suggested that aggregation of SOD1 could be a potential therapeutic target.

7.2.1.2 *Mutant SOD1 is localized inside mitochondria*

The observation of numerous bioenergetic and morphological alterations of mitochondria in SOD1-fALS was puzzling, until it was demonstrated

that a portion of SOD1 (approximately 1% of total SOD1) is localized to mitochondria [66]. Within mitochondria, wild-type SOD1 localizes to the intermembrane space (IMS), where it likely provides protection from superoxide that leaks from the electron transport chain, while the matrix is protected by manganese SOD (SOD2).

Many proteins targeted to the mitochondrial IMS depend on a disulfide relay system that allows for their import, folding, and retention. Within the nascent protein sequence are pairs of cysteine residues that allow transient interactions through intermolecular disulfide bonds with the IMS import machinery, composed of two essential proteins, Mia40 and Erv1 [67]. SOD1 import and retention in the IMS is regulated by oxygen tension that governs folding of the copper chaperone for SOD1, CCS, which in turn is imported by Mia40 [68–70]. Thus, the four-cysteine residues, in particular, the pair that forms the intermolecular bond C57–C146, are critical for SOD1 retention in the IMS.

Mutant SOD1 species are not entirely regulated by CCS or oxygen and are likely retained in the mitochondria by misfolding and aggregation [68]. Substitution of cysteine residues in mutant SOD1 was capable of preventing SOD1 oligomerization in the IMS and alleviating mitochondrial dysfunction in cultured cells [71], confirming the role of cysteine redox chemistry in mutant SOD1 mitochondrial localization and toxicity. Conformation-dependent antibodies raised against misfolded SOD1 have been useful tools in the field. A recent study using these antibodies revealed that multiple conformations of misfolded SOD1 might exist, even within the same motor neurons, in mutant G93A spinal cords [72]. Interestingly, some but not all of these misfolded species were shown associated with mitochondria [73, 74]. However, despite the strong evidence that mutant SOD1 can associate with mitochondria, the relative role of this portion of SOD1 in causing mitochondrial structural and functional damage and, most importantly, motor neuron demise, was difficult to pinpoint, because SOD1 is widely distributed throughout various cell compartments.

Artificial targeting of mutant SOD1 selectively into the mitochondrial IMS in cultured neurons showed that oligomerization of the mutant protein causes mitochondrial dysfunction [71] and mitochondrial transport defects [75]. In motor neuron such as NSC34 cells, mutant SOD1 targeted to the IMS also increased the cell vulnerability to stress [71]. *In vivo*

expression of mutant SOD1 targeted to the mitochondrial IMS affected mitochondrial biochemistry and structure causing muscle atrophy and neurodegeneration [76]. However, in this mouse model, muscle denervation was not evident and a typical ALS phenotype was not observed, suggesting that mutant SOD1 aggregation in the IMS alone was insufficient to cause the full ALS clinical phenotype and that an extra-mitochondrial component of SOD1 is crucial to the pathogenic process.

7.2.1.3 *Mutant SOD1 can affect mitochondria from the outside*

Mitochondria could be affected by mutant SOD1 in the IMS, but also by the presence of the mutant protein on the outer membrane. Several reports identified the presence of misfolded mutant SOD1 deposited on the cytoplasmic surface of the outer membrane of spinal cord mitochondria [74, 77, 78]. Misfolded SOD1 accumulation on the outer mitochondrial membrane (OMM) has been suggested to affect mitochondrial function by interfering with ion conductance through the voltage-gated ion channel (VDAC) [38]. Mitochondrial protein import could also be a target of misfolded SOD1 [79]. Furthermore, binding of mutant SOD1 to Bcl2 was suggested to be a facilitator of mitochondrial association of SOD1, which could also undermine the function of Bcl2 as an anti-apoptotic regulator [80]. Interestingly, association of mutant SOD1 with mitochondria was apparent in CNS, particularly in spinal cord, but not in liver in some studies [81]. This observation has led to the identification of a cytosolic factor, macrophage migration inhibitory factor (MIF), which suppressed SOD1 associating with mitochondria, and ER in liver and other unaffected tissues. MIF levels are low in motor neurons, which could provide a reason for the selective vulnerability of these cells. In fact, increasing MIF levels can enhance survival of mutant SOD1 motor neurons by assisting in the folding of SOD1. Furthermore, ablation of MIF from G85R SOD1 mice exacerbated the disease course [82].

Mutant SOD1 can also affect mitochondria indirectly from the cytosol. For example, mutant SOD1 has been shown to impede with mitochondrial axonal transport and with the trafficking of autophagic vacuoles containing mitochondria targeted for degradation by interfering with the dynein motor complex [42, 83].

7.2.2 *RNA binding proteins: TDP-43 and FUS, unexpected links to mitochondria*

TDP-43 and FUS are mainly nuclear resident, RNA-binding proteins involved in RNA processing, metabolism, and transport. Mutations in both FUS and TDP-43 result in familial ALS/FTLD [84]. Under conditions of cellular stress, such as heat shock, these proteins can be translocated to the cytosol where they participate in stress granule dynamics. In ALS, the nucleocytoplasmic transport regulation may be compromised, causing these proteins to accumulate in the cytosol, where they become prone to aggregation and form inclusions.

Cytosolic mislocalization of FUS and TDP-43 has been suggested to affect mitochondrial function. While TDP-43 has been shown to associate peripherally with mitochondria [85], there are contrasting opinions about the possibility that TDP-43 is actually imported inside mitochondria. In one report, it was suggested that fALS mutants of TDP-43 are imported into the mitochondrial matrix more efficiently than the wild-type protein [86]. In mitochondria, mutant TDP-43 was bound to mitochondrial DNA-encoded mRNAs, specifically decreasing the translation of the ND3/6 components of complex I of the respiratory chain, thereby disrupting its assembly and activity. Remarkably, preventing mitochondrial translocation of TDP-43 using peptides directed to a hydrophobic amino acid sequence critical for its import or removing this motif resulted in reversal of mitochondrial dysfunction and disease phenotypes *in vitro* and *in vivo* [86]. While complex I activity defects have been observed in cultured neuronal cells overexpressing TDP-43 [87], other studies did not observe oxidative phosphorylation impairment or TDP-43 localization in mitochondria of patient-derived fibroblasts and overexpressing cells [88]. Furthermore, alterations of mitochondrial morphology and transport, characterized by smaller size and decreased motility, were observed in the peripheral nerve axons of transgenic mice expressing mutant TDP-43 [53] and in a *Drosophila* model of TDP-43 proteinopathy [89]. These alterations could be consistent with the finding of decreased levels of the mitochondrial transport adaptor Miro1 on the surface of spinal cord mitochondria of transgenic mice expressing mutant TDP-43 [90]. Therefore, whether TDP-43 does, in fact, affect mitochondria from within or at the outer membrane remains to be conclusively established.

Similar to TDP-43, FUS has also been shown to associate with mitochondria and cause mitochondrial fragmentation in *Drosophila* neurons [91]. In this report, FUS was shown to enter the mitochondrial matrix in an Hsp60-dependent manner, since downregulation of Hsp60 modulated FUS mitochondrial localization.

C9Orf72 is a protein with yet unknown function, but large intronic exanucleotide expansions in the intronic region of the gene are responsible for the most common forms of fALS/FTLD and for a significant proportion of sALS cases. The pathogenic mechanisms arising from C9Orf72 expansion include toxicity by dipeptides generated through repeat-associated non-AUG (RAN) translation, nuclear RNA foci, impaired nuclear cytoplasmic traffic, and haploinsufficiency [92]. The involvement of mitochondria by mutant C9Orf72 has not been extensively investigated, but some evidence started to emerge that cells harboring the expansion have mitochondrial bioenergetic impairment [88]. However, the mechanism of this impairment is still unclear and could involve both transcriptional and proteotoxic effects derived from the exanucleotide expansion.

7.2.3 *The ER–mitochondria connection, a pathogenic target in ALS*

Mitochondria and ER are tightly connected organelles, which cooperate extensively in a number of functions, notably intracellular calcium homeostasis and phospholipid biosynthesis. Several lines of evidence have linked ALS to faulty ER–mitochondrial connections [93]. Both FUS and TDP-43 [94] have been shown to activate GSK3β, which disrupts the bridge between ER and mitochondria formed by two proteins, VAPB and PTPIP51, affecting mitochondrial calcium uptake and ATP production. Notably, mutations in VAPB have been linked to fALS [17], further supporting the role of alterations in interactions of organellar membranes in the pathogenesis of the disease.

TDP-43 and FUS mislocalization in the cytosol has also been proposed to trigger endogenous SOD1 misfolding in non-SOD1 fALS and sALS [95]. However, mislocalized TDP-43 or FUS do not co-localize with misfolded SOD1 in the cytosol, suggesting that these proteins may induce SOD1 misfolding indirectly. Importantly, TDP-43 or FUS-induced misfolding of SOD1 has been suggested to propagate across cells through

the extracellular medium, as long as there is SOD1 as seed in the medium and recipient cells [96]. Therefore, FUS and TDP-43 may affect mitochondria–ER interactions directly, but also by altering the normal function and the correct folding of other proteins.

Sigma-1 receptor (SigmaR1) is an ER chaperone that localizes to mitochondrial-associated membranes (MAMs). Activation of Sigma1R regulates calcium transport from ER to mitochondria through IP3 receptors [97]. A missense mutation in SigmaR1, causing an E102Q substitution, was associated with juvenile-onset fALS [98]. Mutant SigmaR1 was found to accumulate in enlarged synaptic terminals and ER structures in spinal cord motor neurons of ALS patients [99]. Moreover, mutant SigmaR1 altered calcium transfer from ER to mitochondria and reduces ATP production [100]. SigmaR1 agonists improved motor neuron function and increased survival in mutant SOD1 mice [101]. However, in that experiment, the effects on mitochondrial function or ER were not directly investigated. Therefore, while the association of SigmaR1 with MAMs is highly suggestive of an effect of the mutant protein on mitochondria [100], the mechanisms of mitochondrial dysfunction remain to be fully elucidated.

7.2.4 *Mitochondrial quality control: VCP and OPTN/TBK1*

Three fALS-related proteins, VCP, optineurin (OPTN), and Tank-binding kinase 1 (TBK1) are directly involved in mitochondrial quality control processes. These are fundamental molecular and cellular pathways, whereby damaged mitochondrial molecular components, such as oxidized and misfolded proteins and lipids, are removed and degraded or, in some cases, when the whole organelle is eliminated by a selective autophagy program, named mitophagy.

7.2.4.1 *VCP and the mitochondrial outer membrane protein degradation systems in ALS*

Mitochondrial membranes are populated by hundreds of proteins, which have important roles in maintenance of mitochondrial structural and functional integrity. Among the proteins that associate with the OMM, which

provides the interface between the cytosol and mitochondria, several have been implicated in neurodegenerative diseases. Notable examples are mitofusin 2 [102], involved in mitochondrial fusion, and Drp1 [103], involved in mitochondrial fission. Furthermore, proteins that play a role in the proteostasis of the OMM, PINK1 and Parkin are involved in familial Parkinson disease, through a loss-of-function mechanism [104].

The ubiquitin-proteasome system (UPS) is directly involved in clearing damaged proteins from the OMM [105, 106]. Parkin is a E3-ubiquitin ligase that ubiquitinates oxidized or misfolded OMM proteins [107], following its recruitment to the OMM, which is triggered by ubiquitin phosphorylation by PINK1 [108]. PINK1 is a kinase that is normally imported and degraded inside mitochondria, but it can accumulate on the OMM of damaged mitochondria, due to incomplete processing and import [109–112]. Ubiquitination of OMM proteins can be performed by other enzymes, such as the mitochondrial ubiquitin ligase MITOL (MARCH5), an E3-ubiquitin ligase permanent resident of the OMM [113], which has been shown to recognize several client proteins, including mitofusins [114, 115] and Drp1 [116–118]. The ubiquitin ligase activator of NF-κB (MULAN) is another mitochondrial ubiquitin-ligase involved in Drp1 ubiquitination [119].

VCP (also known as p97/Cdc48) is an ATPase, whose segregase activity extracts ubiquitinated proteins from membranes, the OMM, as well as the ER, and promotes their degradation through the UPS [120, 121]. VCP can be recruited to the OMM upon protein ubiquitination [122]. VCP mutations cause rare familial diseases, characterized by very complex phenotypes affecting heterogeneous tissues. These disease phenotypes include ALS [123], but also inclusion body myopathy, early-onset Paget disease of the bones, and frontotemporal dementia [124]. Selective VCP translocation to the OMM to extract damaged proteins depends on Vms1 (VCP/Cdc48-associated Mitochondrial Stress responsive 1), a cytosolic protein that senses local mitochondrial stress [107, 125] and binds VCP allowing its translocation to damaged mitochondria [126].

Due to its role in mitochondrial protein quality control, the loss of VCP impairs the clearance of damaged mitochondria, and VCP mutations result in severe accumulation of abnormal mitochondria in transgenic mice [122]. In keeping with the mitochondrial structural alterations,

mitochondrial bioenergetics is also impaired in VCP mutant cells. Metabolic studies suggested that mutations in VCP affect mitochondrial ATP synthesis, resulting in a compensatory increase in glycolysis and mitochondrial biogenesis [127]. Furthermore, fibroblasts of patients carrying three independent VCP mutations showed severe loss of mitochondrial membrane potential and increase in futile mitochondrial oxygen consumption, leading to decreased ATP production [128].

Patients affected by ALS with VCP mutations displayed neuropathological signs typical of ALS, such as loss of motor neurons, intracellular inclusions like Bunina bodies, and corticospinal tracts degeneration [129]. Interestingly, TDP-43 cytoplasmic inclusions containing phosphorylated TDP-43, ubiquitin, p62, and OPTN were detected in spinal motor neurons, further confirming that these aggregates and inclusions may occur downstream of a variety of mutant proteins.

7.2.4.2 *OPTN and TBK1: The selective autophagy pathway of mitochondrial degradation in ALS*

OPTN mutations are associated with fALS and glaucoma [130]. Originally identified as an NF-κB binding partner, more recently OPTN has been defined as an autophagy receptor involved in xenophagy, mitochondrial quality control, and elimination of protein aggregates [131]. OPTN mutations cause rare forms of fALS, but wild-type OPTN is also found to misfold and form inclusions in sALS motor neurons. The observation of OPTN-positive inclusions in post-mortem samples obtained from individuals affected from a variety of neurodegenerative diseases suggests that misfolding and aggregation of OPTN is likely not specific to ALS [132].

OPTN is an adaptor protein connecting the molecular motor myosin VI to secretory vesicles and autophagosomes. fALS OPTN mutations disrupt the interaction with myosin VI, leading to an abnormal OPTN cytoplasmic distribution, inhibition of secretory protein trafficking, ER stress, and Golgi fragmentation [133]. OPTN is directly involved in mitophagy, since it is recruited to damaged mitochondria in a parkin-dependent manner. It was first proposed that parkin is required for OPTN ubiquitination and that without parkin, OPTN fails to be stably associated with damaged mitochondria [134]. However, more recently, it was also

proposed that OPTN recruitment to mitochondria could be directly dependent on PINK1, without the need for parkin, whose involvement could occur later in the process to amplify the autophagic response [135]. Once recruited, OPTN functions as an autophagy receptor for the formation of a phagosome around mitochondria [135, 136]. These findings indicate that mitophagy alterations are involved in ALS and that such alterations could be pathogenic determinants of ALS.

Interestingly, TBK1, another protein linked to fALS and FTLD [137], binds and phosphorylates OPTN at the ubiquitin and LC3 binding domains [138, 139]. The interaction of TBK1 with OPTN and OPTN binding to ubiquitin chains are necessary for TBK1 recruitment and kinase activation on mitochondria. TBK1 phosphorylates OPTN in the Ub-binding domain, promoting OPTN Ub-binding activity and amplifying the signal for autophagy of damaged mitochondria [139]. OPTN and TBK1 are also involved with the function of another fALS-linked autophagy protein, p62/SQSTM1, since activated TBK1 on mitochondria phosphorylates p62, thereby allowing for the final steps of autophagic enclosure of ubiquitinated mitochondria [140].

In cultured cells, inhibition or depletion of TBK1, as well as the expression of fALS mutants of OPTN or TBK1 are able to prevent autophagosome formation upon mitochondrial damage [141], further confirming the functional interaction between TBK1 and OPTN under physiological conditions and the pathogenic role of loss-of-function mutations in fALS.

7.2.5 *CHCHD10, the first mitochondrial protein causative of fALS*

Mutations in coiled–helix–coiled–helix–domain 10 (CHCHD10) have been identified in multiple unrelated pedigrees as causative of an autosomal dominant disease with complex phenotypes that include motor neuron degeneration, FTLD, cerebellar ataxia, myopathy [20, 21], pure motor neuron disease [142], familial late-onset spinal motor neuropathy [143], and familial mitochondrial myopathy [22], and Charcot–Marie–Tooth disease type 2 [144]. Given the rapid expansion of the number of families identified with mutations in CHCHD10, it is likely that they represent a substantial portion of fALS. Several different amino acid substitutions

have been identified, and the prevalence of CHCHD10 mutations was estimated at 1.4–3.5% of ALS and FTD-ALS cases [145], although some of the reported sequence variants have uncertain pathogenic significance [20, 145].

CHCHD10 is a 14 kDa protein localized to the mitochondrial IMS [20, 22]. It is a member of a family of proteins defined by a twin-CX_9C motif, which include several other mitochondrial proteins with different functions, such as Cox17, a copper chaperone for cytochrome c oxidase (COX), and Mia40, a key component of the disulfide relay system for protein import in the IMS. CX_9C proteins require Mia40 to correctly fold and acquire the intramolecular disulfide bonds that allows for their retention in the IMS [146], including the copper chaperone for SOD1 (CCS) [147]. Interestingly, it was proposed that CHCHD10 is involved in mitochondrial bioenergetic functions, since downregulation of its expression in HeLa cells resulted in decreased COX activity and ATP levels [148]. It was also shown that transgenic expression of mutant CHCHD10 in cultured cells causes fragmentation of the mitochondrial network [20, 22]. Furthermore, fibroblasts from patients carrying a CHCHD10 mutation showed moderate respiratory chain defects, which were elicited by growth in the nonfermentable sugar galactose. Muscle biopsies evidenced clear features of mitochondrial myopathy, including COX-negative ragged red fibers and multiple mtDNA deletions [20].

By immuno-electron microscopy, CHCHD10 appears to concentrate in proximity of the inner membrane cristae [20]. Recently, it was proposed that CHCHD10 takes part in the mitochondrial contact site and cristae organizing system (MICOS) complex, where it interacts with mitofilin, the principal component of MICOS and with two other CHCHD10 proteins, CHCHD3 and CHCHD6 [149]. It was shown in cultured cells that CHCHD10 mutations lead to MICOS complex disassembly and loss of mitochondrial cristae. Such structural disarray also causes alterations of the mtDNA, with a decrease in the number of nucleoids, the structures in which mtDNA and proteins interact. It was also shown that repair of mtDNA, after damage imposed by oxidative stress, was impaired in CHCHD10 mutant fibroblasts, possibly leading to accumulation of deleted mtDNA molecules found in patients' muscle. The molecular mechanism leading to mitochondrial dysfunction in CHCHD10 mutants remain to be

fully elucidated, but the presence of missense mutations that do not alter the protein steady-state levels, and yet cause abnormalities, suggest that protein misfolding leading to aggregation or aberrant protein–protein interactions may be involved. In the future, it will be important to confirm the biochemical and pathological findings in tissues directly affected by the disease, such as brain, peripheral nerves, and skeletal muscle. Generation of mutant mice harboring CHCHD10 pathogenic mutations may be useful in studying the pathophysiology of these mutations *in vivo*.

7.3 Conclusions

In many neurodegenerative disorders, there is an issue of protein aggregates/inclusions upon pathological assessment. However, whether the protein aggregates, protein oligomers, or forms of a misfolded protein are the culprit of disease is still unclear. Furthermore, direct evidence correlating these abnormal species of the mutant protein and some pathological phenotype in cells, organelles, or tissue is scarce.

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Chapter 8

Impact of Mitostasis and the Role of the Anti-oxidant Responses on Central Nervous System Disorders

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8.1 Introduction

Mitochondria are the "powerhouse" organelles of the cell, providing adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) of nutritional sources. They are highly dynamic organelles that undergo fission and fusion, move into the cell along the microtubules to generate the mitochondrial network, and likely provide locally the required ATP [1]. Apart from ATP production, mitochondria contribute to plenty of cellular functions including the buffering and sequestering of the intracellular Ca^{2+}; the modulation of the cellular reduction–oxidation (redox) rate, the production of reactive oxygen species (ROS), and also the regulation of cellular transcription rates [2]. By affecting both energy metabolism and apoptosis, mitochondria play an important role in cellular survival. Among different cell types, neurons are particularly dependent on the proper function of

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mitochondria because of their high metabolic demand. Specifically, since the brain requires ~20% of the oxygen and ~25% of the glucose consumed by the human body, mitochondria are crucial in neuronal development, function, and survival [3, 4].

Considering the critical importance of mitochondria for (among others) cellular energy production, it is not surprising that they (differently from other organelles) have developed their own integrated system of mitochondrial chaperones and proteases which, along with the fission/fusion machinery, ensure proper mitochondria dynamics and bioenergetics regulation, also referred to as mitostasis [5].

Mitochondria are unique organelles since they contain their own circular DNA (mitochondrial DNA, mtDNA) and transcription/translation machinery [6]. However, the human mtDNA encodes for only 1% of the mitochondrial proteome, while the nuclear DNA encodes the rest of mitochondrial proteins which are synthesized in the cytosol and must be imported into the mitochondria [6]. The import of newly synthesized peptides and their assembly into the large multisubunit OXPHOS protein machines is followed by a group of independent mechanisms involved in the maintenance of mitochondria integrity and function [7, 8]. Moreover, mitochondria morphology is orchestrated by the continuous processes of fusion and fission that allow for the mixing of mitochondrial content and segregation of damaged mitochondria parts, respectively, in order to enable a selective autophagy known as mitophagy [9] (Figure 8.1).

In addition, the activity of mitochondria, through mitochondrial respiration, is strongly correlated to the generation of ROS [10]. Increased ROS levels can cause oxidative stress, damage mitochondria proteins, and alter mitochondria dynamics [10]. Therefore, ROS need to be maintained low in order to prevent damage, particularly in neurons which are highly vulnerable to oxidative damage [11]. One of the major cellular responses to oxidative and proteotoxic stress is driven mainly by the Nuclear factor erythroid 2-related factor-2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) signaling pathway [12].

The association of mitochondria and oxidative stress with many neurodegenerative disorders underlies the importance of maintenance of mitochondria integrity and function in the neuronal cells [13, 14]. Herein, we discuss the molecular pathways of mitochondrial quality control and the role of oxidative stress in neurodegeneration.

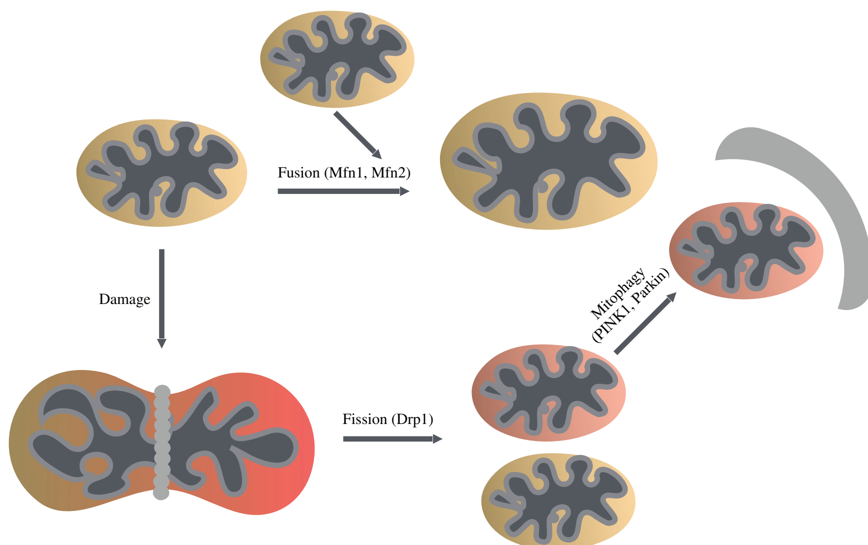

Figure 8.1 Mitochondria dynamics. Mitochondria undergo continuous cycles of fusion and fission. Fusion is driven by Mfn1 and Mfn2, and allows the mitochondria to mix their content and dilute the damage. Fission is largely conducted by Drp1 leading to generation of two daughter organelles. Fission results in segregation of damaged transiently depolarized mitochondria.

8.2 Mitostasis in the nervous system

The mitochondrial proteome is composed of ~1,500 polypeptides, of which only 13 are encoded by the mitochondrial genome. Thus, the vast majority of mitochondrial proteins is synthesized in the cytosol and must be imported into the organelle [6]. The import of proteins which are synthesized in the cytosol is under the surveillance of molecular chaperones, namely the cytosolic Hsp70 and Hsp90 chaperones, along with the mitochondrial mtHsp70 and the multimeric Hsp60–Hsp10 machineries; the effective regulation and tight monitoring of the transport process is essential in order to avoid the formation of protein aggregates or misfolded proteins [8, 15]. Additionally, the oxidation of mitochondrial proteins from ROS can lead to the accumulation of damaged and/or misfolded proteins [16]. Therefore, proteins that become dysfunctional due to, e.g. exposure to oxidative stress, must be either "repaired" or degraded.

The turnover of unfolded or damaged proteins is driven by a complex network of mitochondrial proteases. These proteases are evolutionarily conserved and belong to: (a) the ATP-dependent proteases, namely the LON protease, the Clp Protease Proteolytic (CLPP) subunit, and the mitochondrial AAA (ATPases Associated with diverse cellular Activities) proteases of the inner mitochondrial membrane and matrix, (b) the two ATP-independent proteases, namely ATP23 and HtrA2, and (c) the two oligopeptidases, namely, the Presequence Protease (PITRM1, also known as PreP) and the Mitochondrial oligopeptidase M (MEP, also known as neurolysin) [17].

Several neurodegenerative diseases like Parkinson's (PD), Alzheimer's (AD), Huntington's (HD), amyotrophic lateral sclerosis (ALS), and hereditary spastic paraplegia (HSP) are associated with different mitochondrial defects caused by the dysfunction of one (or more) of the aforementioned proteases.

LON is an ATP-dependent protease that mediates selective degradation of abnormal/mutated proteins [18]. Low LONP (mammalian LON) levels have been implicated in an HSP-dominant form and in ALS [19, 20]. HSP is a group of disorders characterized by degeneration of the motor neurons, progressive spasticity, and weakness of the lower limbs [21]. ALS is a fatal neurological disorder characterized by the loss of upper and lower motor neurons and typically causes death within 3–5 years of onset [22]. Although the substrate recognition mechanism by LON still remains to be elucidated, LONP preferentially degrades unfolded polypeptides, rather than aggregated protein structures [23, 24]. LONP has also been associated with mtDNA regulation either through direct binding to DNA or *via* selective degradation of transcription factor A, mitochondrial (TFAM) mitochondrial transcription factor [25, 26].

The mitochondrial m-AAA protease in human cells exists in two isoforms: one of homo-oligomers and another that forms a hetero-oligomer with paraplegin (SPG7) [27]. m-AAA protease has an important role in the assembly of the respiratory chain enzyme complexes as well as in the maturation of mitochondrial ribosomal components [28]. Mutations of the m-AAA are linked to spinocerebellar ataxia and HSP [29, 30]. These mutated neurons are characterized by mitochondrial oxidative damage and impaired stability of the respiratory complexes [31, 32]. Spinocerebellar ataxia is characterized by degeneration of Purkinje cells with severe clinical symptoms such as tremor and spasms [33].

The HTRA2 protease of the mitochondrial intermembrane space is involved in the degradation of oxidized proteins and in apoptosis. Missense mutations in HTRA2 were reported in patients with hereditary tremor, a predisposing factor for parkinsonism [34]. Also, the PITRM1 zinc metalloprotease has been implicated in AD by being attributed a principal role in the degradation of the amyloid beta (Aβ) peptides [35]. AD is the most frequent form of age-related dementia and it is characterized by the presence of extracellular amyloid plaques which are (mainly) composed of insoluble fibrillar deposits of Aβ aggregates, as well as of intracellular accumulation of Aβ oligomers [36].

8.2.1 *Mitochondria dynamics: Fusion, fission, and motility*

Mitochondria undergo continuous cycles of fusion and fission that coordinate mitochondria morphology. Both processes are regulated by a number of GTPases (guanosine triphosphatases) which are evolutionarily conserved. Mitofusins 1 (Mfn1) and Mitofusins 2 (Mfn2) are GTP-binding proteins of the dynamin superfamily involved in fusion of mitochondrial outer membrane [37]. Downregulation of Mfn1 or Mfn2 in cells leads to mitochondrial fragmentation; additionally, lack of either Mfn1 or Mfn2 implies the total loss of fusion [38]. Mutations of Mfn2 are linked to Charcot–Marie–Tooth neuropathy 2A (CMT2A) [39, 40] which is characterized by muscle weakness, hyporeflexia, and sensory loss in the lower limbs. Mfn2 knockout mice displayed fragmented and absent mitochondria from distal neurites, blocked dendritic outgrowth and formation of axonal projections, as well as enhanced neuronal death [41–43]. In addition, in AD, Aβ aggregates seem to reduce the rates of mitochondria fusion and induce mitochondrial fragmentation [44]. OPA1 is a conserved, large GTPase of the dynamin family involved in cristae remodeling and inner membrane fusion [45]. Mutations of OPA1 lead to degeneration of the optic nerve, known as dominant optic atrophy [46].

Fission is an important process for the generation of new daughter mitochondria; this event is mainly driven by the Dynamin-related protein 1 (Drp1) [47]. Drp1 is highly expressed in the brain, suggesting an important role in neurons. Furthermore, Drp1 is necessary for embryonic development of the mouse brain and for synapse formation in cultured neurons [48]. However, only one case of neuropathy associated with Drp1

mutations has been reported [49]; moreover, in HD, evidences suggest that mitochondrial dynamics are unbalanced towards fission [50, 51].

Another important aspect of mitochondria dynamics is their motility and cellular distribution, especially in neurons which need mitochondria-derived energy (ATP) at sites distant from the cell body [52]. The transport of the mitochondria is a cytoskeleton-based movement via kinesin and dynein motors where the adaptor proteins Rho GTPases Miro1 and Miro2 are required for the attachment of mitochondria to the cytoskeleton [52]. Several lines of evidence support the hypothesis that impaired axonal transport plays an important role in the pathogenesis of a broad range of disorders including ALS, AD, PD, schizophrenia, bipolar disorder, and severe recurrent depression [53–55]. In AD, knockout for presenilin 1 (PS1) in mice impairs kinesin-1-based axonal transport by increased Glycogen synthase kinase 3β (Gsk-3β) activity that enhances the phosphorylation of kinesin-1 light chains (KLC) [56]. This event resulted in a reduction of the amyloid precursor protein (APP) density, of synaptic vesicles, and mitochondria in hippocampal neurons and sciatic nerves [56]. Furthermore, AD neurons of mouse models and patient brains displayed reduced anterograde transport of axonal mitochondria [57, 58]. Thus, axonal transport may alter neuronal function by interfering with both trafficking and distribution of important cargoes, including mitochondria.

The microtubule-associated protein tau, which promotes the assembly and stabilization of the microtubule to the cytoskeleton, has been shown to regulate axonal transport of membranous organelles, including mitochondria [59]. In addition, expression of mutant tau leads to mitochondrial dysfunction, alteration of mitochondrial dynamics, and impaired mitochondrial transport. Expression of human tau in *Drosophila* and mouse neurons blocked the recruitment of Drp1 to mitochondria resulting in mitochondria elongation, dysfunction, and cell death [60].

8.2.2 *Mitophagy*

When an unrepairable mitochondrial damage occurs, selective removal of mitochondria by autophagy (known also as mitophagy) takes place [9]. One of the most described pathways of mitophagy is the Pink1/Parkin-mediated

autophagy [61]. Pink1 is a serine/threonine kinase that acts as a molecular sensor which accumulates on the surface of dysfunctional/depolarized mitochondria. This event directly phosphorylates Parkin and induces its recruitment in the mitochondria, where it ubiquitinates the fusion proteins Mfn1 and Mfn2, Miro1 and Miro2, as well as other proteins of the outer mitochondrial membrane [62–64]. Following Parkin-mediated ubiquitination, the selective autophagy adaptor protein p62/SQSTM1 is recruited to mitochondria and promotes autophagy [65, 66]. Recently, two new Parkin-independent pathways have been described in which Pink1 plays a crucial role in the recruitment of autophagic machinery independent of Parkin [67, 68]. Mutations in Pink1 and Parkin are more prevalent in patients with autosomal recessive PD early onset [69]. PD is the second most common neurodegenerative disease and is mainly associated with the abnormal increase of phosphorylated forms of α-synuclein. The aggregates of α-synuclein form the accumulations known as Lewy bodies which induce the progressive degeneration of dopaminergic neurons [70]. Nevertheless, it is still obscure how Pink1/Parkin mitochondrial dysfunction leads to neurodegeneration. It remains unclear, for instance, whether the Pink1/Parkin pathway regulates mitophagy in neurons during normal conditions. Moreover, the role of Parkin in neurons is controversial since various studies have shown that mitochondrial impairment does not trigger the translocation of Parkin in mitochondria [71]. Also, recent evidence in post-mortem PD brain samples and in mouse models suggests that Parkin is inactivated during PD by post-translational modifications [72].

Altered autophagy or mitophagy seems to contribute to mitochondrial defects in AD brains [73]. Although there is an increased autophagic rate, AD brains still exhibit aberrant accumulation of structurally altered mitochondria, being characterized by reduced size and broken internal membrane cristae [73]. Accordingly, a recent study by Ye *et al.* [58] showed that there is a strong induction of Parkin-mediated mitophagy in both AD neurons of mouse models and patient brains. Moreover, hAPP neurons exhibit increased recruitment of cytosolic Parkin to depolarized mitochondria; notably, the disease progression is followed by the depletion of cytosolic Parkin, suggesting a deficit in mitophagy that likely explains the accumulation of damaged mitochondria [58].

In summary, the association of mitochondrial function and dynamics with a broad range of neurological disorders underlies the central role of this organelle in the function of neuronal cells.

8.2.3 *UPR^{mt}: A Mitochondria-specific unfolded protein response*

The Mitochondrial Unfolded Protein Response (UPR^{mt}) was first described in mammalian cells as a mitochondrial stress response [74]. UPR^{mt} is a form of retrogressive signaling, aiming to maintain the quality control of mitochondria and the functional integrity of the mitochondrial proteome. Accumulation of unfolded, misfolded proteins, or unassembled complexes beyond the folding capacity of the organelle results in the alteration of proteostasis and organelle dysfunction [75]. When this occurs, mitochondria "signal the problem" to the nucleus in order to boost folding and degradation capacity through the transcriptional activation of specific mitochondrial proteases and chaperones. Although UPR^{mt} has been studied in different model organisms, *Caenorhabditis elegans* has been the most useful model for the understanding of this pathway. The first described component of the UPR^{mt} is the C/EBP homology protein (Chop) which heterodimerizes with C/EBPβ and binds to the promoter region of Hsp60 to increase its transcription levels [75]. Chop and C/EBPβ contain at their promoter region two conserved sequences, known as conserved Mitochondrial Unfolded Response Elements (MUREs) [76]. In UPR^{mt}, ClpXP seems to also have a key role, as accumulated unfolded proteins are processed by the ClpXP and are transported across the inner mitochondrial membrane by the matrix ATP-dependent peptide transporter HAF-1 (Mdl1 in yeast) [75, 77, 78]. Deletion of ClpXP disrupts the proteolysis of unfolded mitochondrial proteins, whereas deletion of HAF-1 attenuates its activation during stress [79]. Both proteins are essential for the survival and normal lifespan during mitochondrial stress condition, underlying the important role of ClpXP and HAF-1 in mitochondria protein quality control. Another downstream component of HAF-1 is the bZip transcription factor ATFS-1 (Activating Transcription Factor associated with Stress), which normally is imported into mitochondria and degraded by the LON protease [80]. During mitochondrial stress, ATFS-1 accumulates in the nucleus and activates transcription of UPR^{mt} genes [80]. Another important protein complex necessary for the activation of UPR^{mt} is the

DVE-1/UBL-5 complex that acts downstream of ClpXP/HAF-1. DVE-1 and UBL-5 form a complex that binds to the Hsp60 promoter and other chaperones and proteases during mitochondrial stress [79, 81].

Mutations in the nuclear gene that encodes Hsp60 in humans (HspD1) have been associated with autosomal dominant HSP [82, 83] and with an autosomal recessive inherited hypomyelinating leukodystrophy termed MitCHAP-60 disease [84]. The Hsp60 chaperonin seems to also be important for cellular viability. Knockout mouse for Hsp60 results in early embryonic lethality [85]. These data indicate that the function of the proteins involved in the UPR^{mt} is important for neuron function.

8.3 Nrf2/Keap1 signaling pathway

A critical pathway in maintaining cellular redox homeodynamics is the Nrf2/Keap1 signaling pathway. Nrf2 is a member of the cap'n'collar (CNC) family of transcription factors which stimulates the expression of a wide array of phase II and anti-oxidant enzymes [86]. Under normal conditions, Nrf2 is retained in the cytoplasm by the actin-binding protein Keap1, which functions as an adaptor protein for the Cullin3-based E3 ligase that targets Nrf2 for ubiquitination and degradation by the proteasome [87, 88]. However, cell exposure to electrophiles or oxidants oxidizes Keap1 leading to Nrf2 liberation and translocation to the nucleus, where by heterodimerizing with the small Maf (Musculo Aponeurotic Fibrosarcoma) protein, stimulates the expression of its downstream transcriptional targets by binding to anti-oxidant (AREs) or to electrophile (EpREs) genomic response elements [86, 89–91] (Figure 8.2).

Oxidative stress is strongly implicated in the pathogenesis of many neurodegenerative diseases such as AD, PD, ALS, and HD [92, 93]. In the AD brain, it has been noted that there is a decrease (as compared to normal tissue) in Nrf2 activity [94]. Other studies have shown increased expression levels of the Nrf2 target gene NAD(P)H dehydrogenase (quinone 1) (NQO1) in neurons [95, 96], as well as of the Heme oxygenase-1 (HO-1) in post-mortem temporal cortex and hippocampus of AD brains [97]. Also, Kanninen *et al*. [98] reported reduced mRNA levels of NQO1, the GCL catalytic subunit (GCLC), and the GCL modifier subunit (GCLM), as well as of Nrf2 protein levels in an APP/PS1 AD mouse model. On the contrary, according to a recent study, no significant changes

ARE
NRF2
Keap1
NRF2
ROS
Keap1
NRF2
Cullin3
Ub
Ub
Ub
NRF2
Ub
Ub
Ub

in any of the aforementioned genes were observed in the same mouse model of older age [99]; these contradictory findings can be likely attributed to the stage of the disease.

PD is another neurodegenerative disorder in which oxidative stress is involved [100]. Consistently, it has been noted that Nrf2 is mainly distributed in the nucleus of PD nigral neurons likely indicating an activated state [94]. Moreover, NQO1 was found to be upregulated in astroglial and endothelial cells and also in dopaminergic neurons of human PD brains [101]. In addition, HO-1 protein levels were increased in the dopaminergic neurons, as well as in astrocytes residing within the parkinsonian substantia nigra [102]. Although extensive research has been conducted in mouse models of PD, the data often are not consistent, and more studies should be performed in order to clarify how Nrf2 modulates the pathology of this disease [93].

Compelling evidence suggests that oxidative stress may also underlie the pathogenesis of both ALS and HD. Sarlette *et al.* [103] reported that Nrf2 mRNA and protein levels were reduced in neurons from primary motor cortex and spinal cord post-mortem tissue samples of ALS patients. Proteomic analysis in human HD brain samples obtained from striatum and cortex revealed induction of the anti-oxidant enzyme peroxiredoxines 1, 2, and 6, as well as of the glutathione peroxidases 1 and 6 and increased activity of the mitochondrial superoxide dismutase and catalase, suggesting an ongoing response to oxidative stimuli in HD [104]. In accordance, an initial study showed increased HO-1 protein levels in HD brain relative to normal tissue [105]. Interestingly, it was found that the activation or overexpression of Nrf2 in mouse models delayed the progression, or even the onset, of ALS and HD, thus making Nrf2 a promising therapeutic target for the treatment of both disorders [99]. Taking these data into account and considering that oxidative stress seems to underlie the

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Figure 8.2 Nrf2/Keap1 anti-oxidant response signaling pathway in neurons. Nrf2 is retained in the cytoplasm by the actin-binding protein Keap1 and is targeted for degradation by the proteasome after ubiquitination by the Cullin3-based E3 ligase. Upon increased mitochondria ROS levels, Nrf2 is liberated from Keap1 and translocates to the nucleus, where by heterodimerizing with the small Maf protein binds to the anti-oxidant (AREs) or to electrophile (EpREs) genomic response elements activating thus its downstream transcriptional targets.

pathogenesis of many neurodegenerative diseases, the role of Nrf2 to counterbalance oxidative stress is of great importance for neuronal health.

8.4 Concluding remarks and perspectives

Mitochondria quality control has a key role in mitochondrial functionality and therefore in the normal function of neurons. The several proteolytic modules together with the other pathways involved, such as mitochondria dynamics and the anti-oxidant responses for ROS detoxification, underlie the complexity and the extensive wiring of these networks in order to maintain mitochondria functionality; it should be also noted that the modules involved in mitostasis regulation, cross-talk not only with each other, but also with extra organelle systems, such as the ubiquitin-proteasome system (UPS) in order to maintain mitochondrial proteome stability. In addition, post-translational modifications, like ubiquitination, seem also to be a main regulatory factor of mitochondria quality control.

In the last decade, there was a significant progress in understanding mechanistic details of mitochondria quality control processes. Mitochondria depolarization induces mitophagy, inhibits fusion, and promotes fission, while stress conditions, such as hypoxia, promote mitochondria transport and again trigger fission and mitophagy. However, further studies should be performed to better understand the role of mitostasis in mammals and particularly in neuronal cells. In neurons, mitophagy seems to be activated in the early stages of neuropathologies, but also accumulation of damaged mitochondria can induce the mitophagy deregulation. Moreover, how the balance of mitochondria dynamics is pushed toward fusion or fission in neurodegeneration needs to be further elucidated. Thus, the implication of mitochondrial dynamics and/or mitophagy in CNS disorders needs more investigation in order to understand their contribution to disease onset and/or progression.

Damaged mitochondria produce high levels of ROS, leading thus to oxidative stress, which plays a catalytic role in the pathobiology as well as in the disease progression of most (if not all) major neurodegenerative disorders. Many existing data highlight the Nrf2/Keap1 signaling pathway as a potent target for the treatment of the aforementioned diseases. Considering the

contradictory findings on that topic, as well as the toxic effect that high levels of long-term Nrf2 hyperactivation induce in model organisms (our unpublished findings), it is evident that more studies must be conducted to better understand the Nrf2 functional implication in neurodegenerative diseases.

The much anticipated new discoveries that will precisely elucidate the role of the anti-oxidant responses modules and of mitostasis in neurons homeodynamics will help to develop new therapeutic applications to ameliorate or fight neurodegeneration.

Acknowledgments

We apologize to those authors whose work was not cited due to space limitations. Research studies at the group of IPT are financially supported by the following EU grants: TASCMAR (EU-H2020, GA 634674), MICROSMETICS (FP7-PEOPLE-IAPP 2013, GA 612276), and MEDIHEALTH (H2020-MSCA-RISE-2015, GA 691158).

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Chapter 9

Propagation of Misfolded Proteins in Neurodegeneration: Insights and Cautions from the Study of Prion Disease Prototypes

Robert C. C. Mercer, Nathalie Daude, and David Westaway

9.1 Introduction

Prion diseases are invariably fatal neurodegenerative diseases of humans and other animals that are singular in that their etiology can be sporadic, genetic, or infectious. They are also notable in that the causative agent, the prion, is the only pathogen which lacks an intrinsic nucleic acid genome. The prion protein (PrP) is the central player in these diseases and is a broadly expressed, glycolipid-anchored N-glycosylated protein found in many vertebrates [1]. The structural conversion of the largely α-helical cellular PrP (PrP^{C}) to the disease-associated conformer PrP^{Sc} (scrapie) results in a molecule with significantly more β-sheet secondary structure [2–7]. This three-dimensional transformation produces molecules that are often (but not always) resistant to digestion by broad-spectrum proteases and are poorly soluble in detergents. As PrP^{Sc} accumulates, it induces spongiform change in the central nervous system [8]. Beyond astrocytic gliosis, amyloid plaques formed from PrP^{Sc} can also be present, but are not a ubiquitous feature of these diseases (Figure 9.1).

Human PrP^{C}, encoded by *PRNP* on chromosome 20, is composed of 253 amino acids (this varies depending upon the species) and attains its highest level of expression in neuronal cells [9–11]. The nascent

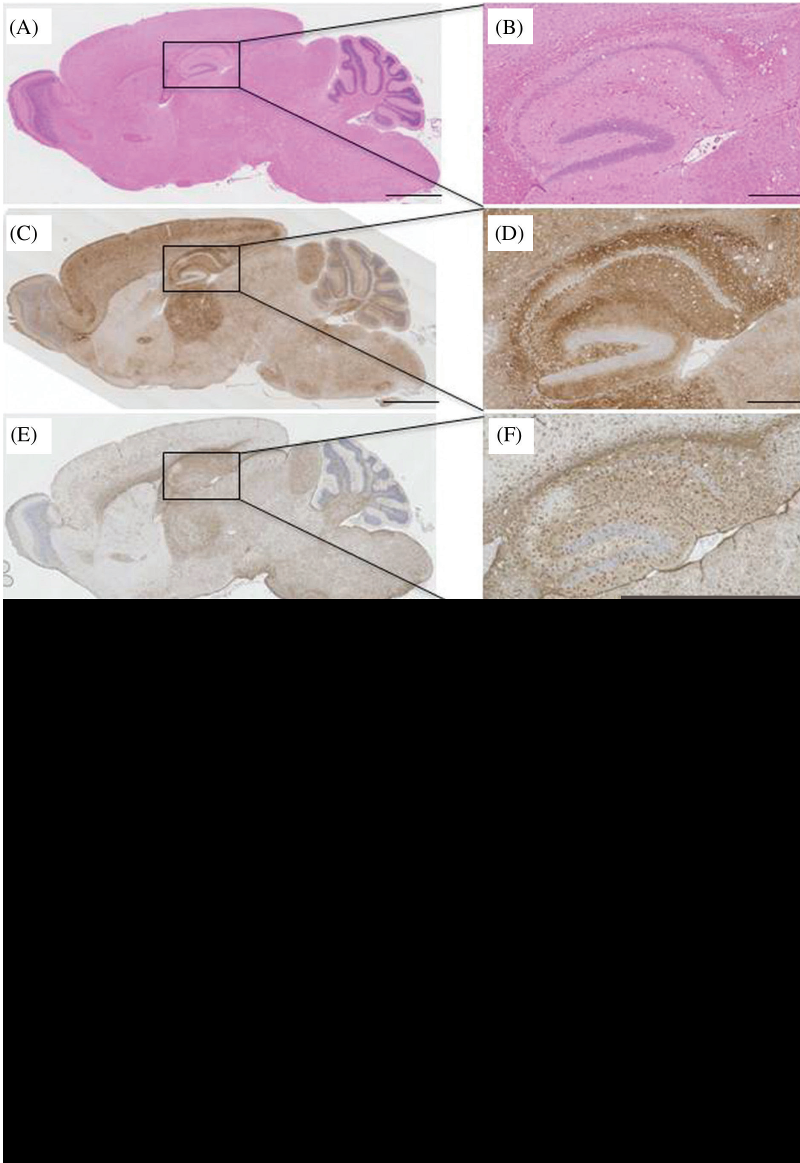

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Figure 9.1 Neuropathological features of prion disease. Sagittal sections from the brain of a terminal-stage, wild-type FVB mouse infected by intracerebral (IC) inoculation with Rocky Mountain Laboratory (RML) (a mouse-adapted prion strain; see below). (A) Hematoxylin and eosin staining revealing spongiform change. (C) PrP^{Sc} staining (brown) after partial

polypeptide is post-translationally processed in the secretory pathway; the first 22 residues direct the protein through the endoplasmic reticulum, where they are cleaved, and ultimately to the cell surface [12]. As the protein transits the secretory system, it can be glycosylated at either or both of two N-linked sites positioned at residues Asn181 and Asn197 [13, 14]. A disulfide bond is formed between Cys179 and Cys214 (in helices 2 and 3, respectively) that further stabilizes the structure [12, 15]. The 23 C-terminal residues serve as a signal for the addition of a glycosyl phosphatidylinositol (GPI) moiety to anchor the protein to the outer leaflet of the cell membrane and they are also removed from the mature protein (Figure 9.2A) [8, 16]. Mature PrP, extending from residues 23–230, is composed of two domains, a so-called disordered N-terminus and a globular C-terminus bearing the N-linked carbohydrate trees. The globular domain consists of three α-helices and two β-strands arranged in a $\beta1–\alpha1–\beta2–\alpha2–\alpha3$ orientation (Figure 9.2A) [17–19]. This domain structure has been resolved for numerous species and is remarkably conserved despite instances of significantly divergent amino acid sequences; for example, an alignment of human and chicken PrP reveals a 43% sequence identity while the structures of the globular domains are all but superimposable [20]. While the N-terminus of PrP, at least in recombinant form, is natively disordered this region nonetheless contains a number of conserved motifs. Following a charged patch come two degenerate hexarepeats that are followed by the octapeptide repeat region. The number of octarepeats (ORs) in healthy animals varies slightly depending on the

Figure 9.1 (*Continued*) hydrolysis to remove PrP^C, antibody SAF83. (E) GFAP staining revealing astrocyte activation largely coinciding with PrP^{Sc} accumulation (scale bar = 2.5 mm). (B, D, and F) High-power views of the hippocampus (scale bar = 500 μm). (G) TgBov XV "bovinized" transgenic mice infected with BSE prions showing striking focal accumulations of PrP^{Sc} in the corpus callosum, IC, terminal-disease stage, antibody 8G8 (scale bar = 2.5 mm). (H) High-magnification view of the corpus callosum (scale bar = 100 μm). (I) "Petblot" analysis using membrane blotted from embedded formalin-fixed brain of wild-type FVB mice infected IC with RML, terminal-stage, antibody SAF83 [123]. (J and K) "Histoblot" of coronal section of wild-type FVB mice infected IC with RML, terminal stage [124]. (J) Ponceau red staining (total protein) and (K) PrP^{Sc} staining, antibody SAF83, scale bar = 2.5 mm.

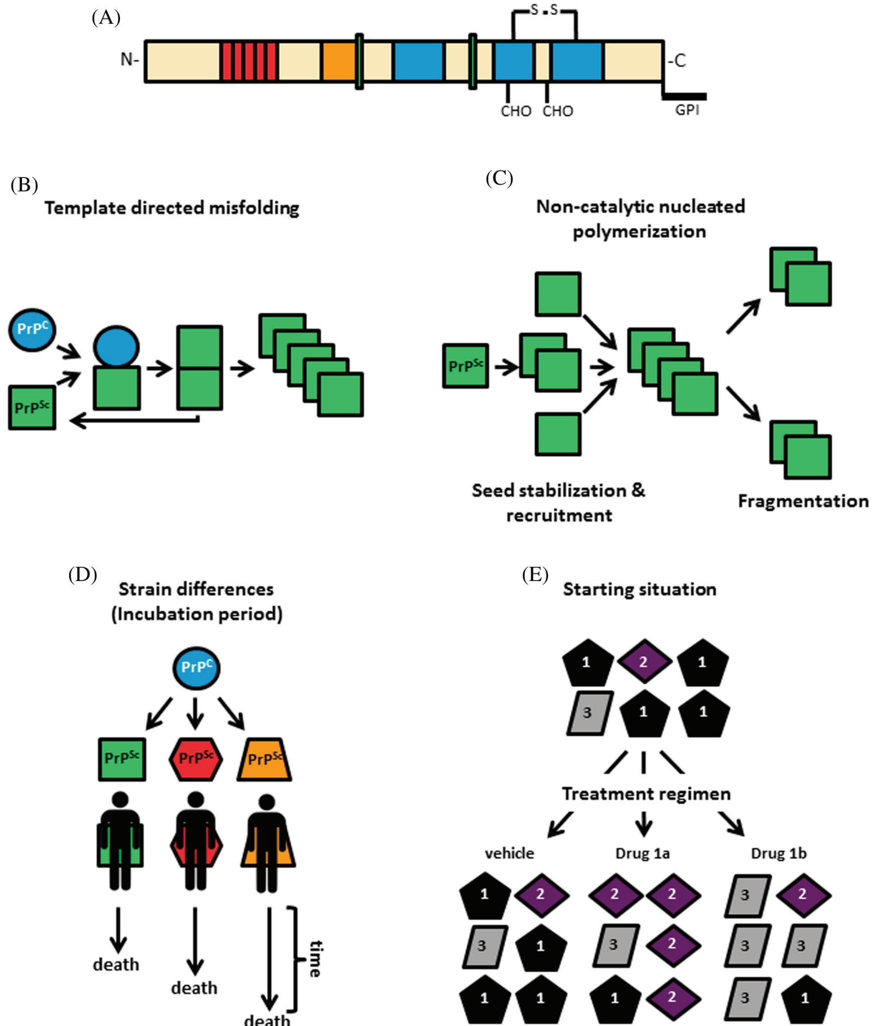

Figure 9.2 Domain structure of PrP and prion-related concepts. (A) Domain structure and post-translational modifications of PrP. Starting from the N-terminus ORs are indicated in red, the hydrophobic region in orange, β-strands in green, and α-helices in blue. The disulfide bond, glycosylation sites, and GPI anchoring are labeled. (B) Template-directed misfolding. In this model, PrP^{C} (blue circle) directly interacts with PrP^{Sc} (green square) forming a heterodimer. The structure of PrP^{Sc} is then adopted by the PrP^{C} and the process continues. (C) Noncatalytic nucleated polymerization. In this model, PrP^{Sc} is continuously present but levels are undetectable and the molecule

species, but five is typical. In mammals, each OR of the general form PHGGGWGQ is capable of binding divalent metal cations, notably Cu^{2+} and Zn^{2+} [21–23]. PrP holoprotein is proteolytically processed at three sites: cleavage at residues 109/110 (mouse numbering system) produces a metabolically stable C-terminal fragment referred to as C1. The corresponding N-terminal fragment, N1, is released into the extracellular environment [24, 25]. Alternatively, cleavage of PrP^{C} holoprotein at residues 88/89 and possibly other sites in the ORs produces a C2 fragment(s); this is of similar length to PrP27-30 produced by *in vitro* digestion of PrP^{Sc} with proteinase K (PK) and fragments observed naturally in some types of infected cells and infected mouse brain [25–28]. Finally, PrP^{C} can be cleaved near the GPI anchor at residue 228 to generate a full-length, unanchored form sometimes referred to as N3 [29, 30].

While the three-dimensional structure of PrP^{C} has been known since the mid-1990s, atomic-level resolution of PrP^{Sc} has yet to be achieved as progress on this front is complicated by the conformer's insolubility and proclivity to aggregate [17, 18, 31]. Initial studies utilizing Fourier transform infrared and circular dichroism spectroscopy indicated that while there was a significant increase in the β-sheet content of PrP^{Sc} relative to PrP^{C}, the molecule still retained some α-helical structure. However, this interpretation has been revisited of late; hydrogen–deuterium exchange

Figure 9.2 (*Continued*) is unstable. Upon stabilization of the seed through recruitment of other PrP^{Sc} molecules, the process continues through fragmentation and generation of additional seeds. (D) Visual representation of incubation time as an example of strain differences. In this example, strains generated from the same PrP^{C} (blue circle) are represented by a green square, a red hexagon, and an orange trapezoid. One criterion used to differentiate between strains is incubation time (duration between infection and death) which is represented here as an example. (E) Selective pressure of drugs on prion strains. In this example, the three strains represented as 1 (black pentagon), 2 (purple diamond), and 3 (grey rhomboid) co-exist but strain 1 has a selective advantage (the dominant strain). Two notional drugs against strain 1, Drug 1a, and Drug 1b are dissolved in a delivery solution (vehicle) and applied to animals. In both cases, strain 1 is depleted but the ratios of strains 2 or 3 begin to shift as these are only partially affected or are unaffected by the drugs. The vehicle-treated control, with ratios and net amount of PrP^{Sc} remaining unaltered, is illustrated as a point of reference.

experiments have since demonstrated that PrP^{Sc} is composed largely of β-sheet secondary structure [2, 32, 33]. X-ray fiber diffraction from PrP27-30 showed that it contains a four-rung β-solenoid structure as its key element [34]. Recent work using an anchorless form of PrP^{Sc} (this results in a more homogenous fibril preparation) has provided, for the first time, a three-dimensional reconstruction of infectious prions through cryo-electron microscopy. These analyses reveal that these prion fibrils consist of two interwoven protofilaments with a four-rung β-solenoid as the underlying structural element, confirming the X-ray diffraction analysis [7, 35]. In terms of re-arrangements of N-terminal to the globular domain, increases in the number of the ORs above the wild-type norm (likely arising by unequal recombination events occurring during meiosis) are associated with the development of genetic prion disease. Depending upon clinical presentation and brain pathology, this can be diagnosed as either genetic Creutzfeldt–Jakob disease (gCJD) or Gerstmann–Sträussler–Scheinker disease (GSS) [36–39]. *Post hoc* analysis with DNA sequencing data in hand suggests that the clinical outcome as gCJD or GSS as well as the age of onset (younger ages correlating with larger expansions) depends largely on the number of supernumerary ORs. Interestingly, the basis of this effect may lie with the altered ability of OR expanded PrP to conformationally respond to changing Cu^{2+} concentrations relative to their wild-type counterparts [40].

9.2 Propagation of PrP^{Sc}

The mechanism by which PrP^{Sc} propagates itself through the conversion of PrP^{C} remains one of the most pressing questions in prion biology. Two models have been proposed that differ in their assumptions about the initial seed. The first, referred to as template-directed misfolding, postulates that once spontaneous conversion from PrP^{C} to PrP^{Sc} occurs, or after introduction of PrP^{Sc} through infection, the energy barrier for conversion is lowered through an interaction between PrP^{Sc} and PrP^{C}. In this scenario, there are now two molecules of PrP^{Sc} that continue to convert PrP^{C} in an exponential manner [41, 42]. While there is a significant energy barrier toward the spontaneous conversion of PrP^{C} to PrP^{Sc}, this would have to be broken in order for spontaneous disease to occur (Figure 9.2B). The second model is known as the noncatalytic nucleated polymerization

model. Here, there is the underlying assumption that conversion of PrP^{C} to PrP^{Sc} is a ubiquitous process and is always present at very low levels but is undetectable and inherently unstable. Upon the formation of a PrP^{Sc} seed of sufficient size, it would be stabilized which would allow for the recruitment of PrP^{C} at a much faster rate upon fragmentation of the resulting amyloid (Figure 9.2C) [43, 44].

There are three well-established methods for the *in vitro* propagation of PrP^{Sc}: protein misfolding cyclic amplification (PMCA), which produces infectious material, and real-time quaking-induced conversion (RT-QuIC), which does not produce infectious material but is establishing itself as a go-to diagnostic. The first report of a cell-free system for prion propagation was extremely inefficient and relied on the use of ^{35}S for detection [45]. For PMCA, the repeated use of sonication steps following periods of incubation greatly enhanced the rate of prion propagation, hence the use of the term "cyclic amplification" in the name [46]. The underlying principle at work is believed to be periods of PrP^{Sc} aggregate growth during incubation which are then fragmented to form additional seeds during sonication which, over the course of many cycles, allows for the exponential conversion of the provided PrP^{C} to PrP^{Sc} [47]. Unlike PMCA, which relies on PK digestion to monitor propagation of PrP^{Sc}, RT-QuIC utilizes the amyloid-binding nature of thioflavin-T (ThT) for detection of amplified PrP^{Sc} and is as sensitive as costly and time-consuming animal bioassays [48, 49]. RT-QuIC has been used to detect prions in accessible fluids and tissues in humans such as cerebrospinal fluid, blood, and, most recently, from nasal brushings poising RT-QuIC to become a clinically applicable ante-mortem diagnostic test for prion disease [50–52]. Another technique of great utility, though with longer turnaround time, is the scrapie cell assay (SSCA) [53–55]. This approach uses living cells, registers infectivity (rather than toxicity or assembly into dye-binding aggregates), and can measure infectious units. This technique has recently been used to investigate the mechanism of slow pathogenesis in *Prnp*$^{0/+}$ heterozygous mice [56].

9.3 Prion strains and species barrier effects

Focusing attention on prion infections, the existence of prion strains is an accepted phenomenon in the field that thus far eludes description at the atomic level but is thought to reflect structurally different subvarieties of

PrP^{Sc} [57]. Operationally, a prion strain can be defined as a prion isolate that, when used to inoculate genetically identical hosts, produces phenotypes divergent from other isolates. Such phenotypes can be defined not only clinically, but also by histopathological analysis of lesion profiles (intensity and localization of spongy change in various brain regions) as well as incubation times (the period between infection and end-stage of disease; Figure 9.2D). Biochemical analyses may also be used as indicators of different strains, though they are not in and of themselves sufficient to define them. In many instances, alterations in the glycosylation pattern of PK-resistant PrP are noted in addition to differences in the size of the PK-resistant fragments visible after *in vitro* de-glycosylation reactions. Thus, the aforementioned size variations of PK-resistant fragments visible after *in vitro* digestion are taken to reflect different PrP^{Sc} conformations that result in more or less accessibility of the PrP residues to PK after the end of the OR region and before the C1 cleavage site; these distinctions in PrP^{Sc} are apparently different despite being generated from the same primary structure [58–60]. A sensitive method to discriminate between PrP^{C} and PrP^{Sc} through the use of differential antibody binding to differentially exposed epitopes, the conformation dependent immunoassay, has also been used to demonstrate structural differences between prion strains without the need for proteolytic digestion [61]. High-resolution structural information of multiple PrP^{Sc} strains, which has yet to be accomplished, will provide insights into the molecular basis of these phenomena [31]. On the other hand, species barriers are effects that can depend upon the amino acid sequence of PrP. In practice, the effect is apparent when prions derived from one species may or may not be able to infect a novel host species, or may only be able to do so after a period of adaptation that can be observed as decreasing incubation times of successive passages of a given agent into the new host species. Notably, in a lab setting, this can be overcome by matching the PrP^{C} expressed in the host with the amino acid sequence of the PrP^{Sc} present in the inoculum. For example, transgenic mice that express hamster or human PrP are susceptible to prions derived from these species while wild-type mice are not [62, 63]. This same type of logic is used in matching PrP^{C} substrate and PrP^{Sc} seed for templated misfolding in *in vitro* PMCA assays (see below) [64].

9.4 Allelic forms of PrP^{C} and internal species barrier effects for infections

The concept of wild-type sequences also requires some qualification in terms of allelic differences present *within* populations of healthy individuals. Sporadic Creutzfeldt–Jakob disease (sCJD) was identified at the start of the 20th century and is by far the most common prion disease of humans, having a worldwide occurrence of approximately one case per million people per year [65]. The cause of sCJD is unknown, though it is commonly thought to arise from the spontaneous generation of PrP^{Sc} that then propagates internally within the host without the involvement of any iatrogenic procedure. Humans have a high-frequency allelic polymorphism in *PRNP* such that codon 129 can code for either a methionine or a valine. Individuals can thus express Met129 PrP^{C}, Val129 PrP^{C}, or can co-express both at close to equimolar levels. These molecules are of course potential substrates for conversion to PrP^{Sc} forms and, of note, individuals that are heterozygous at this position are protected from sCJD relative to homozygotes [66]. A crystal structure of human PrP in complex with an antibody that inhibits prion propagation in cell-culture models, ICSM-18, reveals a packing between PrP molecules that involves residue 129, suggesting that heterozygosity for this polymorphism leads to protection due to inexact docking of PrP^{C} with PrP^{Sc} [67]. A second example of the influence of codon 129 upon prion propagation is seen in the acquired human prion disease, variant CJD (vCJD). vCJD is caused the consumption of cattle infected with bovine spongiform encephalopathy (BSE or "mad cow disease") but, thankfully, has remained quite rare [68–70]. Evidence suggests that it can be horizontally transmitted through blood transfusions and, because of this, strict policies regarding blood donation have been put in place throughout the world [71, 72]. Interestingly, all individuals who have thus far presented with vCJD are homozygous for methionine at codon 129.[a] This has been taken to suggest

[a] Since submission of the chapter, variant CJD has now been described in a Met129/Val 129 patient; Mok T, Jaunmuktane Z, Joiner S, Campbell T, Morgan C, Wakerley B, Golestani F, Rudge P, Mead S, Jäger HR, Wadsworth JD, Brandner S, Collinge J. *N Engl J Med*; 376: 292–294.

that individuals that are heterozygous or homozygous for valine at this position may have yet to, or may never, present with disease. In support of this notion, an analysis of the removed appendixes of people in the United Kingdom demonstrated that 1 in 2000 tested positive for abnormal PrP by immunohistochemistry. Of these, a disproportionate number of positive appendixes belonged to people with the valine polymorphism [73, 74]. A final example of the importance of residue 129 for prion propagation lies with fatal familial insomnia (FFI), a genetic prion disease which is characterized clinically by a progressive and profound inability to sleep which leads to hallucinations and eventual death [75]. This disease has only a single known causative mutation of *PRNP*, Asp178Asn [76]. Interestingly, if the mutated allele encodes for a methionine at position 129, the patient presents with FFI while a valine at this position leads to genetic CJD (gCJD) [77].

A second polymorphism, Gly127Val, was discovered more recently and has, so far, been observed only in the Fore linguistic group that inhabits the northern highlands of Papua New Guinea [78]. This population suffered from an endemic prion disease known as “Kuru” which provided the first evidence of the transmissibility of human prion diseases as it was associated with the consumption of community members during ritualistic mortuary feasts and successfully eradicated by the cessation of such practices [79]. In an astonishing example of rapid human evolution under selection pressure of a common disease (some villages had an incidence of 5–10% [80]), it was found that within the Fore, some individuals harbored an unusual *PRNP* coding sequence polymorphism (Gly127Val) — perhaps a new mutation — that came to have a high allele frequency as it prevented infection with Kuru. The authors hypothesize that even without the cessation of cannibalism, the continued transmission of the disease may well have been prevented because of the ascending frequency of the Gly127Val allele [78]. Importantly, the mutation identified in the Fore was also found to prevent the transmission of sporadic, iatrogenic, and variant CJD prions to transgenic mice homozygous for valine at position 127 [81].

As is the case with any complex biological system, there are exceptions; in this instance concerning the concept that identical primary sequence shared between the host encoded PrP^{C} and that of the invading

PrP^{Sc} facilitates efficient prion conversion. Interestingly, bank voles and transgenic mice expressing bank vole PrP have been recently thrust into the spotlight as "universal acceptors" of prion strains. To date, they have succumbed to every prion inoculum so far assayed regardless of its source and often with extremely abbreviated incubation times [82–85]. Importantly, biochemical and histopathological strain differences are maintained upon passage in bank voles and transgenic mice expressing bank vole PrP. Much work is still to be done, but this new experimental paradigm may prove extremely useful for investigations of the molecular underpinnings of prion strain diversity.

9.5 Prion-like properties of other neurodegenerative diseases

In recent years, the prion concept of templated misfolding of an endogenous neuronal protein has been applied to polypeptides implicated in different neurodegenerative diseases [86, 87]. Here we will briefly summarize the evidence of the self-templating nature of three such proteins for which the evidence of this phenomenon is the most robust: Aβ, tau, and α-synuclein.

Pathological hallmarks of Alzheimer's disease (AD) are extracellular plaques composed of Aβ peptides and intracellular neurofibrillary tangles (NTFs) formed by the microtubule-associated protein, tau [88]. Aβ peptides are generated through the endoproteolytic processing of the amyloid precursor protein (APP) by γ- and β-secretases and first form soluble oligomeric species before forming the amyloid plaques that are characteristic of AD [89]. The first indication that the formation of Aβ pathology could be seeded by the introduction of preformed exogenous Aβ aggregates came from studies on marmosets [90]. This was followed by similar studies using transgenic mice expressing APP carrying a mutation that increases the production of Aβ [91]. An unambiguous demonstration that the induction of this pathology was due to the presence of misfolded and aggregated Aβ was achieved through similar studies that abolished the observed seeding activity of AD patient-derived brain homogenate through immunodepletion, passive immunization, and treatment of the material using formic acid [92]. These mice are also susceptible to the induction of Aβ deposition by intraperitoneal injection [93]. As these transgenic animals would have eventually developed Aβ pathology

without this intervention, these findings could be looked upon as merely accelerating an inevitable process. However, the induction of Aβ plaque pathology was later achieved using transgenic mice that express wild-type APP in which such pathological changes have not been observed [94]. A final demonstration of the prion-like behavior of Aβ comes from the laboratory of Stanley B. Prusiner where it has been shown not only that Aβ derived from patients can induce morphologically different pathologies, similar to prion strains, but that synthetic Aβ can also be used to demonstrate this effect, further likening Aβ to "traditional" PrP prions, where additional macromolecular components beyond misfolded PrP are not required for the templating process [95–97]. It should be noted here that Aβ deposition was observed upon recent examination of the brains of patients that received CJD-contaminated growth hormone or dura matter grafts, suggesting that this pathology may have been concurrently transmitted to these patients [98, 99].

The misfolding of tau is associated not only with AD, but also with a larger group of diseases that have commonly come to be called tauopathies (even though some do not contain mutations in the tau gene, denoted *MAPT*). These diseases include frontotemporal dementia, Pick's disease, corticobasal degeneration, and progressive supranuclear palsy [100]. As for studies outlined above, the first demonstration of the induction of tau misfolding through the introduction of exogenous seeds came from the use of transgenic mice. Here, brain homogenate from mice expressing a mutant form of human tau that causes frontotemporal dementia (Pro301Ser tau) was intracerebrally injected into transgenic mice expressing wild-type human tau (it is important to note that these wild-type human tau expressing transgenic animals do not develop a tauopathy); this treatment resulted in silver stain-positive tau deposits and the hyperphosphorylation of tau which was not observed using control homogenates or when the Pro301Ser derived homogenate was immunodepleted of tau [101]. These studies were later complemented by the demonstration that AD brain homogenate can induce tau misfolding in the brains of wild-type mice (expressing only mouse tau) as assessed by silver and ThT staining, as well as the use of phosphorylation-dependent tau antibodies [102]. As for Aβ, intraperitoneal injection of brain homogenate from Pro301Ser tau mice could also induce tau pathology, though this was done using

presymptomatic Pro301Ser mice [103]. Two groups independently created transgenic mice where mutant (Pro301Leu) tau expression was confined to the entorhinal cortex and demonstrated that this mutant tau can spread in a trans synaptic manner to induce the misfolding of endogenous mouse tau at distant sites [104, 105]. In a recent report, however, one of the groups crossed these mice with tau knockout animals to demonstrate that the mutant tau can comparably spread and accumulate in adjacent brain regions in the absence of endogenous tau (a finding diverging from that seen with prions which require a continual supply of PrP^C substrate). The absence of wild-type mouse tau also markedly blunted the toxicity of the mutant tau (a finding in line with PrP prions). Together, this led the authors to suggest that while misfolded tau has some features in common with prions composed of PrP, it does not strictly meet the criteria of a prion [106]. However, the existence of tau strains has been suggested through the use of HEK293 cells expressing truncated tau that propagate distinct tau aggregate morphologies. These phenotypes can be introduced to naive cells and are maintained over several passages. Interestingly, when these aggregates were injected into the hippocampus of transgenic Pro301Ser mice, the aggregate morphology was maintained over serial passage [107]. However, prion strains made of PrP can be found in situations where the covalent structure of the polypeptide backbone is invariant, whereas establishing a precisely analogous situation for tau — which is subject to a number of natural covalent heterogeneities including complex patterns of mRNA splicing — may prove more challenging.

The final protein we will consider, α-synuclein, plays a central role in three neurodegenerative diseases, namely dementia with Lewy bodies, Parkinson's disease (PD), and multiple system atrophy (MSA) [108]. A transgenic mouse model of synucleinopathy that expresses a mutated (Ala53Thr) human α-synuclein develops motor deficits and displays insoluble and phosphorylated α-synuclein deposits in the brain [109]. Intracerebral inoculation of brain homogenate from sick animals into younger, asymptomatic animals accelerates the development of motor deficits and the appearance of pathological α-synuclein, as do recombinant human α-synuclein fibrils [110, 111]. These findings were extended using nontransgenic mice where it was found that recombinant mouse α-synuclein fibrils could also initiate the development of motor deficits

and dopaminergic neuron loss [112]. The above-mentioned transgenic mice were then used to demonstrate that brain homogenate from two MSA patients could also accelerate these symptoms and pathology in recipient mice, in almost half the time as brain homogenate from a symptomatic mouse [113]. A recent report utilizing these same animals extended these findings through the use of additional MSA patient samples. Surprisingly, they demonstrated that the brain homogenate from PD patients was incapable of such an induction suggesting that different strains of α-synuclein are responsible for the two diseases [114]. Underscoring this observation is the finding that Lewy body extracts from PD patients proved capable of inducing dopaminergic neuron loss in wild-type mice and macaques, though no gross motor deficits were noted [115].

9.6 Re-purposing inhibitors of prion replication and pathogenic pathways?

Prions have served as pathfinders for a deeper understanding of other protein misfolding diseases but when does the analogy break down? To what extent might inhibitors of replication and pathogenesis be repurposed? In the case of replication by templated refolding, there are several clouds on the horizon. First, it is quite likely that the tertiary arrangement of β-structure in infectious PrP is not the same as that found in other diseases [34]. So, the more tailored a structure-guided reagent for PrP is, the less likely it is to be useful for diseases with differing etiologies. Another cautionary message concerns structural heterogeneity in misfolded PrP. Quasi-species of PrP^{Sc} may co-exist, with a predominant species under a given set of conditions reflecting the balance of selection pressures [60]. Be it an antibody or a small molecule, once a therapy is engaged against a predominant template, minor "escapee" species will gain a selective advantage (Figure 9.2E) [54]. While giving two drugs is an option, the number of PrP^{Sc} quasi-species is likely higher. For example, for scrapie (a prion disease of sheep often studied in susceptible mice) at least seven strains are capable of replicating from the same allelic form (*Prnp*^{a}) of PrP^{C} substrate [116]. As the strain phenomenon may be playing a role in the more common neurodegenerative diseases discussed above, caution

should be exercised when using molecules that inhibit the template refolding of seemingly dominant species of Aβ, tau, or α-synuclein as minor species may escape and rise to the fore. Another point to reiterate is that PrP^{Sc}, as defined biochemically and by templating potential, is not necessarily the key toxic species. These reconsiderations now start to define options and approaches for intervention: first, it may be "like déjà vu all over again" for gene therapy concepts considered in the 1990s (albeit now reimagined by the dramatic recent advances in DNA editing technology [117]). This is because PrP^{C} precursor may be a better disease target than PrP^{Sc}, given that cattle and goats seem to do perfectly well without any PrP^{C} and that certain people are heterozygous for frameshift mutations that are putative null alleles [118–120]. Also, whatever the exact toxic species are for PrP, they must nonetheless be derived from the refolding of the PrP^{C} substrate. Going on to consider Aβ, similar debates exist concerning the identity of toxic oligomeric species, so this means that knockdown approaches against *APP* and *MAPT* may yet win the day [121, 122]. Overall, despite some words of caution in the specific instance of replication inhibitors, the application of the prion concept to these more common maladies has been remarkably useful; indeed, the gain in understanding of pathogenesis is stunning with advances almost on a weekly basis. With this in mind, it is a firm hope that the increasing knowledge base about overlaps of other neurodegenerative conditions with prion diseases will go on to provide successful translation at the bedside.

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Chapter 10

Endoplasmic Reticulum Stress Response in Neurodegenerative Diseases

Hyung Don Ryoo

10.1 Introduction

Wild-type and mutant proteins that are prone to misfolding and aggregation underlie many neurodegenerative diseases. Many such diseases manifest in an age-dependent manner, and there is individual variability in the outcome. Emerging evidence indicates that our cells are equipped with various quality control mechanisms that suppress the accumulation of those misfolded protein aggregates, but disease often manifests when such quality control mechanisms decline with age [1, 2]. Thus, there is significant interest in understanding the precise protein homeostasis mechanisms that affect various neurodegenerative diseases.

For normal proteins, folding occurs as newly synthesized peptides emerge from the ribosome. Due to the compartmentalization of eukaryotic cells, ribosomes exist in at least three different forms: ribosomes in the cytoplasm, those on the rough endoplasmic reticulum (ER), and those inside the mitochondria. These different compartments use different sets of chaperones to fold nascent peptides, and if excessive misfolding occurs, the distressed compartments send signals to the nucleus to induce the expression of various quality control genes, including those chaperones that help to reduce the amount of misfolded proteins. The signaling pathway activated by protein aggregation in the cytoplasm is widely referred

to as the heat shock response, and those activated by excessive misfolding in the ER or the mitochondria are referred to as the unfolded protein response (UPR) of the ER and mitochondria, respectively [3–5]. Here, I focus on the relationship between UPR^{ER} and a number of specific neuro-degenerative disorders.

10.2 UPR^{ER} pathways

Most membrane and secretory proteins are synthesized on the rough ER. Thus, mutations in those proteins or other conditions that impair their folding property can impose stress in the ER. Eukaryotic cells are fairly resistant to such conditions, as they have evolved robust UPR pathways that respond to ER stress (henceforth referred to simply as UPR).

The three UPR pathways are particularly well characterized (reviewed in [3]). The transmembrane protein IRE1 mediates one branch of the UPR (Figure 10.1). This ER stress sensor detects misfolded peptides through luminal domain and in turn oligomerizes to activate its cytoplasmic RNase domain. The activated IRE1 helps to reduce ER stress through at least two different mechanisms: one is to cleave mRNAs that encode membrane and

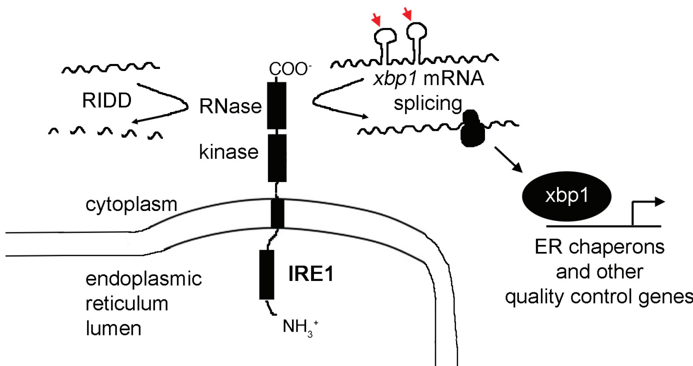

Figure 10.1 IRE1 pathway. IRE1 detects misfolded peptides through the ER luminal domain, and in response, activates the cytoplasmic RNase domain. A specific substrate of IRE1 is the mRNA of *xbp1* (right side), which undergoes mRNA splicing to generate an isoform that encodes an active transcription factor, which induces the transcription of ER chaperones and other quality control genes. IRE1 also targets other mRNAs for degradation (left side), through a process called RIDD.

secretory proteins for decay, thereby reducing the burden of new protein synthesis into the ER, a process that is now widely referred to as RIDD (regulated IRE1-dependent decay) [6, 7].

The other is to cleave the mRNA of the transcription factor XBP1 (or Hac1 in yeast) for mRNA splicing [8–11]. This results in the generation of an active XBP1 splice isoform that enhances the expression of protein folding chaperones of the ER, as well as those involved in misfolded protein degradation in that compartment, also referred to as ER-associated degradation (ERAD) [12]. In addition to these two well-established activities, IRE1 is implicated in the activation of JNK signaling and the regulation of Bcl-2 family proteins [13, 14].

PKR-like endoplasmic reticulum kinase (PERK) is an ER stress sensor that mediates another branch of the UPR. It has an ER luminal domain that is similar to that of IRE1, and in response to ER stress, activates its cytoplasmic kinase domain [15]. PERK's major phosphorylation target is the translational initiation factor eIF2alpha. Phosphorylation of the latter protein in response to stress reduces eIF2alpha's ability to deliver initiator methionyl-tRNA to the ribosome [15, 16]. Thus, PERK helps to attenuate general translation in response to excessive protein misfolding in the ER, thereby reducing the burden on the protein folding system (Figure 10.2). In addition, PERK-mediated eIF2alpha phosphorylation activates a gene expression program through an unconventional mechanism mediated by downstream transcription factors. The best characterized is that mediated by ATF4 [16, 17]. ATF4 is translationally activated when general translational initiation efficiency is paradoxically reduced, as it contains regulatory upstream open reading frames (uORFs) in its 5′ UTR. As the ribosome scans for the ATF4 ORF from the 5′-cap, and as the second uORF overlaps with that of ATF4 ORF in a different reading frame, it is believed that an inefficient translational initiation allows the ribosomes to bypass uORF2 to encounter the ATF4 ORF AUG codon [18]. Once induced, ATF4 induces the expression of various quality control genes, which not only include ER quality control genes, but also those involved in anti-oxidation and amino acid metabolism [19]. Some of these targets, such as Gadd34, Ero-1L, and CHOP, have been reported to accelerate cellular degeneration in response to excessive ER stress [20, 21]. It is noteworthy that a few other transcripts, including CHOP, ATF3, and ATF5, have 5′ UTRs with uORFs

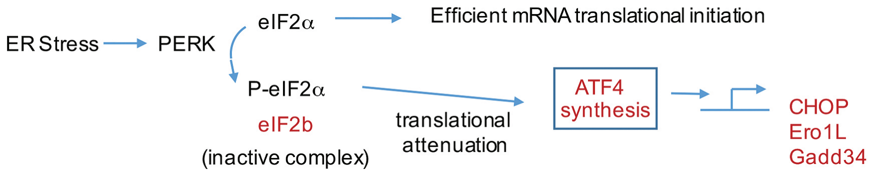

Figure 10.2 PERK/ATF4 pathway. PERK is activated upon ER stress to phosphorylate the translational initiation factor eIF2alpha, thereby prompting this protein to form an inactive complex with its GEF, eIF2B. This results in general translational attenuation, but also paradoxically stimulates ATF4 synthesis. Genes induced downstream of ATF4 include CHOP (transcription factor), Ero1L (ER oxidase), and Gadd34 (phosphatase regulatory subunit).

that allow their translation to be stimulated in response to PERK activation [22, 23]. In addition, certain studies have suggested that PERK somehow activates other transcription factors including FOXO and NF-kB [24, 25].

ATF6 is another factor that mediates yet another branch of the UPR. This unusual transcription factor has a transmembrane domain that is tethered to the ER membrane. Upon ER stress, this protein is trafficked to the Golgi, where it is cleaved by membrane proteases [26, 27]. The cytoplasmic domain of ATF6, which contains a DNA binding domain, can translocate to the nucleus under these conditions to induce ER stress responsive transcripts, which include ER chaperones, as well as UPR regulators such as XBP1 [9, 28].

While most UPR studies focus on these three transcription factors, the literature reports that there are other signaling pathways that mediate gene expression changes upon ER stress. These include Ca^{2+} signaling, as ER is a reservoir of Ca^{2+} that can leak out into the cytoplasm upon stress, and certain studies have suggested that such Ca^{2+} signaling can activate the cell death program [29–31]. With this understanding of the UPR, I will now review the current understandings of the associations between ER stress and a few specific cases of neurodegenerative disorders.

10.3 Parkinson’s disease

Parkinson’s disease (PD) is a disease that is associated with motor symptoms, such as tremors in limbs and face, slowness in overall movement,

and, ultimately, an inability to maintain upright posture. These symptoms are caused by the death of dopaminergic neurons in the substantia nigra. There are a number of causes of PD, a few of which have been associated with excessive ER stress and UPR activation.

One of the underlying causes of PD is alpha synuclein duplication or mutation [32]. In addition, genome-wide association studies indicate that polymorphisms in alpha-synuclein correlate with sporadic forms of PD [33]. It is now widely understood that alpha-synuclein's normal role is to regulate synaptic vesicle trafficking: specifically, alpha-synuclein helps to assemble the SNARE complex for neurotransmitter release [34]. Additional studies support the role of alpha-synuclein at the synapse, including a role in the regulation of synaptic vesicle pool size and reclustering after endocytosis [35, 36].

The disease-causing mutant alleles of alpha-synuclein often mislocalize within cells and is a primary component of Lewy bodies, which are eosinophilic inclusions with radiating fibrils that are found in substantia nigra or the cortex [37, 38]. A number of studies indicate that these mutant alpha-synucleins impose stress in the ER. Such association was first reported by Lindquist and colleagues, who found that mutant alpha-synucleins impair ER to Golgi protein trafficking by associating with Rab1 into the cytoplasmic inclusions [39]. Such idea is further supported by a recent study, which found that alpha-synuclein inhibits the ATF6-branch of the UPR by impairing ATF6 incorporation into COP II vesicles. As ATF6 fails to translocate to the Golgi under this condition, the protein fails to be proteolytically activated as part of the UPR [40]. Such impairment of ER homeostasis has been further supported by a recent study that employed iPSC cells derived from alpha-synuclein mutant patients [41]. PD is frequently studied in another model of dopaminergic neuronal cell death, in which animals are subjected to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treatment, which inhibits mitochondrial electron transport chain. This model is also associated with abnormal UPR signaling, and ATF6alpha mutant mice are more vulnerable to MPTP [42].

PD is associated with other branches of the UPR as well. Substantia nigras of PD patients have elevated levels of ATF4 expression and show signs of IRE1/XBP1 pathway activation [43]. In PC12 culture model, impairment of the ATF4-branch of the UPR makes cells more vulnerable

to MPTP treatment [43]. One of the downstream targets of ATF4 includes Parkin, a gene whose normal function is to help maintain mitochondrial homeostasis and is mutated in certain cases of PD [43].

These studies linking PD to ER dysfunction has led to new efforts to reverse the course of cellular degeneration, which has shown promise in a few PD models. These include the suppression of alpha-synuclein-associated toxicity by enhancing ER to Golgi vesicle trafficking, specifically through the overexpression of Rab1 [39]. In addition, researchers have found that HRD1, a critical ubiquitin ligase that degrades misfolded ER proteins, suppresses alpha-synuclein-associated cell death in a yeast model [44]. Consistently, HRD1 reduced the accumulation of misfolded proteins in patient-derived iPS cells [41]. Delivering spliced XBP1 into substantia nigra to enhance the IRE1/XBP1 signaling reportedly provides protection in a mouse PD model [45]. This idea is further supported by another study, which showed that alpha-synuclein-associated toxicity in rats was suppressed by overexpressing an XBP1 downstream target gene, BiP [46]. Whether these findings will lead to effective clinical therapies remain to be seen.

10.4 Demyelinating diseases

UPR has been associated with a few neurodegenerative diseases that involve demyelination. In retrospect, this should not come as a shock as myelin synthesis requires massive production of lipids and membrane-bound proteins, thereby rendering the ER of the myelin-producing cells vulnerable to ER stress. Here, I will discuss two particularly well-documented cases: Charcot–Marie–Tooth disease 1B, which is associated with the death of Schwann cells in the peripheral nervous system, and Vanishing White Matter Disease (VWM) associated with the loss of central nervous system oligodendrocytes and astrocytes.

Charcot–Marie–Tooth (CMT) neuropathy refers to a group of genetic disorders of the peripheral nervous system, characterized by the loss of muscle tissue and touch sensation in various parts of the body. CMT is further subcategorized, with CMT1, CMT3, and CMT4 involving demyelination. CMT 1B is a specific case caused by mutations in Myelin protein

zero (P0), whose normal function is to help myelin maintain a compact state [47]. In healthy Schwann cells, this protein accounts for 20–50% of total myelin proteins [48]. Among the various mutations in P0, an allele caused by deletion of serine 63 has been characterized in detail in a mouse model. Those studies indicate that the mutant protein fails to traffic to the myelin sheath, and instead, is retained in the ER to trigger UPR activation [49]. Although UPR activation mostly occurs as an adaptive mechanism to help cells survive, the PERK branch of the UPR in this disease model actually helps to accelerate demyelination. Specifically, these studies have found that deletion of a PERK downstream target gene, CHOP, suppresses demyelination [50]. A more recent study has found that genetic or pharmacological inhibition of a CHOP downstream target gene, Gadd34, also improves myelination in this CMT 1B disease model [51]. These studies provide a framework for the development of therapeutic strategies against CMT 1B.

VWM is a disease that involves demyelination of the central nervous system white matter. The disease is caused by various mutations in the five subunits of the eIF2B, whose normal role is to serve as a guanine nucleotide exchange factor (GEF) for the translational initiation factor eIF2 [52, 53]. At the same time, eIF2B plays an important role in the regulation of the PERK/ATF4 branch of the UPR [3, 18, 54]: in this pathway, stress triggers PERK to phosphorylate the alpha subunit of eIF2. Phosphorylated eIF2a, in turn, forms a tight inactive complex with eIF2B. Thus, eIF2 phosphorylation has the same effect as the loss of eIF2B function in cells. Consistent with this idea, various mutations in eIF2B subunits result in reduced mRNA translation, as well as the stimulation of ATF4 synthesis [55, 56]. Although eIF2B plays such essential roles, it is generally thought that the hypomorphic nature of the mutations allow patients to survive, with the symptoms varying from prenatal to adult-onset ataxia and dementia [57]. The loss of white matter in the central nervous system is frequently initiated by trauma and other stress, which may involve eIF2alpha phosphorylation. It is tempting to speculate that the demyelination mechanism in VWM is similar to that established in CMT 1B. However, it remains unclear whether reduced mRNA translation or abnormal ATF4 induction are causal factors in VWM.

10.5 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is characterized by dysfunction or death of motor neurons in the motor cortex, brain stem and the spinal cord. It has been reported that certain sporadic cases of ALS show signs of UPR activation [58]. There are mutations in at least 20 genes that underlie familial cases of ALS, and their proposed functions appear divergent. One of those is an autosomal dominant point mutation allele in VapB, P56S [59]. VapB contains an amino terminal domain called MSP, and a transmembrane domain that anchors the protein in the ER [60–62]. In *Drosophila* and *Caenorhabditis elegans*, it has been demonstrated that the wild-type VapB has its MSP domain cleaved and secreted to act on growth cone guidance receptors such as Ephrin receptors and Lar-like protein tyrosine phosphatase [63, 64]. In addition to these non-autonomous roles, VapB is involved in a number of autonomous roles as an ER protein, including the regulation of phospholipid levels [65, 66]. Consistently, VapB uses its FFAT domain to interact with lipid binding proteins such as Oxysterol binding protein (Osbp) [67]. The P56S mutant equivalents, however, form insoluble aggregates that accumulate in the ER, thereby activating the UPR in model organisms such as *Drosophila* and in mouse motor neurons [63, 68]. Such conditions result in the impairment of MSP secretion and Eph receptor signaling and Osbp mislocalization to the Golgi, as shown in *Drosophila* [63]. Restoring the expression of Osbp in the ER partially suppresses the phenotypes associated with mutant VapB expression [69]. These observations indicate that the ALS-causing mutations in VapB impose stress in the ER and raise the possibility that strategies to restore the function of Osbp and Eph receptor functions can lead to therapeutic effects.

10.6 Autosomal dominant retinitis pigmentosa

Retinitis Pigmentosa (RP) is a genetic disorder that involves progress loss of vision due to the degeneration of photoreceptors. There are many causes of RP, most frequent of which are autosomal dominant retinitis pigmentosa (ADRP) caused by specific mutations in rhodopsin, which encodes the light-sensing proteins of rod photoreceptors. Many such mutations

P

make the encoded protein expressed at low levels, suggesting that the mutations destabilize the protein. Among ADRP-causing rhodopsin mutations, the most frequently found is the P27H allele [70].

Intriguingly, there are dominant mutations in the *Drosophila* rhodopsin-1 gene that underlie age-related retinal degeneration in this organism. While the null allele does not have such dominant effects, a number of missense alleles with such properties have been reported to date [71, 72]. Similar to the human alleles, the *Drosophila* mutants that underlie retinal degeneration are detected at very low levels, suggestive of their instability.

It had been speculated that the instability of mutant rhodopsins might be due to their inability to fold properly. As rhodopsins are membrane proteins that normally undergo folding in the ER, such conditions are likely to impose stress in that organelle. Such speculation was validated first in the *Drosophila* model for ADRP, in which the G69D allele of rhodopsin-1 activated multiple branches of the UPR [31, 73]. Moreover, the P27H equivalent allele was generated with *Drosophila* rhodopsin-1, which also activated UPR, indicative of ER stress [74]. Consistently, rhodopsin P27H allele expression in mammalian cells also activate UPR [75].

Many proteins that fail to fold properly in the ER undergo rapid degradation. One of the major mechanisms by which this occurs is through ERAD, a process that involves ubiquitin ligases such as HRD1, and the proteasome in the cytoplasm. Both the *Drosophila* and human alleles undergo degradation, in part, through ERAD [76, 77]. Another fraction undergoes degradation in the lysosome [77]. At least in flies, the course of retinal degeneration can be significantly delayed through the overexpression of HRD1, indicating that such conditions reduce the amount of toxic misfolded proteins [76].

In addition to the canonical UPR pathways, mutant rhodopsin-1 activates other pathways that contribute to their eventual degeneration. An RNAi screen identified kinases CDK5 and Mekk1 as such contributing factors. These kinases neither affected IRE1 or PERK/ATF4 branch signaling, and they have no known connections with the canonical UPR pathways [31]. These findings suggest that there is a distinct pathway that links misfolded rhodopsins to the cell death machinery. The precise nature of this pathway and their relevance to human ADRP remain unclear.

10.7 Conclusions

In the last two decades, significant advances have been made regarding how eukaryotic cells respond to ER stress. These discoveries, and the assay methods that have been developed, have allowed many studies to uncover the connection between UPR and various neurodegenerative diseases. This review has primarily focused on those diseases whose UPR association is well established. It is noteworthy that there are a number of other neurodegenerative diseases that have been associated with excessive ER stress, which includes Alzheimer's and Huntington's diseases. Future research will help establish precise links between those diseases and UPR, with the ultimate goal of developing new therapeutic strategies.

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Chapter 11

Proteomic Analysis of Huntingtin-Associated Proteins Provides Clues to Altered Cell Homeostasis in Huntington's Disease

Naoko Tanese

11.1 Introduction

Protein misfolding and aggregation are hallmarks of many neurodegenerative disorders. They are characterized by irreversible proteinaceous inclusions termed amyloids that are considered toxic and impair neuronal functions. Age-associated disorders such as Alzheimer's disease (AD) are associated with protein misfolding and propagation of amyloid-like structures containing Aβ peptide.

Huntington's disease (HD) may also be considered a protein misfolding disease. Mutation in the huntingtin (*HTT*) gene causes HD [1]. It is an autosomal dominant trinucleotide-repeat expansion disease in which CAG repeat sequence expands to >35 times. This results in the production of mutant HTT protein with an increased stretch of glutamines near the N-terminus. The normal human *HTT* gene encodes a 350 kDa protein whose functions remain elusive. Investigations of wild-type and mutant HTT proteins have identified roles in transcription, intracellular transport, cytoskeletal structure/function, signal transduction, autophagy, and post-transcriptional gene regulation [2]. HD is characterized by the appearance of amorphous nonfibrillar nuclear inclusions and degeneration of the striatum. Although HTT protein is expressed early in embryos, aging is not an

absolute requirement for the development of the disease. The length of the CAG stretch may affect the type of clinical symptoms. The most classical — uncontrolled jerky movements and gait disturbances — develop by mid-life in most of the patients. Current evidence indicates mutant HTT to cause significant dysfunction of neurons leading to progressive neuronal loss initially in the striatum. Wild-type HTT is essential for development as HTT knockout mice die at day E7.5 [3–5]. Increasing evidence suggests that mutant HTT may alter neurogenesis and development of striatal neurons resulting in neuronal loss. This has led to a view that HD is a neurodevelopmental disorder [6, 7].

To find clues for the mechanism of HD pathogenesis, researchers have conducted experiments to identify proteins differentially associated with wild-type or mutant HTT. These studies have uncovered not only potential new roles for the normal HTT protein, but also provided insights into deregulated cellular pathways caused by mutant HTT.

11.2 Huntingtin and its various cellular functions

Human huntingtin is a large 3144 amino acid protein. It is nearly ubiquitously expressed with the highest level of expression in the brain and the testes. The size and predicted structure of the protein suggest its role as a protein scaffold that coordinates a multitude of cellular functions [reviewed in 2]. The expanded polyQ in HTT has been considered a gain-of-function mutation. Early studies have focused on identifying pathways deregulated by mutant HTT. Recent findings suggest loss-of-function of the wild-type HTT may also play a role in the disease pathogenesis.

Studies have reported pro-survival properties of wild-type HTT. It protects cells from cell death induced by variety of stimuli [8–10]. HTT influences gene transcription by binding to transcriptional regulators including the repressor RE1-silencing transcription factor [11], p53 and CREB-binding protein [12], NeuroD [13], NF-κB [14], and transcriptional activators Sp1 and $TAF_{II}130$ [15]. HTT is involved in vesicular transport of brain-derived neurotrophic factor [16], synaptic precursor vesicles [17], and GABA receptor-containing vesicles [18]. HTT is also involved in ciliogenesis [19], endosomal trafficking [20], and autophagy [21, 22].

11.2.1 *HTT associates with proteins involved in RNA metabolism*

Proteomic analyses of proteins co-purifying with full-length, endogenously expressed HTT in mice [23] [see also 24, 25] uncovered a new role of *HTT* in RNA metabolism. Postnatal day 15 mouse brains engineered to express FLAG-HTT from the endogenous locus [26] were homogenized and fractionated. Soluble proteins from cytoplasmic and membrane-associated fractions were incubated with α-FLAG affinity resin to isolate endogenous FLAG-HTT. Mass spectrometric analysis of HTT purifications identified different functional groups of interacting proteins. Strikingly, significant enrichment of proteins involved in RNA metabolism and protein synthesis was observed. The results supported previous findings that HTT may be involved in some aspect of RNA metabolism.

The HTT protein has not been reported to have a role in RNA metabolism. However, we have located HTT in RNA granules/transport particles in primary cortical neurons [27, 28]. Live cell imaging shows microtubule-dependent movement of HTT in dendrites. HTT co-localizes with mRNA and likely contributes to gene silencing during transport. HTT co-fractionates with translating ribosomes separated by sucrose gradient sedimentation [23]. Translation of mRNAs is a highly regulated process similar to transcription of genes. A number of proteins have been reported to selectively target mRNAs to regulate translation.

The involvement of HTT in post-transcriptional gene expression could explain the specific pattern of cell loss and symptoms seen in HD if select groups of genes/mRNAs are more adversely affected over others. These potential changes need not be large in magnitude. They are likely small to account for the delay in symptom appearance, and normal development seen in affected individuals. An emerging body of evidence suggests that regulated transport and local translation of mRNAs in neurons play a critical role in establishing their connectivity. Our findings implicate normal HTT in these important dynamic processes in neurons. It is possible that mutant HTT perturbs them in some way, contributing to the disease pathogenesis. We think HTT associates with a subset of mRNAs in neuronal RNA granules and regulates transport and local translation of these mRNAs in response to synaptic activity (Figure 11.1).

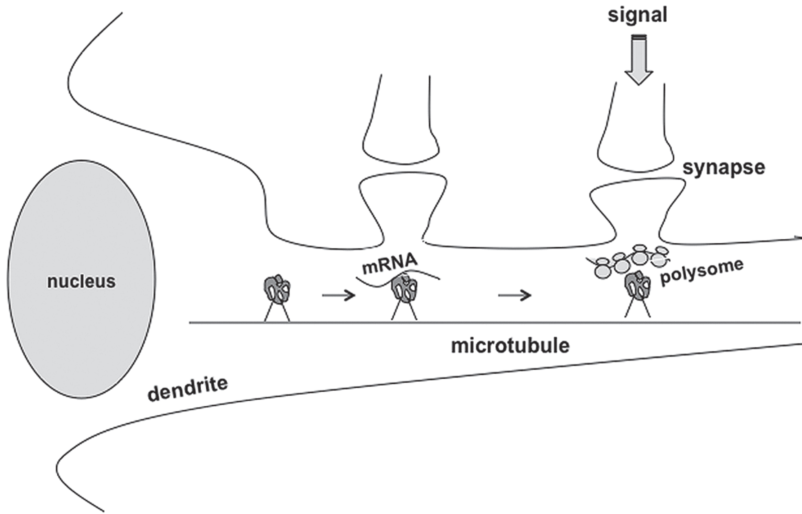

Figure 11.1 Diagram illustrating the association of HTT with RNA transport. RNA granules transport mRNAs along microtubules to a location where translation takes place in response to a specific signal.

11.2.2 *HTT associates with mRNA encoding HTT itself*

Above findings have led to identification of mRNAs that might be present in the purified HTT protein complex [29]. Microarray analyses of co-purifying mRNAs consistently identified *HTT* mRNA as the most enriched RNA in the FLAG-HTT purifications from mouse brains compared with the negative control. RNA-Seq also detected *HTT* mRNA sequences across the entire length of the mouse *HTT* coding region at levels orders of magnitude greater than in the control immunoprecipitate. Immunofluorescence experiments with a fluorescently labeled reporter plasmid containing the 3′ UTR of *HTT* demonstrated co-localization of *HTT* 3′ UTR mRNA with the endogenous HTT protein. Same results were obtained using mouse embryonic stem cells expressing FLAG-HTT.

It is not yet known whether the interaction is direct or through other RNA binding proteins with which HTT co-purifies. This finding has interesting parallels to TDP-43, which regulates its own mRNA levels to keep them within a certain acceptable threshold, thereby preventing the toxic accumulation of the protein [30]. Mutations in TDP-43 are associated

with both familial and sporadic amyotrophic lateral sclerosis (ALS). Like TDP-43 and other RNA binding proteins linked to neurodegenerative diseases, HTT may play a role in RNA metabolism in neurons and share mechanisms and common pathways that lead to their death. Identification and characterization of mRNAs targeted by wild-type and mutant HTT will help to define HTT's role in transport/translation of specific mRNAs.

11.3 HEAT repeats in huntingtin

A discovery-based proteomics approach found HTT co-purifying with a large number of proteins associated with RNA metabolism. Although *HTT* is a large protein made up of many helical (HEAT) repeats with no recognizable RNA binding motifs, HTT may associate with mRNAs directly or indirectly and regulate their expression through transport and translation. Differences in mRNAs that associate with wild-type or mutant HTT could contribute to the tissue (striatum)-specific toxicity found in HD.

It is interesting to note that many proteins that contain HEAT repeats (an acronym for four proteins in which this structure is found: Huntingtin, Elongation factor 3, protein phosphatase 2A, and yeast kinase TOR1) are involved in translation. A genetic screen for neurological mutants in mice identified *listerin1*, an E3 ubiquitin ligase and component of the large ribosomal subunit-associated quality control complex [31]. Human Ltn1 contains 16 HEAT repeats that serve as a linker to position nascent polypeptides targeted for degradation on 60S ribosome subunit [32]. It is tempting to speculate that HTT with its predicted 37 HEAT repeats serve to position/stabilize weak protein interactions and facilitate translation of select mRNAs in a positive or a negative way.

Structural studies of full-length HTT have uncovered clues for how expanded polyQ repeats near the N-terminus confer pathogenic properties on the mutant protein [33]. The amino- and carboxyl-termini of the 3144 amino acid protein appear to interact forming a spherical solenoid composed of five internal domains. The polyQ expansion affects intramolecular interactions that can lead to altered interactions with other proteins. Since HTT associates with many proteins engaged in different cellular pathways, small changes in the platform structure of HTT are predicted to have multiple downstream effects.

11.4 RNA granules are reversible RNA–protein assemblies that may become aggregates

Messenger ribonucleoprotein particles (mRNPs) are reversible self-assembling structures that contain mRNAs and proteins [34]. Studies have found RNA binding proteins implicated in neurodegenerative diseases to localize to RNA granules. They include ALS, spinal muscular atrophy, and fragile X syndrome. These RNA binding proteins (TDP-43, FUS, SMN, Fragile X mental retardation protein [FMRP]) share similar characteristics that affect RNA transport, translation of mRNA, and formation of stress granules [35]. Stress granules are reversible protein–RNA assemblies/aggregates comprised of mRNAs bound by RNA binding proteins and associated with translation machinery. It has been suggested that at high concentrations of RNA binding proteins, stress granules may serve as seed for irreversible toxic aggregate. Interestingly, TDP-43 aggregation is found not only in ALS, but also in inclusions of HD [36]. HTT co-localizes with TDP-43 suggesting a mechanism involving similar reversible protein aggregation in HD.

A new study reports formation of RNA granules derived from translation-stalled polyribosomes in the soma [37]. Using FMRP as a tracer, the authors find mRNA targets of FMRP enriched in RNA granules transported along microtubules to distal dendritic synapses. Fragile X Syndrome results from silencing of the FMRP gene. The finding implicates potentially widespread RNA granule dysfunctions in nervous system disorders.

11.5 HTT associates with mis-spliced *HTT* exon1 mRNA

A mis-spliced mRNA encoding the N-terminal *HTT* exon1 fragment was found specifically in cells and tissues expressing mutant HTT protein [38]. Consistent with the report, we detected both the mis-spliced mRNA containing the exon1 sequence and its encoded polypeptide only in the immunoprecipitates of the mutant HTT protein [29]. This finding raises the question of how HTT complex associates with both mis-spliced and full-length *HTT* mRNA since two transcripts differ in their 3′ UTR sequence. A motif search program (MEME) identified several short sequences in the 3′ UTR from both mRNAs that included a putative binding site for CELF4, a neuronal RNA binding protein reported to bind to

15–20% of the brain transcriptome [39]. CELF4 binds specifically in the 3′ UTR of target mRNAs enriched for synaptic functions. Like HTT, CELF4 is found in RNA granules suggesting a potential link to the HTT complex. Since the full-length and mis-spliced mRNAs have a common 5′ UTR, it is possible that HTT protein associates with both mRNAs through this region. We observed co-purification of wild-type HTT protein with the full-length wild-type *HTT* mRNA. We also found mutant HTT protein to co-purify with the full-length and mis-spliced *HTT* mRNA. The two mRNAs might be differentially regulated by wild-type or mutant HTT protein leading to changes in their levels and contributing to HD pathogenesis.

The discovery has raised a potentially significant role of *HTT* exon1 in the pathogenesis of HD. Transgenic mice expressing HTT exon1 with expanded polyQ sequence were made decades ago and have served as important mouse models of HD [40]. These animals show rapid progression to neurodegeneration and die within 3 months, suggesting mutant HTT exon1 encoded polypeptide is highly toxic. By contrast, knock-in animals or BAC transgenic animals expressing full-length mutant HTT with expanded polyQ show slower progression to disease and live for nearly 2 years [41]. The exon1 *HTT* model has been thought of as an acute case of HD. However, given the new finding, the toxicity associated with the N-terminal polypeptide albeit at low levels could contribute to the long-term disease progression in HD.

11.6 Concluding remarks

Mutations in a variety of genes cause neurodegenerative diseases. RNA binding proteins linked to several neurodegenerative diseases have been localized to dendritic RNA granules. The granules serve to transport mRNA along microtubules to distal sites where local translation takes place in response to specific stimuli. It is tempting to speculate that mutant proteins that accumulate in the granules convert them to irreversible aggregates over time. Recent studies suggest HTT's role in RNA metabolism. Altered neuronal RNA transport and translation may be another dysregulated pathway in HD that contributes to the disease. Detailed knowledge of pathogenic mechanisms is needed to help uncover new targets for therapeutic intervention.

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Chapter 12

Overcoming the Obstacle of the Blood–Brain Barrier for Delivery of Alzheimer's Disease Therapeutics

Eliezer Masliah and Brian Spencer

12.1 Introduction

Alzheimer's disease (AD) is the most common form of progressive dementia characterized by synaptic loss, neurodegeneration of selective neuronal populations, neuro-inflammation, and cognitive impairment [1–3]. Accumulation of intra- and extraneuronal Aβ as well as intraneuronal Tau are believed to be the central players in the neurodegeneration [4, 5]. Currently more than 5 million patients in the United States suffer from AD with another 35 million patients worldwide. This number is expected to double every 20 years due to the increasing aging population. AD is the sixth leading cause of death in this country and the only cause of death among the top 10 in the United States that cannot be prevented, cured, or even slowed [3].

In the initial stages of AD, the neurodegenerative process targets the synaptic terminals [6–8] and then propagates to axons and dendrites, leading to neuronal dysfunction and eventually neuronal death [9]. Neurofibrillary tangle formation with accumulation of phosphorylated Tau is also an important pathological process in AD and has been linked to the cognitive alterations in these patients [10]. Increased immune

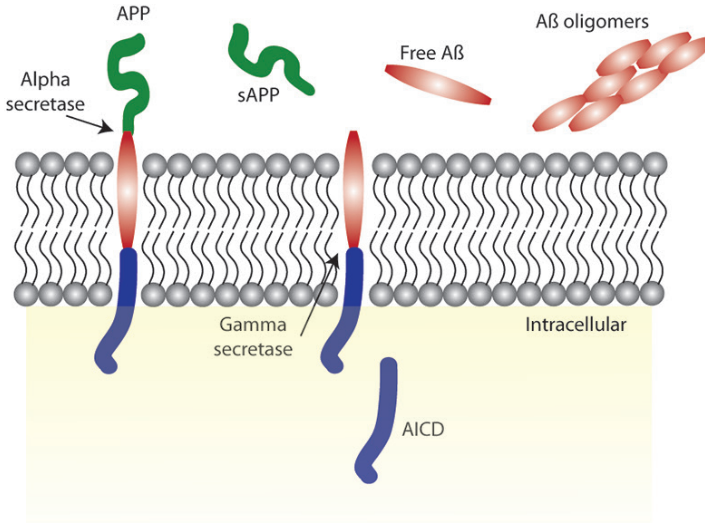

Figure 12.1 Schematic processing of APP by alpha secretase and gamma secretase to release soluble APP (sAPP), APP intracellular domain (AICD), and Aβ.

response by astrogliosis and microgliosis lead to pro-inflammatory cytokine release that contributes to neuronal cell loss [4].

Aβ is formed by the systematic processing of the amyloid precursor protein (APP) transmembrane protein by proteolytic cleavage on the extracellular side of the membrane by b-secretase and by the g-secretase complex at the intracellular side to generate $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ (Figure 12.1) [11]. Genetic and/or environmental factors appear to play a role in the preferential formation of $A\beta_{42}$ fragments over $A\beta_{38}$ or $A\beta_{40}$. The $A\beta_{42}$ fragment is considered more toxic due to its ability to readily assemble into oligomers and higher-order fibrils which themselves present toxic products to the neuron. Aβ monomers and oligomers can also bind to cell-surface receptors promoting signaling pathways and induce neuronal degeneration [12].

$A\beta_{42}$ small-order oligomers may also propagate from neurons to spread the disease [13, 14]. This may explain the progression of the disease and pathological progression of Aβ accumulation and plaque deposition beginning in the entorhinal cortex proceeding to the hippocampus and to cortical areas [15, 16]. This results in progressive memory and cognitive deficits observed in the patients [17].

To date, therapeutic interventions for AD have been primarily limited to treating symptoms without the ability to target the underlying causes of Aβ accumulation. These treatments include acetylcholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and *N*-methyl-D-aspartic acid (NMDA) glutamate receptor antagonists (memantine) [18]. β-Secretase inhibitors have been tested clinically in an attempt to reduce the production of Aβ. These small molecules and peptidomimetic inhibitors have succeeded in reducing plasma and cerebrospinal fluid (CSF) levels of Aβ; however, off-target toxicity and secondary neurodegeneration have been observed [19]. Immunotherapy is one method that has been advanced recently for its ability to reduce the accumulation of Aβ and Tau and potentially treat the underlying cause of AD; however, limited central nervous system (CNS) penetration of antibodies and other biologics appears to have prevented the widespread clinical success in AD [20, 21]. Therefore, developing novel strategies that will facilitate in a controlled manner the trafficking of biological and small molecules across the blood–brain barrier (BBB) will have a considerable impact in the treatment of AD.

The BBB controls the passage of substances from the blood into the CNS. Nonfenestrated endothelial cells form tight junctions preventing the passage of most molecules larger than approximately 500 Da [22]. These brain capillary endothelial cells (BCECs) are notable for their lack of intracellular vesicles compared to endothelial cells of other organs [23, 24]. This restriction in vesicular transport, along with reduced pinocytosis of the plasma membrane, significantly reduces the trafficking of proteins across the BBB. BCECs are regulated by the astrocytic feet and pericytes located on the CNS side of the endothelial cell [25]. Thus, a major challenge for the delivery of protein therapeutics is the transport of large proteins and molecules to the CNS (Figure 12.2).

Transport across the BCECs occurs principally by three main mechanisms: (a) carrier-mediated transporters (CMT) allow the transport of nutrients such as sugars and amino acids from blood to the brain, (b) receptor-mediated transport (RMT) allows the transport of larger proteins and carrier proteins such as transferrin (iron), apolipoprotein (lipids), and insulin from the blood to the brain, and (c) efflux transporters export drugs from the CNS to the blood, e.g. p-glycoprotein and breast cancer resistance protein [26–28] (Figure 12.3). Developing new

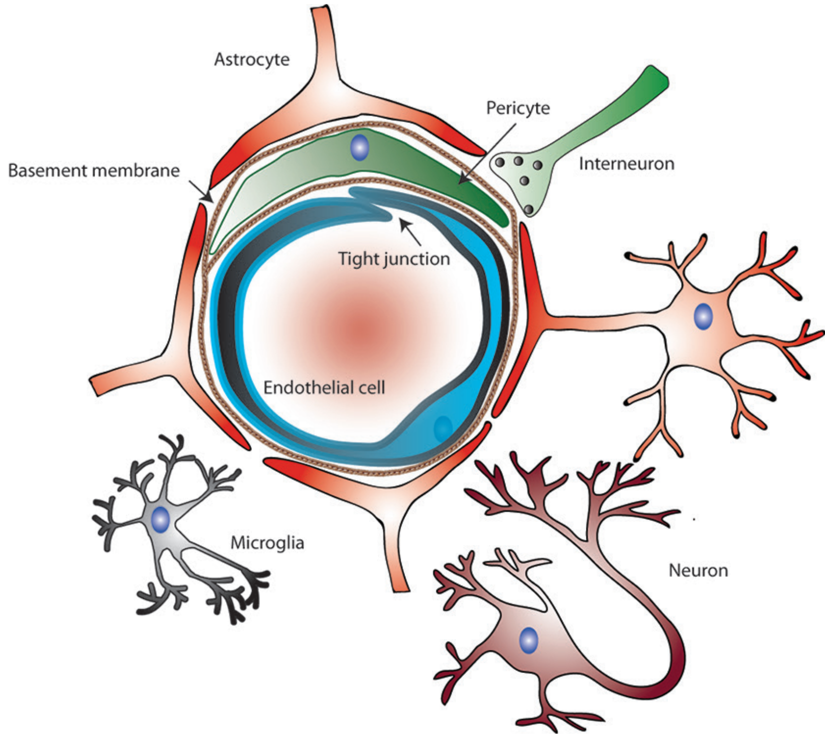

Figure 12.2 Diagram of the BBB depicting the location of the astrocytic feet, pericytes, interneurons, endothelial cells, and basement membrane.

strategies to improve the crossing of antibodies, growth factors and other biological agents as well as small molecules for AD will involve engaging each of these three mechanisms.

12.2 RMT strategies for the transport of agents to treat AD

Receptors expressed on the interior face of the endothelial cell of the vessels at the BBB transport essential proteins, nutrients, and amino acids to the CNS. Glucose and amino acids are transported by CMTs. CMTs typically transport small molecules and selectively recognize size and stereochemistry of molecules, thus these have been less successful for transport of larger protein and therapeutics. To-BBB has utilized the glutathione amino acid

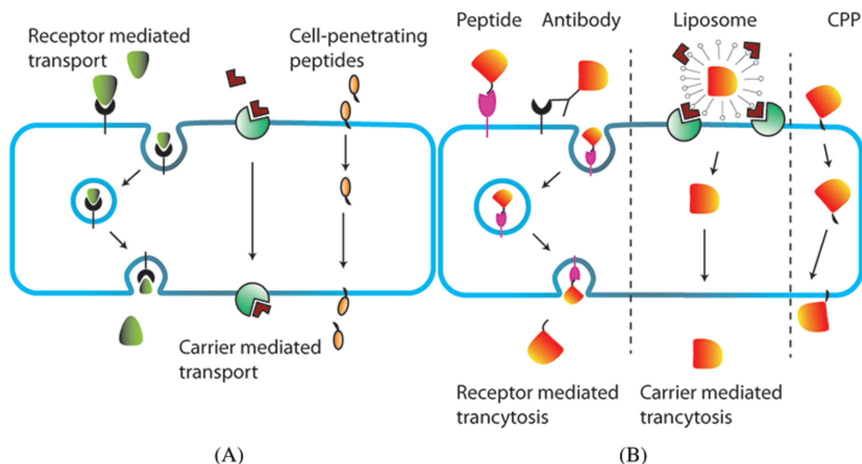

Figure 12.3 Diagram of the BBB transport mechanisms. (A) Endogenous showing RMT, CMT, and cell-penetrating peptides. (B) Manipulation of these transport mechanisms for delivery of a cargo protein across the BBB using a peptide or antibody targeting receptor-mediated trancytosis, liposome encapsulation transport *via* carrier-mediated trancytosis, or cell-penetrating peptide fusion to the target protein.

receptor for transport of reduced glutathione coated pegylated liposomes to transport glucocorticoid, methylprednisone, and antibodies for the treatments of amyotrophic lateral sclerosis, encephalomyelitis, and AD respectively [29–32]. In fact, To-BBB has completed Phase 1/2a clinical trials of doxorubicin delivery for brain metastases of breast cancer using CMT to transport doxorubicin across the BBB [33].

In contrast to the CMT, RMT relies on specific transport receptors binding to partner proteins for transport across the BBB. Size is typically not a limiting factor as endogenous proteins as large as 300 kDa (apolipoprotein B) have been reported transported by RMT [34–36]. On most cells, the binding of a protein to its extracellular receptor prompts internalization followed by fusion with an acidic endosome. The acidic environment causes release of the bound protein, and the receptor is recycled back to the cell surface. In contrast, within the BCEC, the internalized endosomes are transported to the neuronal side of the cells where they release the protein and the receptors are then recycled back to the blood side of the BCEC. Due to the limited number of receptors expressed on

the cell surface of the endothelial cells, this is a saturable process as was observed with the delivery of labeled rh-EPO [37].

For instance, transport of iron by the protein transferrin occurs *via* the transferrin receptor, transport of lipids occurs *via* the proteins, and apolipoproteins *via* the lipoprotein receptors. This occurs by binding of the protein to the receptor on the blood side of the endothelial cell, internalization, and transport to the neuronal side, followed by exocytosis of the protein and release from the receptor. The receptor is then recycled back to the blood side of the endothelial cell. Many investigators have utilized this natural mechanism to transport proteins across the BBB that are not normally transported to the CNS.

The low-density lipoprotein receptor family is a group of cell-surface receptors that bind lipoprotein complexes for internalization to the lysosomes. The family comprises approximately 10 different receptors with the most common examples being low-density lipoprotein receptor (LDLR), low-density lipoprotein-related receptor (LRP), very-low-density lipoprotein receptor (VLDL), megalin, and apolipoprotein E receptor. The receptors are expressed in a tissue-specific manner and primarily bind apolipoprotein complexes. The apolipoprotein, of which the two most prominent members are apolipoprotein B (ApoB) and apolipoprotein E (ApoE), function to bind lipids in the blood stream and target them for lysosomal degradation. Binding of the apolipoproteins to the receptor results in endocytosis and transport to the lysosome where the low pH compartment facilitates the release of the protein complex. The LDL receptor is then recycled to the cell surface. At the BBB, the LDL receptor binds lipoproteins resulting in endocytosis. Following endocytosis by the endothelial cells, a portion of the LDL receptor is shuttled to the apical side of the BBB where presumably the apolipoprotein is released to be taken up by neurons and/or astrocytes (reviewed in [38, 39]).

This RMT process can be co-opted by utilizing an antibody to the receptor and attaching the cargo protein, thus piggy-backing on the RMT. In 1991, Starzyk and co-workers [40] were the first to show that targeting a receptor on the BBB could transport a "cargo" protein to the neuronal side of the BBB. An antibody developed against the transferrin receptor expressed on the BBB was able to transport methotrexate to the CNS.

This same approach has been used to target the transport of proteins and peptides across the BBB efficiently [41, 42].

Alternatively, the process can be co-opted by utilizing as little as the receptor-binding domain of the target protein. These targeting peptides can be as small as 19 amino acids or fewer [43–47]. Thus, binding to the receptor on the endothelial cell is sufficient to trigger endocytosis and transcytosis to the neuronal side. In fact, delivery of naked nanoparticles has been found to be transported to the CNS without the addition of any targeting molecules such as antibodies or receptor binding domains [48, 49]. This BBB transcytosis occurs by nonspecific "sticking" to apolipoproteins in the serum that then themselves bind the LDL-receptor at the BBB and trancytose the whole complex to the neuronal side.

Similar to the apolipoproteins binding to the LDL-R, aprotinin binds to LRP through a Kunitz protease inhibitor domain (KPI). Interestingly, Aβ also contains a KPI and Aβ that lacks the KPI binds poorly to LRP and is not transported across the BBB [50]. Using the KPI as a transport peptide, Demeule *et al.* [51, 52] showed a 10-fold higher transport across an *in vitro* BBB compared to the holo-transferrin molecule. Angiochem has used the KPI domain to deliver siRNA [53], antibodies [54], and a lysosomal enzyme [55] across the BBB in preclinical trials and is currently in phase 2 trials for the delivery of paclitaxel with the KPI domain for malignant gliomas [56].

Delivery of many therapeutic protein and peptides for AD and other neurodegenerative diseases has incorporated one or more of these BBB transport targeting strategies. Antibody therapy directed at either Aβ or Tau has shown promise in animal models and in early clinical trials, however most studies show that of the injected dose, only approximately 1% reaches the CNS [57, 58]. The rest of the protein is probably degraded by proteases in the bloodstream or taken up by other clearance organs such as the liver, kidney, or resident macrophages. Uptake of IgG antibodies into the CNS across the BBB has been a source of interest for some time. Since most large protein require a specific receptor on the surface of the endothelial cells for transport to the CNS and the Fc receptor is not expressed on BBB, it was not known how the IgG antibodies were transported. In 1985, the neonatal Fc receptor (FcRn) was found expressed on

intestinal and vascular endothelium including the BBB. This receptor is composed of the major histocompatibility complex (MHC) class I heavy chain and the β2 microglobulin light chain [59–63]. Binding and transport is restricted to the IgG class of antibodies with little or no uptake of IgM, IgA, IgD, or IgE subclasses [59, 64]. Interestingly, the FcRn binds IgG at an acidic pH (6.0) and releases its cargo at a neutral pH (7.4). The FcRn is localized intracellularly in endosomes and only binds IgG molecules following pinocytosis of the protein at the cell surface [59–63]. With the limited pinocytotic activity of the endothelial cells and the fusion of the endosomes containing the FcRn, there is little wonder that so little IgG is transported into the CNS. However, the FcRn that is bound to IgG can be transported to the CNS side of the endothelial cell, and following fusion with the plasma membrane, the neutral pH of the extracellular environment promotes release of the IgG molecule [65].

Thus, with such an inefficient method for delivering antibodies to the CNS following i.v. infusion, it is a wonder we have therapeutic efficacy in the clinic. In order to improve the CNS uptake of these immunotherapeutics and/or reduce injected dose, several groups have undertaken approaches to fuse BBB transport peptides to the therapeutic antibody. Construction of a bi-functional antibody binding, both the insulin receptor for transport across the BBB and an anti-Aβ binding domain were the first to prove this approach viable [66]. Delivery of this recombinant protein led to reduced plaques in the APP transgenic mouse model of AD. Further attempts to deliver antibodies targeting Aβ across the BBB have targeted the transferrin receptor [67–70].

Interestingly, Yu *et al.* [69] found that bi-functional antibodies that bound too tightly to the transferrin receptor functioned poorly as transport molecules. Instead, antibodies that bound weakly to the receptor were better able to deliver their cargo to the brain side of the BBB. They speculated that the low-affinity antibody may be better able to dissociate from the transferrin receptor after transport across the BBB, whereas the high-affinity receptor still bound to the receptor, recycled back to the blood side with the receptor.

These strategies have relied on the generation of bi-functional antibodies that can target and bind to receptors requiring the generation of large protein for delivery. In contrast, targeting the LDL-R receptor with a peptide

that docks at the receptor binding site can be accomplished with a much smaller moiety. In fact, the LDL-R binding domain from apolipoprotein B is only 38 amino acids and apolipoprotein E is 19 amino acids and both have been shown to be sufficient to transport proteins across the BBB via the LDL-R [46, 47]. Recently, we showed that insertion of the 38 amino acid ApoB LDL-R into a single-chain Fc region antibody could significantly enhance delivery of antibodies across the BBB [43]. In addition to enhanced CNS delivery, the addition of the ApoB LDL-R domain provides a unique cellular uptake mechanism [43].

Once inside the CNS, full-length IgG molecules may bind to their target and be taken up astrocytes and microglia by the Fc receptor expressed on the surface of those cells. Binding to and endocytosis by the Fc receptor results in activation of astrocytes and microglia resulting in release of cytokines and immune activation in the CNS. In contrast, the scFV fused to the ApoB LDL-R domain is taken up by cells by the LDL-R receptor and targeted for degradation by the autophagy pathway without activation of immune cells or cytokine release. Thus, enhanced delivery of scFV without the Fc region of the IgG molecule, while still retaining a mechanism for cellular uptake and degradation, makes these fusion molecules an enhanced therapeutic option.

Delivery of the Aβ endopeptidase neprilysin was also made possible through RMT. We and others have shown that neprilysin can degrade Aβ monomers both *in vitro* and *in vivo* in APP tg mouse models of AD [44, 58, 71–74]. Fusion of the ApoB LDL-R domain to a secreted form of neprilysin has been shown to cross the BBB and reduce the accumulation of Aβ, increase synaptic density, reduce astrogliosis, and improve spatial memory in an AD mouse model [44, 58].

Alterations in NPY expression are associated with Down's syndrome [75], Huntington's disease (HD) [76], and AD [77–82]. Delivery of NPY across the BBB has been facilitated by the addition of the LDL-R binding domain of ApoB resulting in decreased neuronal degeneration and improved spatial memory in the AD mouse model without affecting levels of accumulated Aβ. More importantly, NPY delivery across the BBB increased neurogenesis in the hippocampus, potentially providing an alternative and complementary therapy to Aβ altering biologics [83]. We have previously shown that NPY cleavage by neprilysin produces a new NPY c-terminal

fragment with neuro-protective abilities [84]. Delivering the combination of NPY and neprilysin using RMT transport may provide a one–two punch for therapy for AD.

An alternative receptor-mediated BBB transport involves the use of the envelope protein from the Rabies virus. The Rabies G (RVG) protein binds to acetylcholine receptor on neuronal cells for viral uptake in the CNS and for propagation from neuron to neuron. For uptake across the BBB, presumably the RVG protein binds to the a7 subunit of the AchR on the BCEC for transport [85, 86]. Addition of a 9 amino acid arginine sequence to the RVG peptide facilitates the binding of nucleotide sequences allowing the transport of siRNA across the BBB [85]. This could be an effective mechanism for reducing expression of Aβ in AD.

12.3 Cell penetrating peptides

Unlike transport receptors which are expressed on the endothelial cells of the BBB as well as some other cells, cell-penetrating peptides (CPPs) function to transport protein or peptides into cells in a nonspecific, receptor-independent manner. Cell-penetrating peptides are short stretches of amino acids ranging from approximately 8–28 amino acids in length [87]. These peptides were first identified in 1998 with the characterization of the HIV protein TAT. To date, these peptides have been isolated from a variety of source proteins including: virus, bacteria, insect, mammal, and even synthetically generated [87].

Several cell-penetrating peptides have been utilized to deliver proteins across the BBB following intravenous delivery distribution [88–90]. Delivery of single-chain antibodies (scFV) to the CNS has been accomplished with the fusion of penetratin at a rate comparable to RMT, suggesting that CPP may be useful for another option for improving antibody efficacy to the CNS [91]. In fact, addition of a cell-penetrating peptide can facilitate the absorption of proteins across the small intestinal epithelium [92]. Thus, experimental evidence exists for the presence and use of cell-penetrating peptides for nonspecific transport of proteins from the gut to the blood and from the blood to the brain.

12.4 Conclusion

Several therapeutics for AD have been developed and tested both *in vitro* and *in vivo* in animal models of AD and proved effective. The challenge has been and continues to be delivery to the CNS or the affected regions of the brain. Clearly research in the area of delivery across the BBB by CMT, RMT, or CPP has shown some promise in the lab, now the question is can those promises be transferred to the clinic? Recently, Roche developed an antibody conjugate targeted to Aβ using the transferrin receptor for transport across the BBB [68]. Based on previous reports of increased brain penetration of this method of transport as opposed to the traditional IgG immunotherapy approach, presumably more antibody would be able to reach the CNS of the patient [57].

The advantage of delivering immunotherapeutics by RMT or any other transport mechanism can be either increased efficacy with increased antibody concentrations in the brain from the same injected dose or decreased injected protein resulting in decreased cost and decreased injected total protein. Decreasing the total protein injection could potentially reduce off-target effects, particularly when targeting proteins that are not unique to the CNS. This is particularly important to consider when delivering proteases or growth factors.

Many of the biologics being investigated for potential therapeutics for AD will not cross the BBB endogenously and will need to be either delivered by intracranial injection, infusion, or by cell therapy injection into the CNS. Alternatively, they can be modified to make use of the transport mechanisms mentioned above. The advantage of identifying several alternative transport mechanisms is the option of targeting multiple pathways or proteins in the CNS simultaneously. RMT and CMT are both saturable receptors as is the FcRn receptor. Thus, targeting one receptor for transport will quickly overwhelm the transport kinetics at the BBB. Delivery of multiple targets (e.g. proteases, growth factors, antibodies) may better approached by targeting multiple transport pathways (e.g. transferrin receptor, LDL-R, glutathione receptor).

Identification of more and more specific BBB receptors is needed. The receptors on the BBB currently utilized for transport are the most abundant and were the first to be discovered to transport exogenous

proteins; however, they may not the best for long-term or CNS region-specific transport. Many of the receptors we currently target are highly expressed on other tissues such as the liver, muscle, and spleen, in particular. Thus, targeted proteins are taken up and degraded by these tissues before they even reach the BBB for transport. Therefore, only a small portion of the injected dose reaches the CNS. We may be able to identify receptors that are more highly enriched on the BBB or receptors that are highly enriched in certain regions of the CNS such as the cortex, hippocampus, or cerebellum, thus allowing targeted treatment of certain diseases.

In fact, the identification of novel transport receptors has recently yielding several new targets. Using an unbiased phage display approach, Stanimirovic's group has identified two new antibodies, FC5 and FC44 that bind to receptors on BCEC and transport by RMT across the BBB [93, 94]. More recently, with protein array to identify proteins uniquely expressed on BCECs, they found three new potential targets for future work: basigin, Glut1, and CD98hc. Using an antibody targeted to CD98hc, they were able to transport BACE1 across the BBB. CD98hc and Glut1 were similarly expressed on human BCEC and mouse BCEC making them attractive targets [95].

Finally, the clinical data we obtain from the first in clinic treatment for AD for BBB transport technology will help in developing therapeutics for other neurodegenerative and neuro-targeted diseases. Proteinopathies make up a large class of neurodegenerative diseases including AD but also Parkinson's disease (PD), Lewy body disease (LBD), multiple systems atrophy (MSA), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Immunotherapy and/or delivery of proteases for these disease may also prove helpful if we can bridge the BBB with these proteins. In fact, we have shown that delivery of antibodies targeted for transport by the LDL-R can effectively reduce the accumulation of a-synuclein in mouse models of PD [43, 91]. We have also delivered an α-synuclein protease, neurosin, fused to the ApoB LDL-R binding domain for the treatment of multiple systems atrophy (MSA) to an α-syn tg mouse model of MSA [45]. Similarly, the RVG peptide has been used to deliver siRNA targeting a-synuclein across the BBB in an α-syn tg mouse model of PD [96].

Targeting therapeutics for transport across the BBB allows for the efficient and widespread delivery of many biologics for neurodegenerative diseases without the need for invasive delivery techniques. Clinically, this may lead to reduced cost, increased patient well-being, and increased efficacy. More work can be done in the field, but we are on the cusp of seeing these innovations reap their rewards.

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Chapter 13

Immunotherapies for Alzheimer’s Disease

Einar M. Sigurdsson

13.1 Introduction

Several clinical immunotherapy trials are ongoing, targeting the two major hallmarks of Alzheimer’s disease (AD), the amyloid-β (Aβ) peptide, and the tau protein. Of the two, Aβ immunotherapies are more advanced as the feasibility of targeting this peptide as potential therapy was discovered several years earlier [1, 2]. Furthermore, targeting the tau protein, which is mainly found intracellularly, with antibodies was thought by many not to be possible. The purpose of this review is to give a brief overview of the current status of these two related fields with some thoughts about possible mechanisms of action, which are likely to overlap to some extent for these two related targets.

13.2 Aβ immunotherapies

Although the outcome of many of the active and passive Aβ immunotherapy trials has been rather disappointing, several trials are ongoing (Table 13.1). The hope is that targeting Aβ may work better in the early stages of the disease or prophylactically than in moderate to late-stage AD.

13.2.1 *Epitopes to target*

Four antibodies are in Phase 3 trials, solanezumab, gantenerumab, aducanumab, and crenezumab, which target various forms of Aβ [3–6]. The first

Table 13.1 Aβ immunotherapies in clinical trials.

Aβ Antibody (Isotype)	Aβ Epitope	Stage	Company
Solanezumab (IgG1)	16–26	3	Eli Lilly
Gantenerumab (IgG1)	1–11	3	Roche/Chugai
Crenezumab (IgG4)	16–26	3	Genentech/AC Immune
Aducanumab (IgG1)	3–6	3	Biogen/Neurinmune
BAN2401 (IgG1	Conformational	2	Biogen/Eisai/BioArtic Neurosc
AAB-003 (effectorless)	1–6	1	Janssen/Pfizer
LY3002813 (IgG2A)	3pyroglutamate	1	Eli Lilly
LY2599666*	?	1	Eli Lilly
MEDI1814	41–42	1	AstraZeneca/Eli Lilly
SAR228810 (IgG4)	Conformational	1	Sanofi
Pegylated-Fab of LY2599666*	?	1	Eli Lilly
Aβ vaccine			
CAD106	1–6	2/3	Novartis
Affitope AD02	Mimics 1–6	2	AFFiRis AG
UB311	1–14	2	United Neuroscience
ACI-24	115	1/2	Johnson & Johnson/AC Immune
LU AF20513	1–12	1	H Lundbeck/Otsuka

* Discontinued.

three are all IgG1 antibodies, whereas crenezumab has an IgG4 backbone which minimizes microglial activation. Solanezumab binds primarily monomeric Aβ targeting the middle region of the peptide, whereas the other three recognize mainly various aggregated forms of Aβ. Unfortunately, solanezumab recently failed in its most recent Phase 3 trial which focused on individuals in the early stages of AD [7]. However, as the other three antibodies recognize more forms of Aβ, it is hoped that they may be more effective than solanezumab. In phase 2/3 is an active vaccine, CAD106, containing multiple copies of Aβ1-6 linked to a virus-like particle [8]. One antibody and two vaccines are in Phase 2 trials; BAN2401, a conformational antibody which targets large, soluble Aβ protofibrils [9], Affitope AD02, a vaccine which mimics the N-terminus of Aβ [10], and UB 311, a vaccine containing Aβ1-14 linked to a T-helper

epitope [11]. Another vaccine, ACI-24, is in Phase 1/2 against Alzheimer's and Down's syndrome and contains multiple Aβ1-15 sequences in a liposome preparation [12]. Additionally, several antibodies and one vaccine are in Phase 1 trials. These include: (1) AAB-003 which is derived from the discontinued bapineuzumab against the N-terminus of Aβ and designed to be less likely to activate microglia [13]; (2) LY3002813 which binds to a pyroglutamate form of Aβ [14], (3) LY2599666, an antibody with an undisclosed mechanism which is being directly compared to solanezumab [15], (4) MEDI1814 which has an unknown profile [16]; (5) SAR228810 with an effectorless IgG4 backbone that targets primarily soluble protofibrillar and fibrillar species [17], and Lu AF20513, a vaccine which contains three repeats of Aβ1-12 with tetanus toxin T-helper epitope [18].

13.2.2 *Mechanism of action*

The Aβ antibodies are thought to primarily work extracellularly by targeting both the Aβ plaques as well as smaller aggregates and soluble forms [19] (Figure 13.1).

Microglial phagocytosis of the antibody–Aβ complex is likely an important clearance mechanism for the IgG1 antibodies [20], whereas the IgG4 antibodies or related effectorless antibodies should mainly act by neutralizing the Aβ forms they bind to and thereby preventing their toxicity and further amyloid buildup in plaques or vessels. All Aβ antibodies may also disassemble pre-existing aggregates [21–23]. Prior studies in animals revealed that the Fc portion of the antibody is not required for efficacy [22]. However, antibody-mediated microglial phagocytosis is likely to improve efficacy but may be associated with safety concerns particularly in individuals with high amyloid burden such as those with the apoE4 genotype [24, 25].

It should be noted though that intracellular mechanism of action may be in play as well analogous to what has been observed for tau antibodies. Culture studies have detected Aβ antibody uptake into cells [26], and accumulation of intracellular Aβ aggregates may be an early pathological event in the disease [27].

We should know within a few years if the other Phase 3 Aβ antibodies will be effective, and in about a decade if the earlier stage Aβ

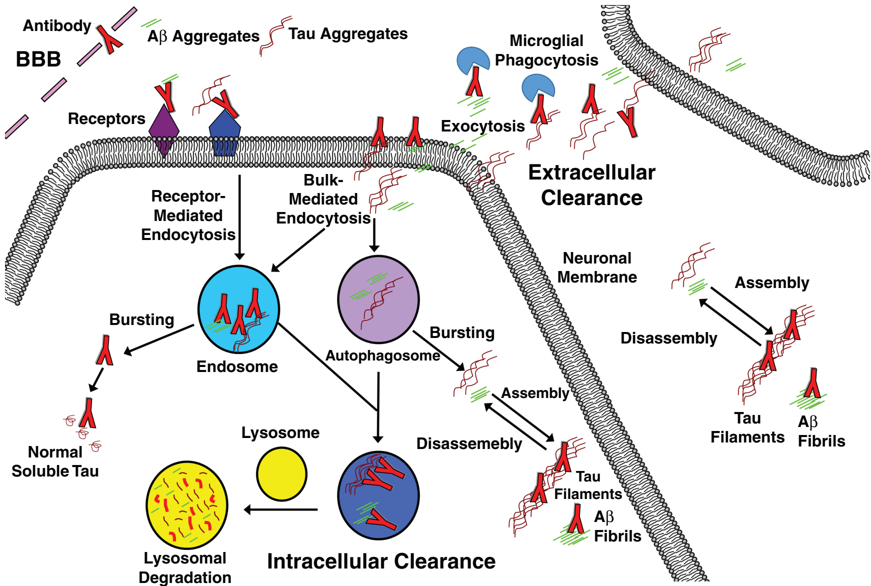

Figure 13.1 Clearance and prevention of spread of pathological Aβ peptide and tau protein by antibodies. Both extra- and intracellular clearance pathways are likely to be involved. The extent of each one is likely to depend on various factors such as antibody properties, the size of the pool of the pathological aggregates within each compartment, and the stage of the disease. Neuronal uptake may be predominantly determined by the charge of the antibody. As most of Aβ aggregates are found extracellularly, that pathway is likely to predominate for Aβ clearance. Most of tau aggregates are found intracellularly but many antibodies may primarily reside extracellularly. In the early stages of disease, the pathologies should be primarily intracellular. Within the neuron, the antibodies are primarily found within the endosomal/lysosomal system but we have detected some tau antibodies in the cytosol. Normal tau protein resides in the cytosol and antibody detection there may require it to bind to a normal tau epitope. It can be envisioned that antibodies may promote lysosomal degradation by disassembling the aggregates allowing access of degrading enzymes. However, some antibodies may promote aggregation which may reduce spreading but can also have detrimental effects associated with buildup of fibrils. Extracellularly, microglial phagocytosis of tau- or Aβ-antibody complex should be the predominant clearance pathway but direct antibody-mediated disassembly of the aggregates may be involved to some extent.

antibodies/vaccines are. Approval for clinical use of any of these antibodies will likely spur development of safe vaccine alternatives, which can be more widely used [28]. An Aβ vaccine may also be more effective than monoclonal antibodies because of more stable antibody levels and broader targets related to its polyclonal response.

Table 13.2 Tau immunotherapies in clinical trials.

Tau Antibody (Isotype)	Tau Epitope	Stage	Disease	Company
BMS-986168 (IgG4)	9–18	1	PSP	Bristol-Meyer-Squibb
C2N 8E12	?	1/2	PSP, AD	C2N Diagnostics/Abbvie
RO 7105705	?	1	AD	AC Immune/Genentech
LY3303560	?	1	AD	Eli Lilly
Tau vaccine				
ACI-35	393–408 [P-396/404]	1	AD	AC Immune/Janssen
AADvac-1	294–305	2	AD	Axon Neuroscience

13.3 Tau immunotherapies

Compared to Aβ, the tau immunotherapy field is still in its infancy, and mechanistic understanding of the efficacy and safety of the various approaches and how those may be improved is not well established. With less research into tau biology over the years compared to Aβ and multiple more epitopes to target, because of its size and post-translational modifications, there is a clear need to support research in this important area. However, as time is of the essence, several clinical trials on tau vaccines and antibodies have already been initiated (Table 13.2) and additional ones are in late-stage preparation [29]. Even though these trials are underway, it is imperative to continue preclinical work in this area to clarify our understanding of the mechanisms involved and to address various challenges.

13.3.1 *Epitopes to target*

Some insights have been obtained regarding which epitopes may be best to target, although other properties of antibodies such as affinity, charge, and isotype are likely to be important as well. The epitope that has received the most attention, phosphoserine 396, 404, which was the key part of the immunogen in our original report [1], has now been confirmed to be a feasible target in several studies [30–40]. Such a vaccine is being employed in one of the ongoing clinical trials [29, 39]. A few studies have compared the efficacy of antibodies binding to different regions of the tau protein [33, 36–38, 40–42], providing additional clarification although the

differences observed may not only be epitope-dependent but can be influenced by other properties of the antibodies such as affinity, charge, and isotype. Other obvious variables that apply when comparing different studies include: the model, age of animals, tau protein expressed and expression levels, dose, number and route of injections, and adjuvant used for active immunizations.

For a detailed insight into ongoing tau immunotherapy programs that are actively seeking a clinical candidate, see recent review [29]. Five Phase 1 and one Phase 2 trials are currently in progress. Three of those are active and three are passive immunotherapies. The active trials consist of testing: (1) KLH-linked Tau294-305 in alum adjuvant in AD patients [43, 44], in phase 1 and 2 trials, and (2) the phosphoserine 396,404 epitope in a liposome adjuvant in an unidentified subject group in a Phase 1 trial [39, 45]. The passive Phase 1 trials are assessing: (1) an antibody against Tau9-18 in healthy subjects and those with an AD related tauopathy, progressive supranuclear palsy (PSP) [46–48]; (2) a tau antibody in PSP patients of unidentified epitope, which based on published work by the group may recognize amino acids 25–30 of the tau protein [42, 49]; (3) a tau antibody of unidentified epitope in AD subjects [50], that based on prior work by the groups involved may be targeting phosphoserine 409 [51], and (4) a tau antibody of unidentified epitope for the treatment of AD [52], which may be targeting a conformational epitope based on a recently published patent of a humanized tau antibody [53]. Many other promising tau immunotherapies are actively being studied, but it is not yet clear if those will pursue clinical trials (for review, see [29, 54–56]).

13.3.2 *Mechanism of action*

In theory, the tau antibodies can interact with their target both extra- and intracellularly (Figure 13.1). It is conceivable that extracellular clearance may be safer but less efficacious than intraneuronal clearance and/or sequestration to prevent secretion and further spread of tau pathology. Our published findings support the efficacy argument [34, 35], as well as preliminary findings from others, showing improved life expectancy in tangle mice that express intracellularly a tau antibody fragment, compared to an identical fragment that was directed into the secretory pathway [57]. The

importance of each pathway likely depends on the availability of the epitope being targeted within each compartment as well as the properties of the antibodies, mainly their charge which influences their entry into cells. It is well established in other immunotherapy fields that manipulating the isoelectric point of antibodies with bioengineering can greatly influence their cell permeability [58]. Generally speaking, acidic antibodies should have lower cell penetrance. The tau antibodies that we have generated and studied in some detail are easily taken up into neurons in various culture and *in vivo* assays as assessed by different techniques. These antibodies have an isoelectric point at or near the neutral range (pH 6–8). In all likelihood, tau antibodies that have been reported not to be taken up into cells/neurons are acidic. Such antibodies would be negatively charged and be repelled from the negatively charged proteoglycans/sialic acid residues on the cell surface. For this reason, their half-life may be longer and therefore to some extent make up for their limited access to the intraneuronal pool of tau aggregates.

Within these different compartments, several different clearance pathways are possible. Extracellularly, the most prominent scenario is likely microglial phagocytosis of the antibody–tau complex [36, 59–61]. Intracellularly, the majority of the clearance is presumably *via* the endosomal/lysosomal pathway, in which tau antibodies have been detected complexed to tau aggregates [1, 34, 36, 62–64]. This binding may disassemble the tau aggregates, thereby allow better access of lysosomal enzymes to degrade those assemblies.

13.4 Other considerations for Aβ and tau antibody therapies

It is not clear whether antibodies with the highest affinities are necessarily the best ones to clear Aβ or tau pathology, and this may depend on the epitope and if they are acting intra- and/or extracellularly. Very strong binding to particular epitopes may prevent degradation of Aβ or tau assemblies and/or promote aggregation which could then seed further aggregation and be detrimental. High affinity could also reduce the half-life of the antibody as the bound antibody could not be reused. On the other hand, formation of large antibody–Aβ/tau complexes could be helpful up to a point by sequestering smaller aggregates, thereby slowing

the spread/progression of the disease. It is conceivable that relatively low-affinity antibodies that still maintain high degree of specificity may be preferable as therapeutic antibodies by tilting these diverse pathways towards degradation instead of furthering aggregation. Binding to certain sites may also prevent seeding as Aβ/tau assembly would be prevented. Lower-affinity antibody may come off the molecule with pH changes in endosomes and be exocytosed and could therefore interact with other Aβ/tau molecules. This could result in longer half-life of the antibody and lower dose for efficacy. Such examples exist from other fields [58]. Ideally, this needs to be explored with antibodies against the same epitope that only differ in their affinities toward it. However, hints of this possibility have been seen with tau antibodies against different epitopes with low-affinity antibody toward a conformational epitope showing efficacy in clearing tau pathology, whereas high-affinity antibody against total tau was not effective in the same mouse model [41]. Comprehensive data that we have generated over the last few years on 4E6 and 6B2 against the same epitope region (Tau386-408) in multiple models supports this scenario, with the lower-affinity antibody 4E6 consistently being effective in clearing tau aggregates with resulting cognitive improvements, whereas 6B2, the higher-affinity antibody, is ineffective in the same models [35]. Targeting both normal and pathological Aβ/tau may also reduce efficacy as binding to the normal peptide/protein will sequester the antibody, which reduces the amount of antibodies that can bind to the pathological form.

There are some indications that isotype differences can influence antibody efficacy. This was shown 13 years ago for Aβ antibodies [65] and more recently for a couple of tau antibodies with similar affinity against the same epitope [37]. For extracellular clearance, this would be expected based on isotype-dependent microglial phagocytosis. However, these findings need to confirmed with antibodies that are identical except for their isotype. As the summary of the Aβ clinical trials indicates, isotype is clearly thought to be important within that field and likely to influence antibody efficacy/safety profile. For tau antibodies acting primarily extracellularly, the same issues apply except vascular side effects of microglial activation are less likely because vascular tau pathology is much less

prominent than Aβ angiopathy. At this point, it is unclear how/if antibody isotype influences antibody uptake into neurons. We have shown such uptake to be important for efficacy of tau antibodies and to be primarily mediated *via* low-affinity FcII/III receptors [34, 35]. Binding to these receptors could in theory be influenced by antibody isotype but has not been well studied.

13.5 Imaging studies to assess brain penetration and target engagement of Aβ or tau antibodies as well as associated clearance of aggregates

A few Aβ targeting β-sheet dye derivatives are already in clinical use [66] and have been employed to verify the presence of Aβ plaques in individuals being considered for clinical trials of Aβ targeting therapies and to assess Aβ clearance. For tau, at least five small-molecule β-sheet dye derived positron emission tomography imaging ligands have shown promise in pilot clinical studies and new derivatives are continuously being made [67–71]. It is likely that these will be used in some of the ongoing and upcoming clinical trials on tau immunotherapies to verify the presence of tauopathy and to monitor treatment efficacy. As most of Aβ is found extracellularly, such dye derivatives are likely to continue to be the gold standard for *in vivo* Aβ imaging. However, antibody derivatives that have shown promise in animal models should be more specific for tau pathology than these β-sheet binders that inevitably will have some affinity for other amyloids [64, 72]. Furthermore, such antibody-based probes may allow detailed information on the pathological tau epitope profile of each imaged subject, which could then direct personalized immunotherapy strategy targeting those specific epitopes. We have successfully used IVIS live imaging of transgenic mice to show that tau antibodies and their derivatives enter the brain and into neurons to bind to intraneuronal tau aggregates [64]. The success of such noninvasive imaging is verified by histological analysis that detects the antibody probe associated with intraneuronal tau aggregates [64]. We are now evaluating the feasibility of using such antibody fragments as PET brain imaging ligands to detect tau pathology and to monitor treatment efficacy.

13.6 Conclusions

As this succinct overview indicates, these are exciting times for Aβ and tau immunotherapies but much remains to be done to advance these fields into approved therapies. Ongoing mechanistic studies should nicely complement the clinical trials and provide important insights for further development of this promising approach. This is particularly the case for the tau field which is less advanced. The Aβ trials are much further along and were supported by extensive preclinical studies. Those will hopefully result in clinically approved compounds in the near future. However, targeting both Aβ and tau should result in more efficacious therapies. Furthermore, as tau pathology correlates better with the degree of dementia than Aβ burden, targeting tau is likely to be more efficacious than clearing Aβ when clinical symptoms have become obvious.

Acknowledgments

E.M.S. was supported by NIH grants NS077239 and AG032611 and a pilot grant from the Michael J. Fox Foundation. He is an inventor on various patents on immunotherapies and related diagnostics that are assigned to New York University. Some of those focusing on the tau protein are licensed to and are being co-developed with H. Lundbeck A/S.

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Chapter 14

Role of the Microbiome in Polyphenol Metabolite-Mediated Attenuation of β-amyloid and tau Protein Misfolding in Alzheimer’s Disease

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Hanna Księżak-Reding, Ali Sharma, Breanna Valcarcel,
and Giulio M. Pasinetti

14.1 Introduction

Alzheimer’s disease (AD) is one of the top public health challenges facing the Western world and developing countries due to ever-increasing aging population [1]. There is, however, no effective means to prevent or to treat AD. There have been more than 100 drug trials targeting specific AD pathogenic mechanisms since 1998, all of which have failed [2]. Since AD is a complex disease involving multiple interrelating pathogenic mechanisms, redirecting therapeutic strategies to simultaneously target multiple pathogenic mechanisms may increase the likelihood of success.

Polyphenols are receiving increasing attention for their potential role in preventing the onset and/or progression of preclinical AD into frank AD dementia [3–6]. In particular, preclinical evidence [7–16] has demonstrated the efficacy of certain polyphenols, acting either individually or in combination, to modulate multiple diverse mechanisms relevant to AD, implicating the potential for novel development of polyphenols for multi-target engagement in AD. In this chapter, we mainly focus on the role of select grape-derived polyphenols (GDP) that might protect against key

AD pathologic mechanisms that are critical to the onset and progression of interrelating AD neuropathologies underlying cognitive deterioration. In particular, we will discuss the roles of polyphenols in interfering against aberrant protein misfoldings that are critical for the generation of neurotoxic aggregates and polyphenol-mediated restoration of synaptic plasticity responses that are critical for learning and memory. We will also discuss the important issue of polyphenol bioavailability following oral administration as the majority of GDP components are biologically available as phenolic metabolites, such as polyphenol metabolites and phenolic acids.

14.2 Grape-derived polyphenols

Polyphenols belong to a structural class of organic compounds with phenolic structural features. Polyphenols are found abundantly in fruits, such as berries and grapes, vegetables, tea, and other plant sources. Polyphenols have been found to possess a variety of health benefits, including cancer prevention [17], heart disease risk reduction [18], and protection against neurodegenerative disorders [19]. Primary polyphenolic constituents include flavonoids (e.g. flavonols, flavanols, anthocyanidins, etc.), phenolic acids (e.g. stilbenes, resveratrol), lignans (e.g. pinoresinol), etc. Previous bio-guided fractionation and bioavailability studies from our research group revealed that grape seed polyphenol extract (GSPE) is primarily comprised of two flavanol (flavan-3-ols) compounds, catechin and epicatechin, as well as gallic acid, a phenolic acid compound. We also found that a commercially available Concord grape juice (CGJ) is rich in flavanols, flavonols (e.g. quercetin, etc.) [7, 20, 21], anthocyanidins (e.g. malvidin, cyaniding, etc.), as well as phenolic acids (e.g. gallic acid, vanillic acid, etc.) (Figure 14.1) [22]. In preclinical studies, we found select bioavailable, bioactive, and brain-penetrating GDPs from CGJ, particularly those among the flavonoid subclass found in CGJ and GSPE, effectively prevent age-related cognitive deterioration in models of neurodegeneration [7, 23]. Moreover, our laboratory and others also found that select flavanol metabolites may benefit cognition, in part, by attenuating abnormal β-amyloid and tau misfolding, which ultimately promoting synaptic plasticity and

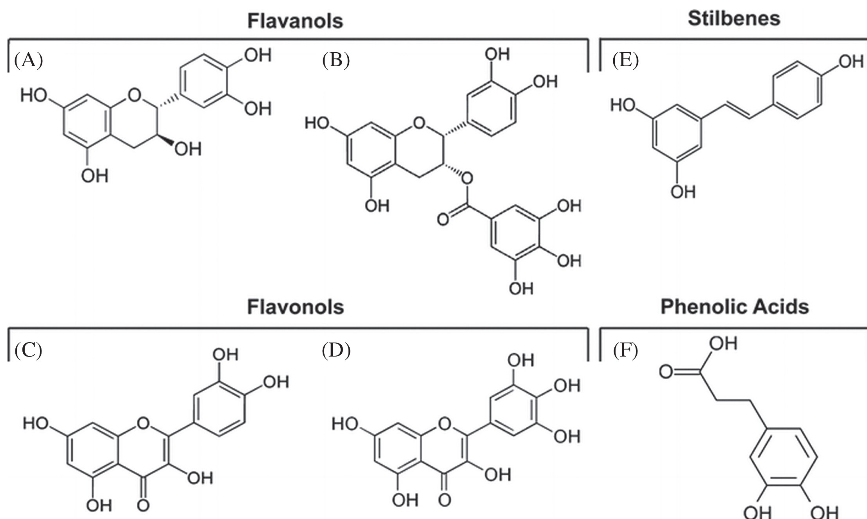

Figure 14.1 Representative structures of polyphenols from grape and grape-derived products. Representatives of flavanols: (A) catechin and (B) epicatechin gallate. Representatives of flavonols: (C) quercetin and (D) myricetin. Representative of stilbenes: (E) resveratrol (RSV). Representative of phenolic acids: (F) dihydrocaffeic acid.

cognitive functions in age-related dementia, such as AD. Consistent with these observations, clinical studies [24] demonstrated that 16 weeks of dietary supplementation with CGJ (15–21 oz. per day) significantly improved cognitive function in mild, cognitively impaired older subjects who were at high risk of developing AD dementia. As further discussed below, our studies provided for the first time the impetus to further develop select bioactive, brain-targeting polyphenol metabolites to prevent AD pathology and promote cognitive function [23, 25].

14.3 GDP and protein misfolding in AD

AD is characterized neuropathologically by the accumulation of extracellular plaques composed of β-amyloid (Aβ) protein, intracellular neurofibrillary tangles of hyperphosphorylated tau protein, and loss of neurons [26]. A major hypothesis regarding the pathogenesis of AD is that abnormally elevated Aβ content in the brain of AD patients leads to the formation of insoluble Aβ fibrillar aggregates, which are major constituents of senile

plaques associated with neuronal loss in AD [27]. This amyloid hypothesis is supported by substantial genetic [28] and preclinical evidence [29], including positive treatment effects of passive immunotherapies in mouse models of AD [30]. Nonetheless, growing evidence suggests that cognitive deterioration in AD is directly linked to the accumulation of extracellular soluble oligomeric Aβ species, rather than amyloid plaque deposition in the brain [31–37]. Oligomeric Aβ, including high-molecular weight (HMW) oligomeric Aβ as well as low-*n* oligomers ranging from dimers to octamers, induces synaptic degeneration and disruption in synaptic plasticity such as decreases in long-term potentiation (LTP) [38, 39], all of which contribute to mechanisms underlying the onset and progression of dementia in AD [35, 40–47]. As accumulation of Aβ and various oligomeric forms of Aβ appear to be critical factors in AD dementia, therapies aimed at inhibiting Aβ oligomerization are therefore increasingly being pursued.

GDP from bioactive dietary products such as GSPE have been shown to directly interfere with Aβ misfolding. Various biochemical methods have been used to investigate the effect of GSPE in various stages of Aβ oligomer formation. Photo-induced cross-linking of unmodified proteins (PICUP) is one such method which is used to evaluate the initial peptide-to-peptide interactions that are necessary for spontaneous oligomerization of Aβ peptides [48]. GSPE has been shown to significantly inhibit initial Aβ–Aβ interactions and block the formation of the Aβ dimer, pentamer, hexamer, and octamer [25, 49]. The method of circular dichroism (CD), used to characterize the conformational transition of Aβ from random coil to β-sheet [50], has also demonstrated that GSPE prevents the conversion of Aβ from random coils into ordered secondary structures (β-sheet) [25, 49, 51]. Visual confirmation, through the use of a transmission electron microscope (TEM), of the effect of GSPE on Aβ fibril formation clearly showed that GSPE significantly reduced the number, length, and width of the Aβ fibrils and that at higher concentrations of GSPE, amorphous aggregates are formed under TEM, rather than fibrils and/or globular structure [25, 49, 51].

GSPE is a complex mixture of flavonoids comprised of monomeric catechin (C), epicatechin (EC), and their gallic acid esters (e.g. catechin gallate and epicatechin gallate), as well as oligomeric and polymeric proanthocyanidins (PACs) comprised of multiple monomeric C, EC catechin

gallate, and/or epicatechin gallate that are generated and covalently linked through C4 → C8 or C4 → C6 interflavan bonds [52]. PACs are the most abundant and complex polyphenols in grapes and grape-derived products (Figure 14.2A). To further tease apart the role of monomeric and complex polyphenols in modulating protein misfolding, we fractionated GSPE into monomeric, dimeric, and PAC GSPE components (Figure 14.2B). Using a combination of PICUP, CD, and TEM assays, we found that each of the three GSPE subfractions is capable of inhibiting Aβ oligomer and fibril formation, with monomers exhibiting the highest potency [49]. More recent work showed the computer modeling of the interactions between Aβ and GSPE monomers, dimers, and oligomers (Figure 14.2C–H; courtesy of Hayden *et al.* [49]). Besides GSPE, which is largely composed of catechin and epicatechin, additional GDPs, such as tannic acid, myricetin, and quercetin, have also been shown to interfere with Aβ fibril formation or to destabilize preformed Aβ fibrils [53, 54].

A number of preclinical studies have been conducted to investigate the anti-aggregation activity of GDP. Oral administration of GSPE to transgenic Tg2576 mice (which model AD-type Aβ neuropathology) significantly reduced Aβ plaque formation in the brain while simultaneously improved animals' spatial memory function [55]. The anti-aggregation activity of GSPE *in vivo* is further supported by the observation that Aβ*56, a 56-kDa Aβ oligomer previously shown to induce memory dysfunction in rodents, was significantly reduced following GSPE treatment [56]. Treatment of Tg2576 mice with a monomeric fraction of GSPE improved spatial memory function and reduced Aβ-mediated neuropathology in the brain [23]. Two studies using polyphenol-rich wines that are made from Cabernet Sauvignon and muscadine grapes showed that dietary supplementation with either of these test wines in Tg2576 mice significantly suppressed Aβ oligomerization and improved cognitive function [57, 58].

Compared to Aβ, much less work has been conducted to investigate the effect of polyphenols on the misfolding of microtubule-associated tau. Abnormal misfolding of tau leads to the aggregation of tau into paired-helical filaments (PHFs), which are ultimately deposited as neurofibrillary tangles in brain cells. The formation of PHFs together with the deposition of neuritic plaques in the brain are the defining neuropathological features

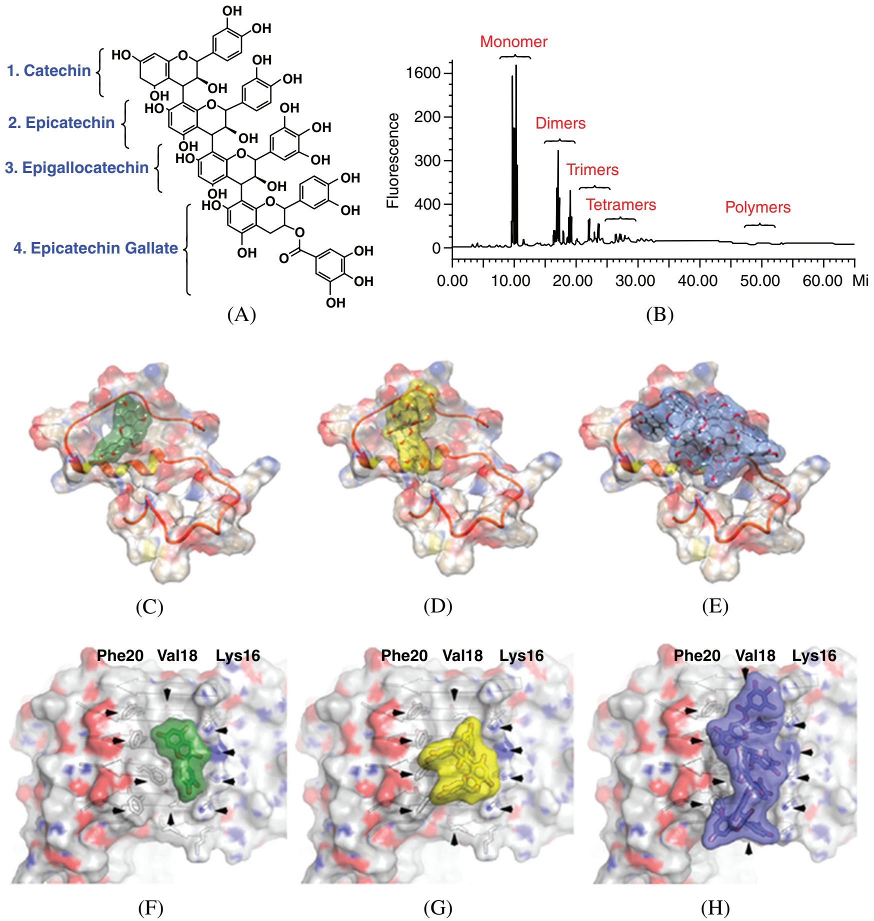

Figure 14.2 Composition of GSPE and models of interaction with Aβ40. (A) Molecular structure of a typical pro-anthocyanidin comprised of catechin/epicatechin base units and their derivatives. (B) Normal-phase HPLC analysis of *GSPE* demonstrates the presence of monomeric and polymeric units. (C)–(E) Models of complexes between Aβ40 and GSPE monomers, dimers, and oligomers: (C) docked monomer, (D) docked dimer, and (E) docked oligomer. The peptide backbone is illustrated in orange and red. (F)–(H) Monomer (F), dimer (G), and oligomer (H) interacting with backbone atoms of an Aβ fibril. Fibril side chains are shown in gray and extend from β-strands that are oriented equatorially, relative to the fibril axis. Hydroxyl groups form hydrogen bonds to Lys16 of Aβ. Polar interactions may occur among the aromatic rings of GSPE and the hydrophobic patches formed by Val18 and Phe20 of Aβ (courtesy of Hayden *et al*. [49]).

of AD. Brain PHF deposition is also a defining neuropathological feature for a number of neurodegenerative disorders, such as dementia pugilistica, progressive supranuclear palsy, among others, which are collectively referred to as tauopathies. We have investigated the effects of GSPE in modulating tau misfolding using the synthetic Ac-306VQIVYK311 tau peptide, a short peptide segment found in the microtubule-binding region of the tau protein that is essential for tau polymerization [59]. In *in vitro* CD studies, we showed that incubation with GSPE significantly inhibits β-sheet conformation of synthetic tau peptide (Figure 14.3A) and disassociates preformed Ac-306VQIVYK311 tau–tau peptide aggregates [60]. Moreover, incubation of GSPE with PHFs isolated from the brain of patients with AD led to a stepwise unfolding and de-aggregation of PHFs from the original typically organized tight fibril structures to a loosened assembly (Figure 14.3B, top-right panel), which increases in the accessibility of PHFs to proteolytic degradation by proteases such as trypsin (Figure 14.3B, bottom panel). Consistent with our *in vitro* evidence, we demonstrated that dietary supplementation with GSPE also protected against the formation of tau fibrils. Specifically, in the transgenic ey-gal4/SM6-TM6B *Drosophila* model of tauopathy, treatment with GSPE starting at the larval stage significantly attenuated mutant-tau-mediated abnormal eye phenotypes in adult flies [61]. In JNPL3 transgenic mice expressing a human tau protein containing the P301L mutation driven by the mouse prion promoter, oral administration of GSPE for 6 months significantly reduced the accumulation of sarcosyl-insoluble aggregated tau in the spinal cord (Figure 14.3C), coincidental with the attenuation of the severity of motor impairment that normally occurs with aging in this mouse model [65].

14.4 GDP attenuates AD neuropathology while promoting synaptic plasticity

Alterations in synaptic structures and functions, which long precede neuronal loss, are increasingly viewed as some of the earliest events in the initiation of AD-type cognitive decline [62–65]. Numerous studies have demonstrated significant alterations in the structure of dendrites and

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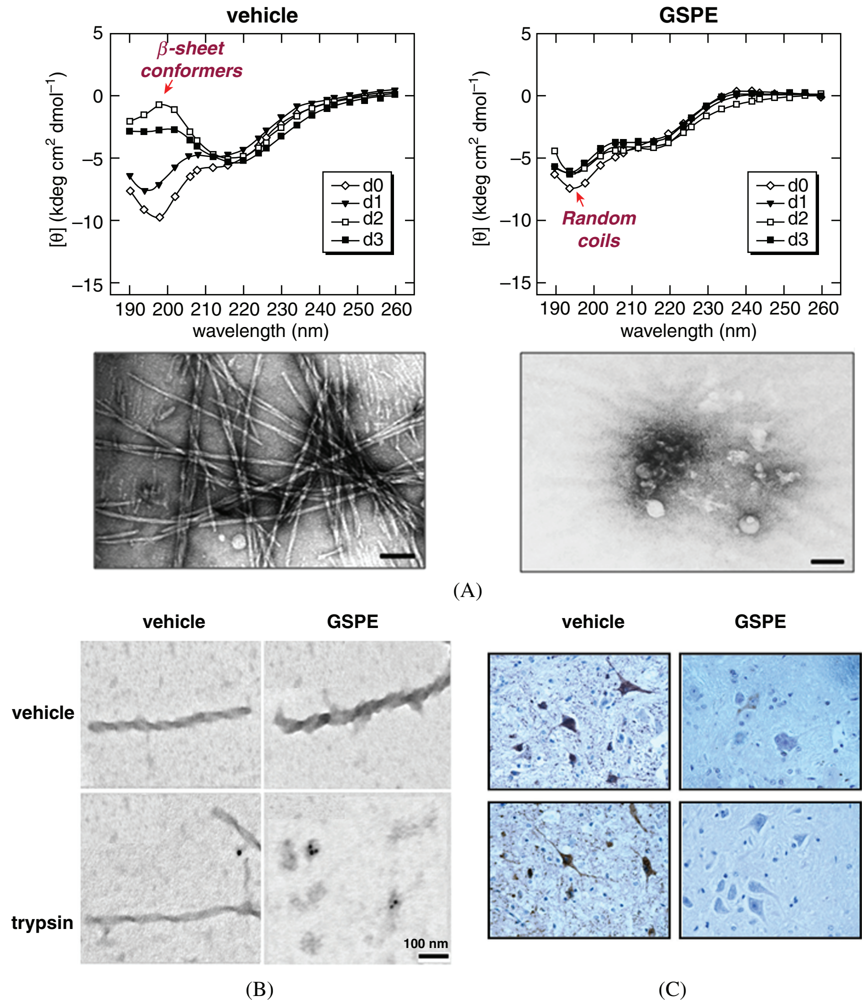


Figure 14.3 GSPE and aberrant tau aggregation. (A) Impacts of the GSPE on spontaneous aggregation of tau peptide into ordered, oligomeric conformers. Top panel: CD spectroscopy assessments of tau secondary structure dynamics. Synthetic tau peptide aggregation into ordered α-helix and β-sheet conformers in the absence of GSPE (left panel) or presence of equimolar GSPE (right panel); arrow indicates spectral characteristics of ordered conformers or random coils. d0, d1, d2, and d3 indicate that samples were taken on days 0, 1, 2, and 3 following incubation for CD testing. Bottom panel: Electron microscopy morphological assessment of tau fibril formation in the absence or presence of equimolar GSPE. Scale bars

spines both in AD and in mouse models of AD, including dystrophic dendrites, aberrant sprouting, curvature of dendritic processes, and loss of dendritic spines with accompanying synaptic loss in hippocampal and neocortical pyramidal cells [66–69]. Both HMW oligomeric Aβ (oAβ) and low-*n* Aβ oligomers (ranging from dimers to octamers) induce synaptic degeneration, synaptic plasticity disruption, and decreases in LTP [38, 39], all of which contribute to mechanisms underlying the onset and progression of dementia in AD [35, 40–47]. The loss of synapses and dendritic spines correlates with increased levels of soluble oAβ both in human post-mortem brains [70–72] and in transgenic mouse models of AD [44, 73–75]. Most importantly, synaptic loss shows the most robust correlation with cognitive decline in AD [62, 64, 65, 76, 77]. Therefore, preventing synaptic loss and conversely restoring synaptic function may provide a viable strategy for early protection/intervention to slow AD progression and preserve cognitive function.

Select grape-derived dietary polyphenols are capable of attenuating cognitive deterioration and reducing brain neuropathology in animal models of AD [55, 58, 78]. In pharmacokinetic studies, we have characterized a panel of biologically available polyphenol metabolites that are derived from these bioactive grape-derived dietary products and are capable of accumulating in brain tissues [55, 58, 78]. We have initiated a series of studies to investigate whether these brain-targeting polyphenol metabolites might modulate AD-type synaptic dysfunction in the brain of Tg2675 mice. Using *ex vivo* hippocampal slices from Tg2576 mice, we have demonstrated that select brain-targeting polyphenol metabolites, such as

Figure 14.3 (*Continued*) indicate 100 nm. (B) GSPE treatment promotes the sensitivity of PHFs to proteolytic digestion. PHFs from AD were treated with vehicle or GSPE at 100 μM for 1 h and were subjected to trypsin digestion for 10 min at RT. Samples were immunogold labeled with anti-tau antibody (AH-1). Note the expansion of the filament width and 60% reduction in PHF quantity with GSPE treatment. Addition of trypsin further lowered the quantity of PHFs to less than 14%. (C) GSPE treatment reduces the number of neurons with tau inclusions in the spinal cords of JNPL3 mice. Spinal cord sections from ventral horns stained with a panel of antibodies against phosphorylated tau PHF-1 (top panel) and AT8 (bottom panel). Note the reduced number of phosphorylated tau-bearing neurons in the spinal cord of treated as compared to untreated JNPL3 mice.

3′-*O*-methyl-epicatechin-5-*O*-β-glucuronide (3′-*O*-Me-EC-Gluc) and cyanidin-3′-*O*-glucuronide (cyanidin-Glc) that are found accumulated in the brain following oral administration of GSPE and CGJ, respectively, are capable of restoring LTP at physiologically relevant concentrations in hippocampal slices derived from Tg2576 mice (Figure 14.4A–C).

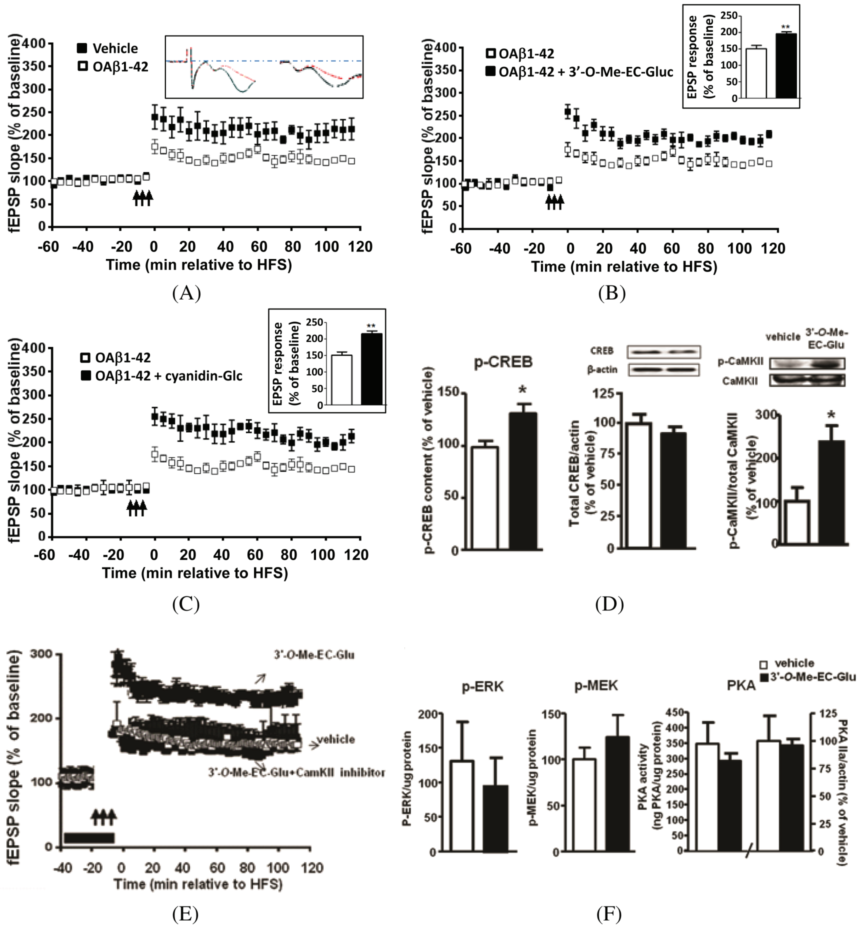


Figure 14.4 Select brain-penetrating polyphenolic metabolites from GSPE and CGJ are bioactive in preventing acute oAβ-induced LTP impairment through CREB signaling. (A)–(D) *Ex vivo* hippocampal slices from wild-type (WT) mice were acclimated in oxygenated artificial cerebrospinal fluid and challenged with (A) 200 nM of oAβ, (B) 200 nM oAβ co-treated with 3′-*O*-Me-EC-Gluc (300 nM), and (C) 200 nM oAβ co-treated with cyanidin-Glur (300 nM) for 1 h before recording. The fEPSPs were recorded from the CA1 region.

Mechanistic investigation demonstrated that the beneficial effect of 3′-*O*-Me-EC-Gluc in restoring LTP in the hippocampal formation of Tg2576 mice is associated with the promotion of cAMP response element-binding protein (CREB) signaling (Figure 14.4D). The CREB signaling pathway is critical for LTP and memory formation [79]. We and others have previously demonstrated that age-related development of AD-type cognitive dysfunction in Tg2576 mice, as well as in humans, is associated with brain synaptic dysfunction, which has been attributed, in part, to impairments in CREB signaling in select brain regions, particularly in the hippocampal formation. Consistent with this observation, we found that 3′-*O*-Me-EC-Gluc, which is capable of restoring LTP the hippocampal formation of Tg2576 mice (Figures 14.4A and 14.4B), is also effective in restoring CREB signaling in the same brain region. We monitored the level of [Ser133]-phosphorylated, active CREB ([Ser133]-P-CREB) as a direct reflection of CREB activation in the hippocampal formation of Tg2576 mice. We showed that treatment with 300 nM of 3′-*O*-Me-EC-Gluc significantly increased cellular [Ser133]-P-CREB contents in treated compared to control (vehicle-treated) Tg2675 hippocampal slices, without changing the cellular content of total CREB protein (Figure 14.4D). CREB can be activated by many kinases, and we found this increase in CREB signaling is associated with significantly increased

Figure 14.4 (*Continued*) The fEPSP slopes (% of baseline) were plotted as a function of time. The arrows indicate the beginning of tetanus used to induce LTP. Inset in (A), representative EPSP traces for vehicle (left) and oAβ (right) treated slices: Red trace represents pre-LTP and black trace represents 60 min following tetanus stimuli. Insets in (B) and (C), average magnitude of LTP during the last 5 min of recording. Data represent mean ± SEM, $**P<0.01$. (D) 3′-*O*-Me-EC-Gluc improves synaptic plasticity through activation of CamKII signaling pathway. CREB signaling pathway analysis of hippocampal slices from old Tg2576 mice perfused with 300 nM of 3′-*O*-Me-EC-Gluc. Levels of phospho- and total CREB, levels of phospho- and total CaMKII in brain slices from Tg2576 mice following treatment compared to the vehicle treated slices. (E) LTP response in hippocampal slices from old AD mice following 300 nM 3′-*O*-Me-EC-Gluc treatment in the presence or absence of 50 μM of CamKII inhibitor CK59. The arrows indicate the beginning of tetanus to induce LTP. (F) The levels of phosphoproteins of Erk1/2 (Thr185/Tyr187) and MEK (Ser222), PKA activity, and protein content of PKA IIa subunit in the treated slices.

levels of [Thr286]-phosphorylated, active Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), without influencing the level of total CaMKII (Figure 14.4D). To test for a potential cause-and-effect relationship between promotion of CREB signaling and restoration of LTP, we treated hippocampal formations of Tg2576 mice with 300 nM of 3′-*O*-Me-EC-Gluc in the presence (or absence of 50 μM of the CaMKII inhibitor, CK59). We observed that co-treatment of Tg2576 hippocampal slices with CK59 completely abolished 3′-*O*-Me-EC-Gluc-medicated restoration of LTP (Figure 14.4E), confirming that 3′-*O*-Me-EC-Gluc-modulated restoration of LTP in Tg2576 mice is mechanistically mediated, in part, by promoting the CaMKII/CREB signaling pathway. Multiple pathways can lead to the activation of CREB. Neither extracellular signal-related protein kinase/mitogen-activated protein (Erk/MAP) kinase nor the protein kinase A (PKA) pathways were involved in the 3′-*O*-Me-EC-Gluc-mediated activation of CREB, as reflected by the lack of changes in the phosphorylation status of Erk1/2 (Thr185/Tyr187), MEK, or PKA (both by PKA activity assay and PKA IIa protein content, Figure 14.4F). Consistent with our *ex vivo* hippocampal slice evidence, dietary supplementation with a polyphenol-rich preparation containing extracts from grapes and blueberries with a high content of flavonoids, stilbenes, and phenolic acids can reverse age-induced spatial memory deficits in Tg2576 mice, coincidental with the promotion of brain CaMKII and nerve growth neurotrophic factor (NGF) signaling pathways [80]. In a rodent model of chronic cerebral hypoperfusion which is one of the causes of vascular dementia and a contributing factor for AD, application of resveratrol, a well-characterized stilbenoid found in the skin of grapes and other berries, can restore synaptic plasticity through PKA-CREB signaling pathway [81]. Therefore, select GDP may activate CREB through modulation of diverse signaling pathways, which ultimately leads to the expression and release of brain-derived neurotropic factor (BDNF). BDNF binds to pre- and postsynaptic TrkB receptors and triggers glutamate release, PI3K/mTOR signaling, and immediate early genes (IEGs) expression which can lead to enhanced translational efficiency and the expression of scaffolding proteins, increased receptor density, and, ultimately, synaptic efficacy (Figure 14.5).

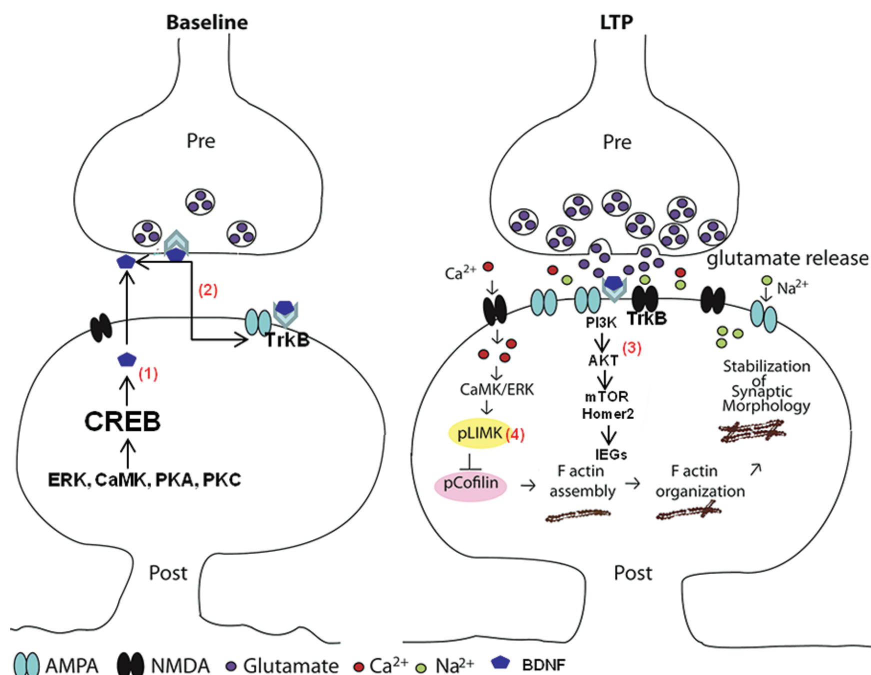


Figure 14.5 GDP and synaptic plasticity. (1) Select polyphenols activate the CREB signaling pathway and promote the expression and release of BDNF. (2) Binding of BDNF to TrkB triggers glutamate release. (3) Select polyphenols promote BDNF-mediated activation of PI3/mTOR signaling and IEG synthesis. (4) Select polyphenols enhance cofilin-mediated F-actin disassembly through modulation of cofilin phosphorylation. Promotion of Arc expression and cofilin dephosphorylation in turn promote actin filament dynamics and reorganization, leading to synapse growth and increased receptor density. Adapted from Spencer *et al*. [91].

14.5 Role of the microbiome in brain GDP bioavailability

In spite of the increasing efforts committed to clinical testing of polyphenols for AD intervention, clinical development of polyphenols for AD is hindered by the limited knowledge of polyphenol bioavailability, specific forms of polyphenols (including polyphenol metabolites) that are capable of accumulating in the brain, bioactivities of these brain-targeting polyphenol forms in engaging AD mechanisms, their underlying mechanisms

of actions, and off-target engagements that might impede with potential clinical development.

GDPs are extensively metabolized during absorptive and post-absorptive xenobiotic metabolism [82, 83], and orally consumed polyphenols are found *in vivo* primarily in their metabolite forms. Therefore, information generated from naturally occurring “precursor” forms in *in vitro* bioactivity and mechanistic studies is largely physiologically irrelevant. Moreover, AD-type neuropathology largely involves the central nervous system. Therefore, it is important to determine which forms of polyphenol metabolites can penetrate the blood–brain barrier (BBB), accumulate in the brain, and exert their bioactivities.

For instance, a 2-week oral treatment of Sprague–Dawley (SD) rats with a bioactive dietary polyphenol preparation (BDPP) comprised of GSPE, CGJ, and resveratrol, delivered daily by intragastric gavage resulted in the accumulation of a panel of polyphenol metabolites in the brain where the major metabolites are methylated, glucuronidated, or glucosidated. These biologically available polyphenol metabolites are derived from xenobiotic metabolism that occurs during the gastrointestinal absorption and/or post-absorptive processing of polyphenol “precursors” that are present in BDPP (Table 14.1).

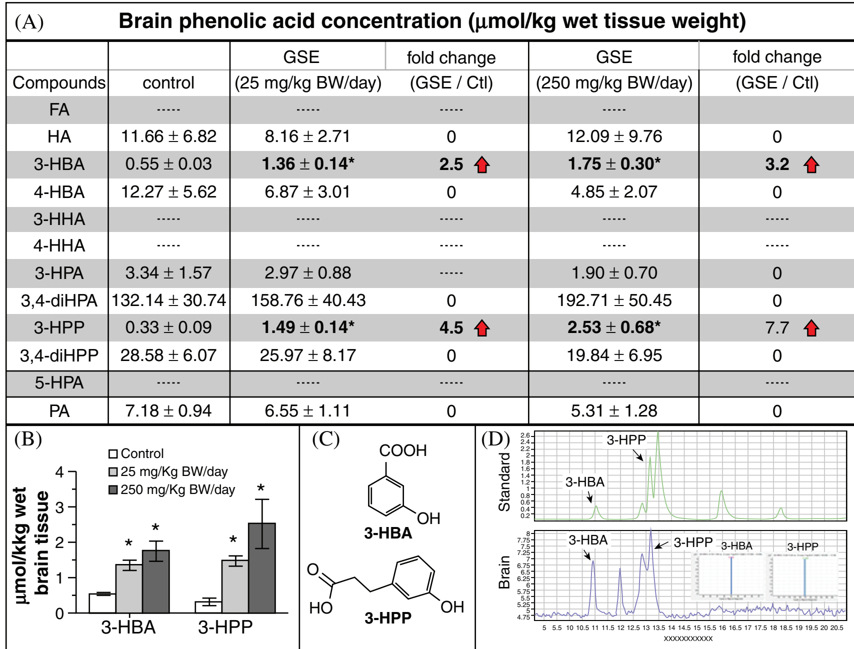
Besides xenobiotic metabolism, the intestinal bacterial population also plays a key role in promoting the bioavailability of dietary compounds by metabolizing dietary compounds into more simple, readily absorbable forms. For instance, oral administration of GSPE to rats led to the identification of 12 phenolic acids known to be generated by microbiota metabolism of anthocyanidins [20] (Figure 14.6). Moreover, GSPE treatment significantly increased the content of two of the phenolic acids in the brain: 3-hydroxybenzoic acid and 3-(3′-hydroxyphenyl) propionic acid, both of which can potently interfere with the assembly of β-amyloid peptides into neurotoxic β-amyloid aggregates that play key roles in AD pathogenesis [20].

Symbiotic and pathogenic microorganisms including bacteria, fungi, and archaea live in our intestine. There is a growing interest in the potential contributions of intestinal microbiota, particularly among the intestinal bacterial population, in human health and/or disease [84]. Approximately 1,000 different bacterial species, dominated by bacteria from bacterial

Table 14.1 Identification of 16 brain-targeting polyphenol metabolites following oral administration of GDP.

	Polyphenol Metabolites				
	Oral Admin with BDPP Components			Oral Admin with BDPP Components	
	Blood	Brain		Blood	Brain
Cyanidin-3-*O*-glucoside	+	+	Peonidin β-glucuronide	+	+
Cyanidin-glucuronide	+	+	Peonidin 3-*O*-glucoside chloride	+	+
Catechin-5-glucuronide	+	+	Petunidin glucoside	+	+
3′-OME-catechin-5-glucuronide	+	+	Petunidin glucuronide	+	N/A
Delphinin-3-*O*-glucoside	+	+	Quercetin-3-*O*-glucuronide	+	+
Delphinin-glucuronide	+	+	OME-quercetin-glucuronide	+	+
Epicatechin-5-glucuronide	+	+	OME-resveratrol-glucuronide	+	N/A
3′-OME-epicatechin-5-glucuronide	+	+	Resveratrol	+	+
Malvidin-3-*O*-glucoside	+	+	Reveratrol-3-*O*-glucuronide	+	+
Malvidin-glucuronide	+	N/A			

phyla *Firmicutes, Bacteroides, Actinobacteria*, and *Proteobacteria*, are found among human intestinal microbiota. Intestinal microbiota from a given person typically contains ~100 distinct bacterial species. However, there is tremendous diversity among individuals' intestinal microbiota with respect to the composition of specific bacterial species and the density (number) of bacteria that are present for each of these bacterial species. Indeed, such interpersonal differences in intestinal bacteria composition have been associated with the presence or absence of an increasing number of health issues, including metabolic syndrome, obesity, diabetes, immunological diseases, and cardiovascular diseases [84]. For example, a recent report of eight healthy subjects revealed high variability in the concentration of a number of phenolic acids in urine after subjects



(A) **Brain phenolic acid concentration (μmol/kg wet tissue weight)**

Compounds	control	GSE (25 mg/kg BW/day)	fold change (GSE / Ctl)	GSE (250 mg/kg BW/day)	fold change (GSE / Ctl)
FA	-----	-----	-----	-----	
HA	11.66 ± 6.82	8.16 ± 2.71	0	12.09 ± 9.76	0
3-HBA	0.55 ± 0.03	**1.36 ± 0.14***	**2.5** ↑	**1.75 ± 0.30***	**3.2** ↑
4-HBA	12.27 ± 5.62	6.87 ± 3.01	0	4.85 ± 2.07	0
3-HHA	-----	-----	-----	-----	-----
4-HHA	-----	-----	-----	-----	-----
3-HPA	3.34 ± 1.57	2.97 ± 0.88	-----	1.90 ± 0.70	0
3,4-diHPA	132.14 ± 30.74	158.76 ± 40.43	0	192.71 ± 50.45	0
3-HPP	0.33 ± 0.09	**1.49 ± 0.14***	**4.5** ↑	**2.53 ± 0.68***	7.7 ↑
3,4-diHPP	28.58 ± 6.07	25.97 ± 8.17	0	19.84 ± 6.95	0
5-HPA	-----	-----	-----	-----	-----
PA	7.18 ± 0.94	6.55 ± 1.11	0	5.31 ± 1.28	0

Figure 14.6 Detection of GSPE-derived phenolic acids in the brain. Rats were treated with GSPE at 250 mg/kg BW/day, 25 mg/kg BW/day, or vehicle (control) for 11 days, and animals were sacrificed 6 h after the last dose of GSPE/vehicle treatment. (A) Detection of phenolic acids in perfused brain specimens from vehicle-treated control and GSPE-treated groups. Data are displayed as mean ± SEM; *P<0.05, GSPE-treated compared to vehicle-treated control animals. Dark arrows indicate increased fold-change in GSPE-treated *versus* vehicle-treated control brain specimens. (B) Dose-responsive accumulation of 3-HBA and 3-HPP in brain specimens from animals treated with a daily dose of 250 or 25 mg/kg BW/day GSPE. Bar graphs present mean ± SEM; *P<0.05, GSPE-treated compared to vehicle-treated control animals. (C) Molecular structure of 3-HBA and 3-HPP. (D) Representative HPLC resolution/detection of 3-HBA and 3-HPP from molecular standards (top spectrogram) and a brain specimen (bottom spectrogram). Inset: MS–MS analysis of 3-HBA and 3-HPP from brain specimens following HPLC resolution.

consumed a set dose of a polyphenol-rich botanicals (350 mL of CGJ) [85]. Moreover, in a microbial bioconversion study using an *in vitro* fecal batch fermenter designed to simulate conditions in the distal colon, it was reported that large inter-individual variation in the capability of the gut microbiota from 10 healthy human donors to convert polyphenols from

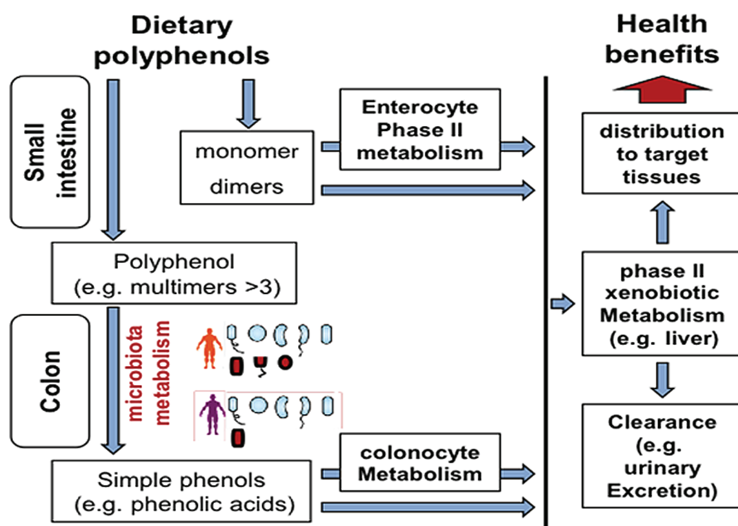


Figure 14.7 Interpersonal differences in gut microbiota composition affect health benefits of GDP. After oral consumption, monomeric (and to lesser extent, dimeric) polyphenols are absorbed by the small intestine, with or without enterocyte modification by phase II metabolism (e.g. glucuridation). Most dietary polyphenols, particularly multimeric ones, are not absorbed by the small intestine and are passed to the colon where microbial metabolism converts them into simple phenols, such as phenol acids. Some of these phenolic acids are absorbed, with or without colonocyte modification by phase II metabolism. Absorbed polyphenol metabolites and phenolic acids may undergo additional phase II metabolism, primarily by the liver, before they are delivered to target tissues, such as blood cells and the brain. The schematics depict interpersonal microbiota diversity with "red" bacterial strains representing those required to generate select bioactive phenolic acids capable of modulating the cellular/molecular mechanisms underlying AD pathology and cognitive function and show that these differences will affect the efficacy of GDP in AD. Moreover, next-generation probiotics designed to promote bioconversion of these select bioactive phenolic acids will enhance the efficacy of GDP.

black tea, red wine, and grape juice to phenolic acids [86]. In view of the increasing evidence supporting development of polyphenol interventions as part of a readily applicable, long-term strategy for AD prevention and/or treatment [87], the interpersonal differences in subsets of gut microbiota are critical for the processing and bioavailability of dietary polyphenols and affect bioavailability of bioactive phenolic metabolites (Figure 14.7), in particular, phenolic acids, to peripheral and brain tissues. This, in turn,

will greatly impact the efficacy of select polyphenol components in modulating AD disease phenotypes.

14.6 Clinical intervention and future directions

Recent clinical evidence suggests that dietary supplementation with polyphenols derived from different sources may help preserve cognitive function in older subjects with age-associated memory complaints [24, 88, 89]. Multiple clinical studies were conducted to test the safety and short-term efficacy of resveratrol either alone or in combination with other dietary supplements in subjects with mild cognitive impairment (MCI) or subjects at various stages of AD (ClinicalTrials.gov Identifier: NCT00678431; NCT01716637; NCT02837107; NCT01219244; NCT01504854). Besides safety and tolerability, the most encouraging finding was reported in the Phase 2 double-blind, randomized, placebo-controlled trial that resveratrol treatment in AD patients may be effective in stabilizing key molecular biomarkers of AD and improving some AD clinical symptoms [90]. In view of increasing evidence supporting protective roles of polyphenols in AD, new studies funded by the NIH-National Center for Complementary and Alternative Medicine (ClinicalTrials.gov Identifier: NCT02033941), by the Alzheimer's Association (ClinicalTrials.gov Identifier: NCT02502253), and by the Department of Defense (ClinicalTrials.gov Identifier: NCT02915237) are in progress to investigate the potential efficacy of, respectively, dietary supplementation with a GSPE for treating AD with a combination of GSPE, CGJ, and resveratrol in cases of mild cognitive impairment, which is a population at high risk for progression to AD, to attenuate their development into frank AD dementia, as well as to treat cognitive deficits in Veterans with Gulf War Illness.

As GDP are receiving increasing attention for their potential role in preventing the onset and/or progression of preclinical AD into frank AD dementia, a wealth of preclinical studies both *in vitro* and *in vivo* were carried out to investigate the mechanisms of action related to AD pathology. As mentioned above, GDPs are extensively metabolized during absorptive and post-absorptive xenobiotic metabolism [82, 83] and are found *in vivo* primarily in their metabolite forms. Therefore, the information generated from naturally occurring "precursor" forms in *in vitro* bioactivity and

mechanistic studies, which comprised a majority of the conducted *in vitro* studies, is largely physiologically irrelevant. Moreover, most of the GDP are administered as nutraceuticals, and, therefore, their ADME-Tox in clinical settings are much less stringent. It is possible that GDP may interact with drug-metabolizing enzymes such as cytochrome P450, UDP-glucuronosyltransferases, and sulfotransferases, which are essential for the metabolism of many medications.

Collectively, we believe that further characterization of GDP in pharmacokinetic and pharmacodynamic studies as well as further mechanistic investigations will significantly improve our understanding of the design of future translational studies in AD and other age-related neurodegenerative disorders. This is of extreme interest especially because most of the GDP exist as complex mixtures and it is highly conceivable that "cancellation effects" may take place via complex *in vivo* pharmacological responses which may ultimately negatively influence efficacy. This is currently the top research priority in our laboratory and will ultimately illuminate specific mechanisms of action of GDP for future translational efficacy in the clinical setting.

Acknowledgments

These studies were supported by discretionary funding from the Icahn School of Medicine at Mount Sinai to Dr. Giulio Maria Pasinetti and by the Geriatric Research Education and Clinical Centers (GRECC) of the James J. Peters Veterans Affairs Medical Center. The funding was also provided, in part, by the NIH-NCCAM grant P01AT004511-01 as part of the Icahn School of Mount Sinai Center of Excellence for Research in Complementary and Alternative Medicine for Alzheimer's Disease to Dr. Pasinetti. In addition, Dr. Pasinetti holds a Career Scientist Award in the Research and Development unit and is the Director of the Basic and Biomedical Research and Training Program, GRECC, James J. Peters Veterans Affairs Medical Center. We acknowledge that the contents of this book chapter do not represent the views of the U.S. Department of Veterans Affairs or the United States Government. The current study was also supported, in part, by the Altschul Foundation and VA Grant 1I01BX000870-01A2 to Dr. Giulio Maria Pasinetti.

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