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Tau Biology

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Tau Biology

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Preface

The isolation of tau protein as a heat-stable protein from the microtubule fraction of porcine brain was first reported in *PNAS* in 1975. The protein was shown to be essential for microtubule assembly, and most early work on tau were done in the context of microtubule assembly and the microtubule network.

In 1986, several groups reported that a major component of neurofibrillary tangles, one of the major pathological hallmarks of Alzheimer's disease (AD), was hyperphosphorylated tau. Since then, studies of this protein expanded to examining mechanisms of tau phosphorylation, aggregation, and degradation. It was in this era that lovers of tau, so-called tauists, were born. Mutations in the tau gene were found in frontotemporal dementia with parkinsonism-17 (FTDP-17) in 1998, highlighting the pathophysiological function of tau, as well the neurotoxic effects of tau aggregation. While therapeutic approaches targeting β-amyloid failed to show efficacy in slowing the progression of dementia, the last 20 years has witnessed an increase in attention to the possibility that targeting tau might be a better approach to tackle AD – this possibility provides a major stimulus for present-day research on tau.

In this book, we portray pioneering work alongside cutting-edge research to illustrate the promise of tau as a target for the treatment of AD. Tau is well-known as a microtubule-associated protein that is normally localized in axons. In the diseased brain, neuronal loss is prominently associated with fibrillary deposits of hyperphosphorylated tau in the remaining neurons. Important questions remain regarding the pathways and mechanisms responsible for the observed neuropathology and whether tau aggregation represents a toxic step that triggers neuronal death. New technologies are helping researchers to address these long-standing questions. This book is intended to provide the reader with basic knowledge on the biology of tau in order to fertilize new ideas that may facilitate the development of new treatments for AD, as well as improve our understanding on the role of tau in brain aging, since age is the greatest risk factor for AD.

In Part I "Structure and Role of the Tau Molecule," the authors discuss the structure, posttranslational modification, regulation of splicing, and degradation of tau. Since tau is a disordered protein in aqueous solution, even after heat treatment, studies on its structure have proved challenging. Tau biochemistry is also still poorly understood, especially with respect to its metabolism and axonal sorting. This section also considers new knowledge about tau hyperphosphorylation, how it becomes insoluble, and aggregates to give rise to neurofibrillary tangles (NFT), a pathological hallmark of the

AD-affected brain. The section concludes with a description of the posttranslational modifications of tau that appear to promote its aggregation.

"Tau localization and function" is the subject of Part II, where the physiological role of tau in neurons and the other neural cell types is described. Under physiological conditions, tau expression is largely restricted to axons where it regulates axonal transport. The recent demonstration of tau in the postsynaptic compartment is important because it suggests tau plays a physiological function in both the axon and the synapse. The precise function of synaptic tau still awaits further definition; it will be interesting to understand how tau fulfils such a role, whether by binding to other signaling, trafficking, or structural proteins. Even more surprisingly, tau is reportedly secreted from dendritic sites to affect proximal neurons and has recently been shown to contribute to ribosomal DNA stabilization, opening new possibilities for onco-therapy.

Part III is dedicated to "tau and disease-related proteins," such as ApoE and amyloid β (Aβ), which have roles in AD. The chapters in this section focus on the relationship between tau and AD-related proteins, which provides insights into mechanisms that may underlie the clinicopathological development of sporadic AD. In addition, studies on dementia with Lewy body (DLB), the second leading cause of cognitive impairment in the elderly, are covered since tau deposition is a common accompaniment of DLB, probably sharing cause-effect properties with synuclein.

"Tauopathies, pathology, drivers, and markers" are considered in Part IV since pathological observations are informative regarding the causes and/or drivers of disease. The chapters in this section include descriptions of the diverse, clinicopathological presentations of tauopathies. Since noninvasive imaging modalities will continue to help investigators better understand the role of secondary risk factors, such as stress, trauma, diabetes, and inflammation, as causes or drivers of tauopathy, space is given to description of developments in the arena of tau PET and fMRI that are likely to aid longitudinal development of tau pathology in parallel with the measures of impaired brain function.

The last section of this book addresses "tau aggregation and therapy." In its native form, tau is an unfolded protein, suggesting that it must be highly soluble, but in tauopathies, it appears as β-sheeted aggregates. Also interesting is that under physiological conditions, tau can phase-separate, thus increasing the local concentration of tau and facilitating the formation of tau "droplets" (membraneless organelles, e.g., stress granules) which may inadvertently stimulate the disease-linked aggregation pathway. This section also discusses how the tau aggregation process may be linked to neurodegeneration, as well as the propagation of the tau aggregation process from the entorhinal cortex to the hippocampus and neocortex, as is thought to be the case in clinical cases of AD; the work described forms a promising basis for developing disease-modifying treatments.

The editors are glad to be able to share their enthusiasm for tau pathology with the reader and look forward to seeing new developments in understanding the biology of this multifaceted protein which may lead to effective therapeutic approaches for AD and other tauopathies.

This book would not be possible without the interest and dedication of its individual authors – the editors thank them heartily for their time in preparing timely reviews of their specific interests in tau.

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Lille, France — Luc Buee

Contents

Part I

Structure and Role of the Tau Molecule

Ordered Assembly of Tau Protein and Neurodegeneration

1

Michel Goedert and Maria Grazia Spillantini

Introduction

Ordered assembly of a small number of proteins into filaments characterises the majority of cases of age-related neurodegenerative diseases, including Alzheimer's and Parkinson's. Most cases are sporadic, but a small number is inherited in a dominant manner. Huntington's disease is always inherited. Work carried out over the past 35 years established a causal role for filament formation in inherited forms of disease. By extrapolation, it appears likely that ordered assembly into filaments is also central for neurodegeneration in sporadic cases of disease. Tauopathies, which are characterised by the assembly of microtubule-associated protein tau, are the most common proteinopathies of the human nervous system (Table 1.1). They include Alzheimer's disease (AD), Pick's disease (PiD), chronic traumatic encephalopathy (CTE), tangle-only dementia, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD) and several rarer diseases.

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Tau Protein

Tau monomers belong to the family of intrinsically disordered proteins that, upon ordered assembly, form structured amyloid filaments [14, 37, 85]. Their expression is largely confined to central and peripheral nerve cells, where they are highly enriched in axons [5]. However, tau assemblies are observed in both nerve and glial cells in a number of neurodegenerative diseases. Since assembly is concentration-dependent, it remains to be established if tau assemblies can form *de novo* in glial cells or if glial tau pathology requires the uptake of seeds from neurons.

Tau protein can be divided into an N-terminal region, a proline-rich domain consisting of two separate parts, the repeat domain and a C-terminal region. The N-terminal region projects away from the microtubule surface and is believed to interact with components of the neuronal plasma membrane. An interaction between exon 1 and annexins may help to explain the axonal localisation of tau [28], which may also be mediated, at least in part, by the axon initial segment [57]. Exon 1 of human tau contains a primate-specific sequence, which has been proposed to mediate interactions with neuronal proteins [78]. The PXXP motifs in the proline-rich region are recognised by SH3 domain-containing proteins of the Src family of non-receptor kinases, such as Fyn [56]. The repeat domain and some adjacent sequences mediate interactions between tau and

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Table 1.1 Neurodegenerative diseases

Alzheimer's disease
Parkinson's disease
Dementia with Lewy bodies
Frontotemporal dementias (including Pick's disease)
Progressive supranuclear palsy
Corticobasal degeneration
Chronic traumatic encephalopathy
Argyrophilic grain disease
Tangle-only dementia
Multiple system atrophy
Huntington's disease
Motor neuron diseases
Prion diseases

microtubules. Electron cryo-microscopy (cryo-EM) has shown that each tau repeat binds to the outer microtubule surface and adopts an extended structure along protofilaments, interacting with alpha- and beta-tubulin [1, 50]. Single-molecule tracking revealed a kiss-and-hop mechanism, with a dwell time of tau on individual microtubules of about 40 ms [46, 65]. Despite these rapid dynamics, tau promotes microtubule assembly. Microtubules have stable and labile domains. Tau is most abundant in the labile domain, which has led to the suggestion that it may not stabilise microtubules, but enable them to have long labile domains [6, 71]. Less is known about the function of the C-terminal region.

Although it lacks a typical low-complexity domain, full-length tau has been reported to undergo liquid-liquid phase separation, which has been suggested to initiate aggregation and neurodegeneration [84, 92]. RNA-binding proteins may influence these processes [2]. Tau is subject to a number of post-translational modifications, including phosphorylation, acetylation, methylation, glycation, isomerisation, O-GlcNAcylation, nitration, sumoylation, ubiquitination and truncation [37].

Tau Isoforms

Six tau isoforms ranging from 352 to 441 amino acids in length are expressed in adult human brain from a single *MAPT* gene (Fig. 1.1a) [31]. They differ by the presence or absence of inserts of 29 or 58 amino acids (encoded by exons 2 and 3, with exon 3 being only transcribed in conjunction with exon 2) in the N-terminal half and inclusion, or not, of the 31 amino acid microtubule-binding repeat, encoded by exon 10, in the C-terminal half. Inclusion of exon 10 results in the production of three isoforms with four repeats (4R) and its exclusion in a further three isoforms with three repeats (3R). The repeats comprise residues 244–368, in the numbering of the 441 amino acid isoform. In adult human brain, similar levels of 3R and 4R tau are expressed [32]. The finding that a correct 3R and 4R tau isoform ratio is essential for preventing neurodegeneration came as a surprise. 2 N isoforms are underrepresented in comparison with isoforms that include exon 2 or exclude exons 2 and 3; 2 N, 1 N and 0 N isoforms make up 9%, 54% and 37%, respectively. Big tau, which carries an additional large exon in the N-terminal half, is only expressed in the peripheral nervous system [20, 34].

The expression of isoforms is not conserved. Thus, in adult mouse brain, 4R tau is exlusively present, whereas adult chicken brain expresses 3R, 4R and 5R isoforms [89]. One hyperphosphorylated 3R tau isoform lacking N-terminal repeats is characteristic of developing vertebrates. In mice, the switch from 3R to 4R tau occurs between postnatal days 9 and 18, with tau phosphorylation decreasing over time [81]. However, isoform switching and phosphorylation are regulated differently. Adult 4R isoforms are better at promoting microtubule assembly than the 3R isoform expressed during development, which is also more phosphorylated than both 3R and 4R tau in adult brain [32]. This is consistent with the need for a more dynamic cytoskeleton during the development of nerve cells.

Tau Assemblies

Brain tau can assemble into filamentous inclusions [7, 37]. The repeats and some adjoining sequences form the filament core, with the N-terminal half and the C-terminus giving rise to

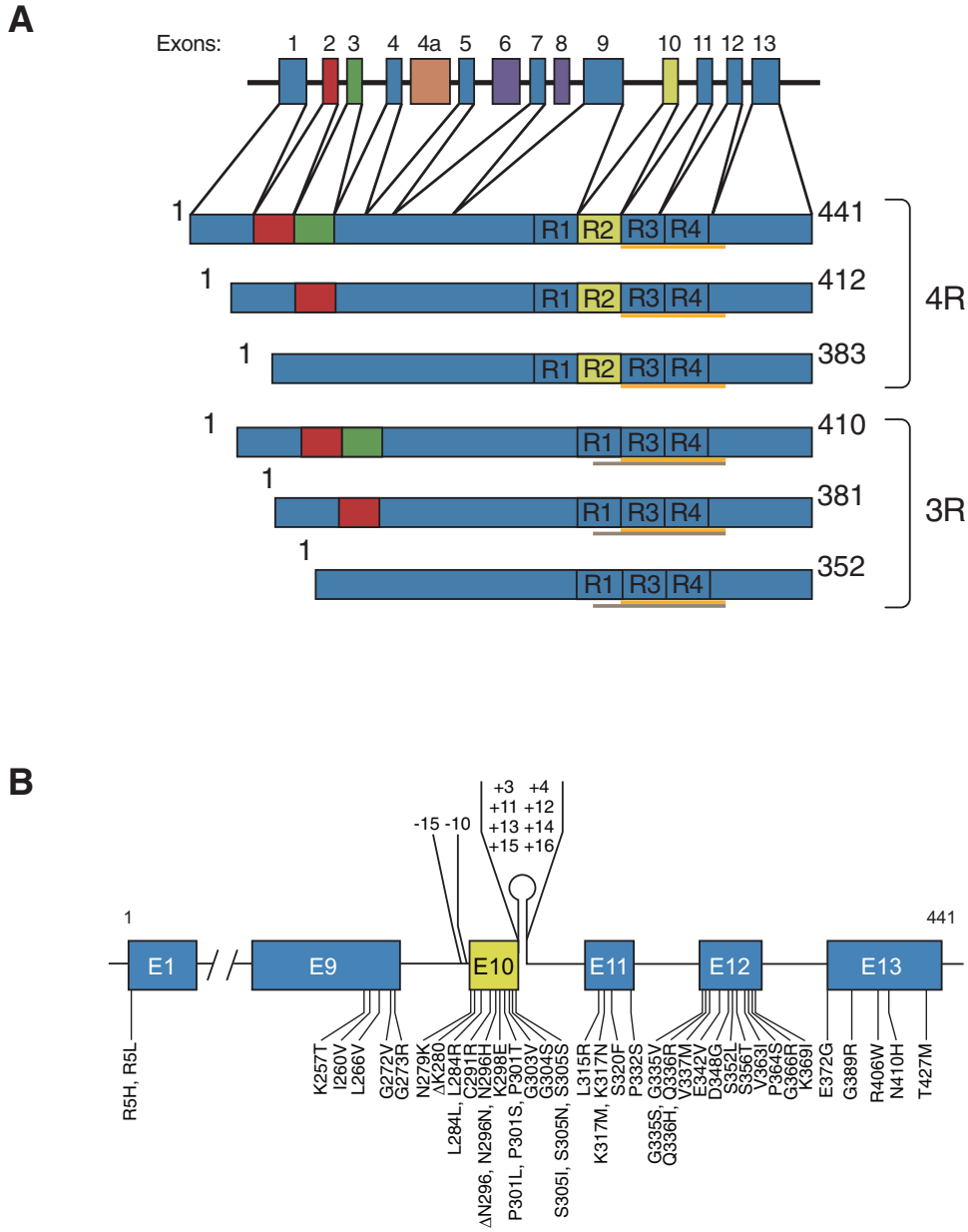

Fig. 1.1 Human brain tau isoforms and disease-causing *MAPT* mutations

(**a**) *MAPT* and the six tau isoforms expressed in adult human brain. *MAPT* consists of 14 exons (E). Alternative mRNA splicing of E2 (red), E3 (green) and E10 (yellow) gives rise to six tau isoforms (352–441 amino acids). The constitutively spliced exons (E1, E4, E5, E7, E9, E11, E12 and E13) are shown in blue. E6 and E8 (violet) are not transcribed in human brain. E4a (orange) is only expressed in the peripheral nervous system. The repeats (R1-R4) are shown, with three isoforms having four repeats (4R) and three isoforms with three repeats (3R). The core sequences of tau filaments from Alzheimer's disease (G273/305-E380) determined by cryo-EM are underlined (in orange); the core sequences of tau filaments from Pick's disease (K254-F378 of 3R tau) are also underlined (in grey). (**b**) Mutations in *MAPT* in FTDP-17T. Fifty coding region mutations and ten intronic mutations flanking E10 are shown

the fuzzy coat [30, 87, 88]. Tau filaments from brain, and those assembled *in vitro* from expressed protein, have a cross-β structure characteristic of amyloids [4]. Since the region that binds to microtubules also forms the core of tau filaments, physiological function and pathological assembly may be mutually exclusive.

Phosphorylation negatively regulates the ability of tau to interact with microtubules and filamentous tau is abnormally hyperphosphorylated [44]. However, it remains to be proved that phosphorylation is the trigger for tau assembly in human diseases. Alternatively, a change in conformation as part of assembly may lead to hyperphosphorylation. Since tau is hydrophilic, it is not surprising that unmodified and full-length protein requires cofactors, such as sulphated glycosaminoglycans, nucleic acids or fatty acids, to assemble into filaments [35, 48, 68, 86]. Cofactors other than heparin and/or post-translational modifications may cause the assembly of tau in human brain [25, 26].

Besides phosphorylation, other post-translational modifications may also play a role. Early studies on tau acetylation reported that it can promote both phosphorylation and assembly [15, 59]. However, subsequent work suggested an inverse correlation between acetylation and phosphorylation, with acetylation inhibiting tau assembly [11, 17]. These discrepancies may have resulted from the use of enzymes that acetylated different residues. Site-specific acetylation of K280 has been shown to enhance heparin-induced tau aggregation *in vitro*, while reducing microtubule assembly [40]. Unlike phosphorylation, acetylation occurs on lysine residues.

In AD, CTE, tangle-only dementia and other tauopathies, all six tau isoforms are present in disease filaments (Table 1.2). Pick bodies are made of only 3R tau. In PSP, CBD, AGD and other diseases, isoforms with 4R tau are found in the filaments. The morphologies of tau filaments vary in different diseases, even when they are mainly made of the same isoforms.

Table 1.2 Neurodegenerative diseases with abundant Tau inclusions

3 + 4R Tauopathies
Alzheimer's disease
Amyotrophic lateral sclerosis/parkinsonism-dementia complex (Guam and Kii peninsula)
Anti-IgLON5-related tauopathy
Chronic traumatic encephalopathy
Diffuse neurofibrillary tangles with calcification
Down's syndrome
Familial British dementia
Familial Danish dementia
Gerstmann-Sträussler-Scheinker disease
Niemann-Pick disease, type C
Nodding syndrome
Non-Guamanian motor neuron disease with neurofibrillary tanges
Postencephalitic parkinsonism
Primary age-related tauopathy
Progressive ataxia and palatal tremor
Tangle-only dementia
Familial frontotemporal dementia and parkinsonism (some *MAPT* mutations, such as V337 M and R406W)
3R Tauopathies
Pick's disease
Familial frontotemporal dementia and parkinsonism (some *MAPT* mutations, such as G272 V and Q336R)
4R Tauopathies
Age-related astrogliopathy
Argyrophilic grain disease
Corticobasal degeneration
Guadeloupean parkinsonism
Globular glial tauopathy
Hippocampal tauopathy
Huntington's disease
Progressive supranuclear palsy
SLC9a-related parkinsonism
Familial frontotemporal dementia and parkinsonism (some *MAPT* mutations, such as P301L and P301S, all known intronic mutations, and many coding region mutations in exon 10)

Genetics of MAPT

The relevance of tau inclusion formation for neurodegeneration became clear in June 1998, when dominantly inherited mutations in *MAPT* were

shown to cause a form of frontotemporal dementia that can be associated with parkinsonism (FTDP-17T, also known as familial FTLD-tau) [43, 70, 76]. Abundant filamentous tau inclusions are present in either nerve cells or in both nerve and glial cells. Aβ deposits, a defining feature of AD, are not characteristic of FTDP-17T. This work established that a pathological pathway leading from monomeric to assembled tau is sufficient for causing neurodegeneration and dementia.

Sixty mutations in *MAPT* have been identified in FTDP-17T (Fig. 1.1b). Filaments are composed of either 3R or 4R tau, or of both 3R and 4R tau. *MAPT* mutations account for approximately 5% of cases of FTLD and are concentrated in exons 9–12 (encoding R1-R4) and the introns flanking exon 10. They can be divided into those with a primary effect at the protein level and those affecting the alternative splicing of tau pre-mRNA. There is no obvious correlation between known mutations and post-translational modifications of tau.

Mutations that act at the protein level change or delete single amino acids, reducing the ability of tau to interact with microtubules [41]. Some mutations also promote the assembly of tau into filaments [36, 63]. Mutations with a primary effect at the RNA level are intronic or exonic and increase the alternative mRNA splicing of exon 10. This affects the ratio of 3R to 4R isoforms, resulting in the relative overproduction of 4R tau and its assembly into filaments [43, 76]. One mutation (ΔK280) has been reported to cause the relative overexpression of 3R tau and its assembly into filaments [82].

Assembled tau shows different isoform patterns and filament morphologies, depending on the mutations in *MAPT* [29]. Mutations V337 M in exon 12 and R406W in exon 13 give rise to insoluble tau bands of 60, 64 and 68 kDa and a weaker band of 72 kDa. Following dephosphorylation, six bands are present that align with recombinant tau, like what is seen in AD [33]. By electron microscopy, paired helical filaments (PHFs) and straight filaments (SFs) are present. The brains of many individuals with missense *MAPT* mutations in exons 9–13 (K257T, L266V, S305N, G272V, L315R, S320F, S320Y, P332S, Q336H, Q336R, K369I, E372G and G389R) are characterised by abundant Pick bodies made predominantly of 3R tau. As in sporadic PiD, insoluble tau shows strong bands of 60 and 64 kDa. However, variable amounts of 68- and 72 kDa bands are also present. A third pattern is characteristic of *MAPT* mutations that affect the alternative mRNA splicing of exon 10, resulting in the relative overproduction of 4R tau (intronic mutations and exonic mutations N279K, L284L, L284R, ΔN296, N296D, N296H, N296N, S305L, S205N and S305S). Insoluble tau runs as two strong bands of 64 and 68 kDa, and a weaker band of 72 kDa; following dephosphorylation, three bands are present that align with recombinant 4R tau (isoforms of 383, 412 and 441 amino acids). A similar pattern of pathological tau bands is observed for mutations in exon 10, such as P301L and P301S, which have their primary effects at the protein level. Assembly of 4R tau has also been described for mutations I260V in exon 9, K317N in exon 11, E342V in exon 12 and N410H in exon 13, showing that it is possible to alter 3R and 4R tau mRNAs through mutations located outside exon 10.

The effects of *MAPT* mutations can vary. Neighbouring mutations in exon 12 (G335S, G335V, Q336H, Q336R and V337M) give rise to structurally distinct assemblies and exert different functional effects. Mutation G335S is characterized by abundant filamentous tau inclusions in nerve cells and glial cells, in the absence of Pick bodies [77]. Mutations Q336H and Q336R give rise to what is essentially a familial form of PiD, with abundant Pick bodies in nerve cells [69, 80], whereas mutation V337M produces a neuronal filamentous tau pathology like that of AD [70, 75]. These findings on *MAPT* mutations in three adjacent codons reinforce the view that the mech-

anisms underlying the formation of neurofibrillary lesions and Pick bodies are closely related. Recombinant tau with the G335S, G335V [64], or V337 M mutation shows a greatly reduced ability to promote microtubule assembly. By contrast, mutations Q336H and Q336R increase the ability of tau to promote microtubule assembly. Mutations G335V and V337M fail to increase heparin-induced assembly into filaments significantly, whereas mutations Q336H and Q336R increase the assembly of 3R, but not 4R, tau.

The architecture of *MAPT* on chromosome 17q21.31 is characterized by two haplotypes as the result of a 900 kb inversion (H1) or non-inversion (H2) polymorphism [79]. Inheritance of the H1 haplotype of *MAPT* is a risk factor for PSP, CBD, Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), but not for PiD [3, 16, 22, 61, 66, 90]. The association with PD and ALS is particularly surprising, since they are not characterized by tau inclusions.

Based on genome-wide association studies for PSP and CBD, it has been shown that association with an allele at the *MOBP/SLC25A38* locus results in elevated levels of appoptosin, a protein that activates caspase-3, which can cleave tau [93]. This may cause aggregation of 4R tau. Additional loci were unique to PSP or CBD. Association of the H1 haplotype with PSP had a higher odds ratio than that between apolipoprotein E epsilon 4 (APOEε4) and AD [42]. APOEε4 is the major risk factor allele for late-onset AD [19]. H1 expresses more exon 10-containing mRNA than H2, especially in subcortical regions [9, 54]. Moreover, H2 is associated with increased expression of exon 3 of *MAPT* in grey matter, suggesting that inclusion of exon 3 may protect against PSP, CBD, PD and ALS [10]. In experimental studies, exon 3-containing tau isoforms (those with both N-terminal inserts) have been found to assemble less than those lacking this exon [94]. Even though all six tau isoforms give rise to PHFs and SFs, known mutations in *MAPT* do not cause AD. They give rise to FTLD-tau. Tau with an A152T substitution has been reported to be a risk factor for AD [18], as well as for PSP, CBD and unusual tauopathies [18, 49, 53, but see also 67].

Heterozygous microdeletions of chromosome 17q21.31 give rise to a multisystem disorder with intellectual disability, hypotonia and distinct facial features (17q21.31 microdeletion syndrome or Koolen-de Vries syndrome) [51, 73, 74]. In addition to *MAPT*, three protein-coding genes (*CRHR1*, *SPPL2C* and *KANSL1*) and two putative genes (*MGC57346* and *CRHR-ITI*) are found in this region. Deletions arise on the H2 haplotype through low-copy, repeat-mediated, nonallelic homologous recombination.

The 17q21.31 microdeletion syndrome is caused by haploinsufficiency of *KANSL1*, which encodes a chromatin modifier that influences gene expression through the acetylation of lysine 16 of histone H4 [52, 95]. A 50% reduction in tau levels does therefore not appear to have a detrimental effect on development of the human brain.

Neurodegeneration and Propagation

Disease-causing mutations in *MAPT* have made it possible to produce transgenic rodent lines that form abundant tau filaments and exhibit neurodegeneration (Figs. 1.2 and 1.3) [37]. Tau assembly correlates with neurodegeneration. Reducing assembly and increasing aggregate degradation are therefore therapeutic objectives. Since assembly is concentration-dependent, decreasing the level of soluble, monomeric tau is likely to result in reduced assembly [21, 55]. However, the molecular species of assembled tau that are responsible for neurodegeneration remain to be identified [58]. At a cellular level, it has been reported that the removal of senescent brain cells leads to a reduction in both tau assembly and neurodegeneration in transgenic mice [8]. In tauopathies, as in most neurodegenerative diseases, inclusion formation manifests many years before clinical symptoms. In future, it will therefore be important to identify individuals at risk of disease. Early diagnostics,in particular imaging of tau inclusions, is therefore likely to play an important role [38].

Transgenic mouse lines were also essential for the identification of the prion-like properties of

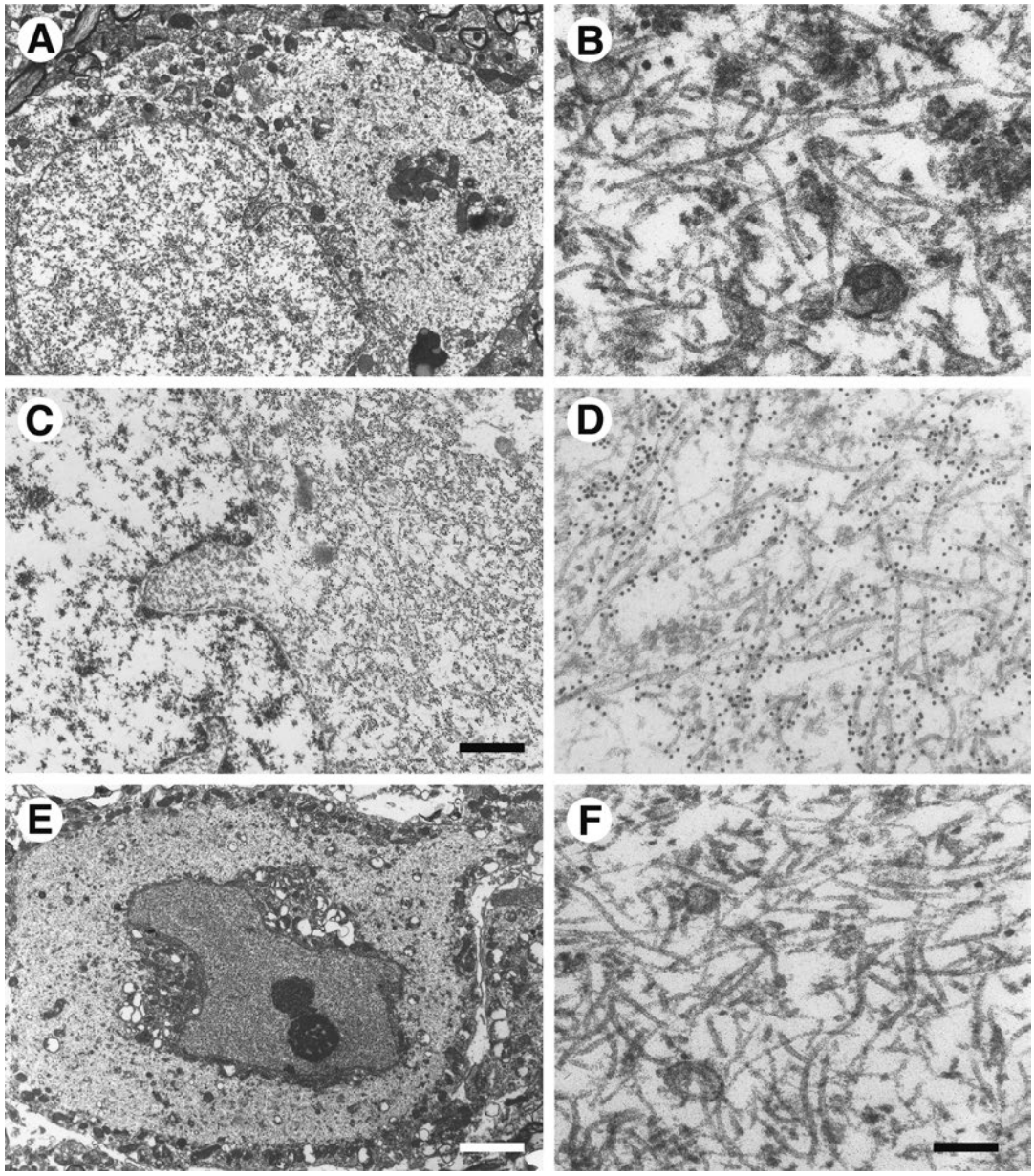

Fig. 1.2 Electron microscopy and immunoelectron microscopy of nerve cells in brain and spinal cord from mice transgenic for human mutant P301S tau (**a**, **b**), Cerebral cortex; (**c**, **d**), Brainstem; (**e**, **f**), Spinal cord. (**b**, **d**, **f**), Higher magnifications of parts of the cytoplasmic regions from (**a**, **c**, **e**). The electron micrographs in (**c**, **d**) show immunogold labelling of filaments using the phosphorylation-dependent anti-tau antibody AT8. Scale bars in (C) and (E) must be in micrometers: C, 1.5 m; E, 5.5 m (for **a**, **e**); F, 300 nm (for **b**, **d**, **f**)

assembled tau *in vivo* (Fig. 1.4) [62]. Aggregation of hyperphosphorylated tau was induced following intracerebral injection of tau seeds from mice transgenic for human mutant 0N4R P301S tau into transgenic mice expressing wild-type non-aggregated 2N4R tau and, to a lesser extent, following intracerebral injection into wild-type mice [12]. Tauopathy then spread to connected brain regions, indicative of seed endocytosis, seeded aggregation, intracellular transport and

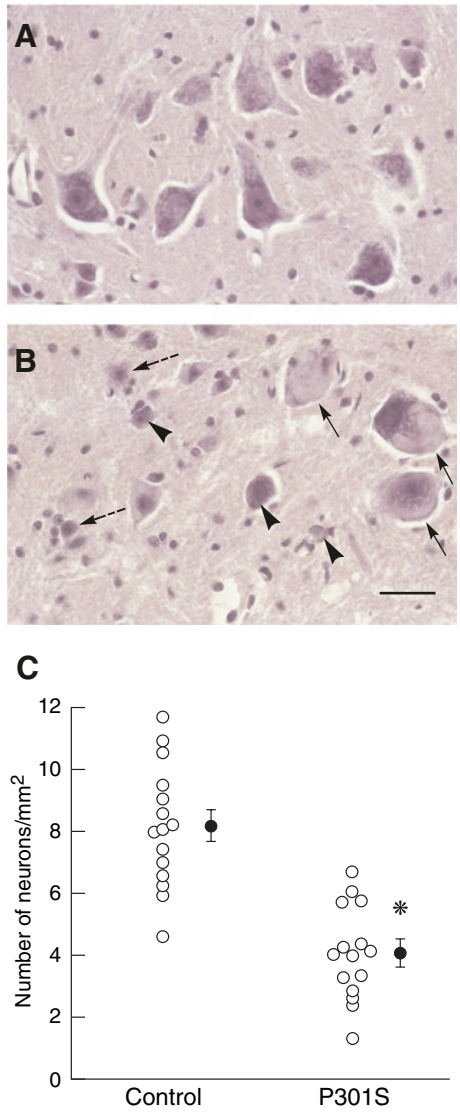

Fig. 1.3 Nerve cell loss in spinal cord of mice transgenic for human mutant P301S tau
(**a**, **b**), Haematoxylin and eosin-stained sections of the ventral grey matter of the lumbar spinal cord (L2-L3) from 6-month-old non-transgenic (**a**) and transgenic (**b**) mice. Swollen, abnormal material-containing motor neurons are indicated by arrows. Arrowheads point to atrophic motor neurons, with dashed arrows pointing to pyknotic cells that are surrounded by glial cells, suggestive of neuronophagia. (**c**) Graph showing the density of motor neurons (expressed as number of neurons per millimetre square) in the anterior horn of the lumbar spinal cord of 6-month-old non-transgenic (Control) and transgenic (P301S) mice. Nerve cell numbers were determined in five consecutive sections from each animal, with the density of motor neurons from each section being represented by a circle. The results are expressed as the means ± S.E.M. (n = 3). $*p < 0.0001$. Scale bar in (B) must be in micrometers: 60 m (for **a**, **b**)

release of tau seeds. This work was complemented by studies, which showed that short tau filaments have the greatest seeding activity (Figs. 1.5 and 1.6) [45]. Seeded aggregation of tau was dependent on the ability of expressed, monomeric tau to aggregate [23].

Distinct conformers of assembled tau appear to exist, reminiscent of prion strains. They may explain the variety of human tauopathies. Inclusions formed and spread of pathology occurred after intracerebral injection of brain homogenates from cases of AD, tangle-only dementia, PSP, CBD and AGD into a mouse line transgenic for wild-type 4R tau [13]. PiD, the filamentous inclusions of which consist of only 3R tau, was an exception. Inclusions formed at the injection sites, but spreading was not observed. However, PiD is only rarely a pure 3R tauopathy, since it can be associated with AD-type tau pathology. We therefore cannot exclude that the activity in the PiD homogenate, which induced aggregation at the injection site, may have been due to the presence of a small amount of aggregated 4R tau.

Sequence requirements for seeded tau aggregation *in vivo* remain to be defined. Tau assemblies reminiscent of those in the corresponding human diseases were observed following the injection of brain homogenates from patients with PSP, CBD and AGD, which are 4R tauopathies [13]. Although these findings are consistent with the existence of distinct tau aggregate conformers, the definition of such conformers must be structural.

High-Resolution Structures of Tau Filaments from Alzheimer's Disease

By cryo-EM, high-resolution structures of Tau filaments were obtained from the frontal cortex of four individuals with AD, three sporadic and one inherited (Fig. 1.7) [25, 27]. The cores of tau filaments are made of two protofilaments consisting of residues G273/304-E380, which adopt a combined cross-β/β-helix structure (Fig. 1.8). Murine and human tau are identical in sequence in this region. The N-terminal part of the cross-β structure includes hexapeptide ^{306}VQIVYK^{311},

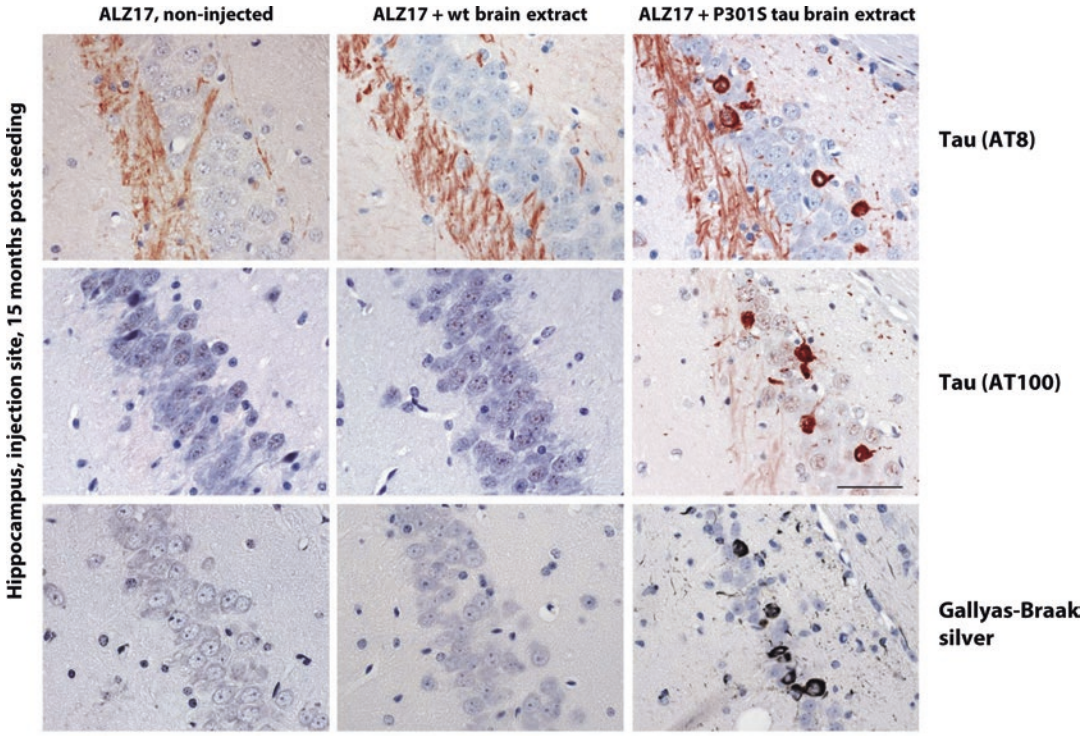

Fig. 1.4 Induction of filamentous tau pathology in mice transgenic for 2N4R wild-type human tau (line ALZ17) following injection with brain extract from symptomatic mice transgenic for 0N4R P301S tau
Staining of the hippocampal CA3 region of 18-month-old ALZ17 mice with anti-tau antibodies AT8 and AT100 and Gallyas-Braak silver. Non-injected (left), 15 months after injection with brain extract from non-transgenic control mice (middle) and 15 months after injection with brain extract from 6-month-old mice transgenic for human P301S tau (right). The sections were counterstained with haematoxylin. The Scale bar must be given in micrometers

which is essential for the oligomerisation of recombinant tau and its assembly into filaments [72, 83]. It packs against ^{373}THKLTF^{378}, in agreement with the predicted heterozipper interaction between ^{306}VQIVYK^{311} and ^{375}KLTFR^{379} [60]. Constructs K18 and K19 end at E372 [39]; they can therefore not give rise to the human brain tau folds determined thus far.

Each protofilament is made of eight β-strands, five of which give rise to two regions of antiparallel β-sheets, with the other three forming a β-helix (Fig. 1.9). The C-terminal residues of R1 and R2 form part of the first β-strand. R3 contributes three and R4 four β-strands, with the final β-strand being formed by 12 amino acids after R4 (residues K369-E380). Strands β1 and β2 pack against β8, β3 packs against β7, with β4, β5 and β6 giving rise to the C-shaped β-helix.

PHFs and SFs are made of identical protofilaments, but differ in inter-protofilament packing, showing that they are ultrastructural polymorphs. PHF protofilaments are arranged base-to-base and SF protofilaments back-to-base. In PHFs, protofilaments are stabilised by backbone hydrogen bonds between their ^{332}PGGGQ^{336} sequences. Moreover, the side-chains of K331 from one protofilament project towards the side-chains of Q336 and E338 of the other protofilament, suggesting additional interactions that stabilise the protofilament interface. Furthermore, in the protofilament interface of the PHF, extra densities between the side-chains of K331 of one protofilament and the backbone of V337 of the other have been observed. They may correspond to a solvent molecule or a post-translational modification of K331, such as mono-methylation [25].

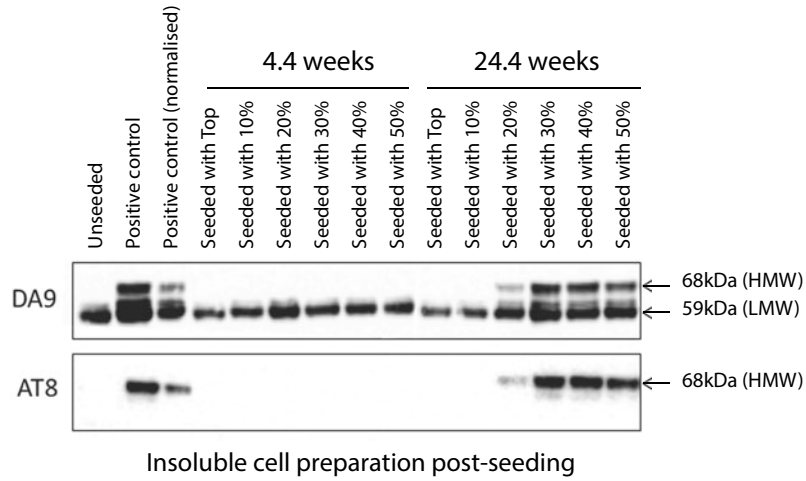

Fig. 1.5 Seeding of tau assembly with sucrose gradient fractions from the brains of mice transgenic for human mutant 0N4R P301S tau in a cell-based assay
The mice were aged 4.4 weeks (no symptoms, no tau filaments) or 24.4 weeks (symptoms, abundant tau filaments). Sucrose gradient fractions were used to seed assembly of tau in HEK cells expressing 1N4R P301S tau. The pellet from a 1000,000 g spin of the seeded cells was analysed by immunoblotting for total tau (antibody DA9) and tau phosphorylated at S202/T205 (antibody AT8). Filamentous tau runs at 68 kDa (high molecular weight, HMW) and non-filamentous tau at 59 kDa (low molecular weight, LMW). As a positive control, sarkosyl-insoluble tau extracted from unfractionated brains of symptomatic transgenic P301S tau mice was used for seeding; the normalized positive control consisted in seeding with sarkosyl-insoluble tau that had been normalised for total tau levels relative to those of the sucrose gradient fractions. Seeding correlated with the presence of the 64 kDa band in 24.4-week-old mice (20–50% sucrose gradient fractions). No seeding was observed upon addition of sucrose gradient fractions from 4.4-week-old mice

In SFs, the protofilaments pack asymmetrically. Their backbones are nearest each other between residues ^{321}KCGS^{324} of the first and ^{313}VDLSK^{317} of the second protofilament. The inter-protofilament packing appears to be stabilised through the region of additional density that interacts with the side-chains of K317, T319 and K321 of both protofilaments. This density may correspond to residues ^{7}EFE^{9}, which constitute the N-terminal region of the discontinuous epitope of conformational anti-tau antibodies ALZ-50 and MC-1 (the C-terminal epitope is ^{313}VDLSKVTSKC^{322}) [47]. A similar density also interacts with K317, T319 and K321 in PHFs, where it does not contribute to the protofilament interface.

High-Resolution Structures of Tau Filaments from Pick's Disease

By cryo-EM, high-resolution structures of Tau filaments were determined from the frontotemporal cortex of an individual with PiD [24]. Two types of filament could be distinguished: a majority of narrow Pick filaments (NPFs) and a minority of wide Pick filaments (WPFs) (Fig. 1.10). The core of NPFs is made of a single protofilament that consists of residues K254-F378 of 3R tau, which adopt an elongated cross-β structure. Murine and human tau are identical in sequence in this region, with the exception of residue 257 (K in human, R in mouse tau). WPFs are formed

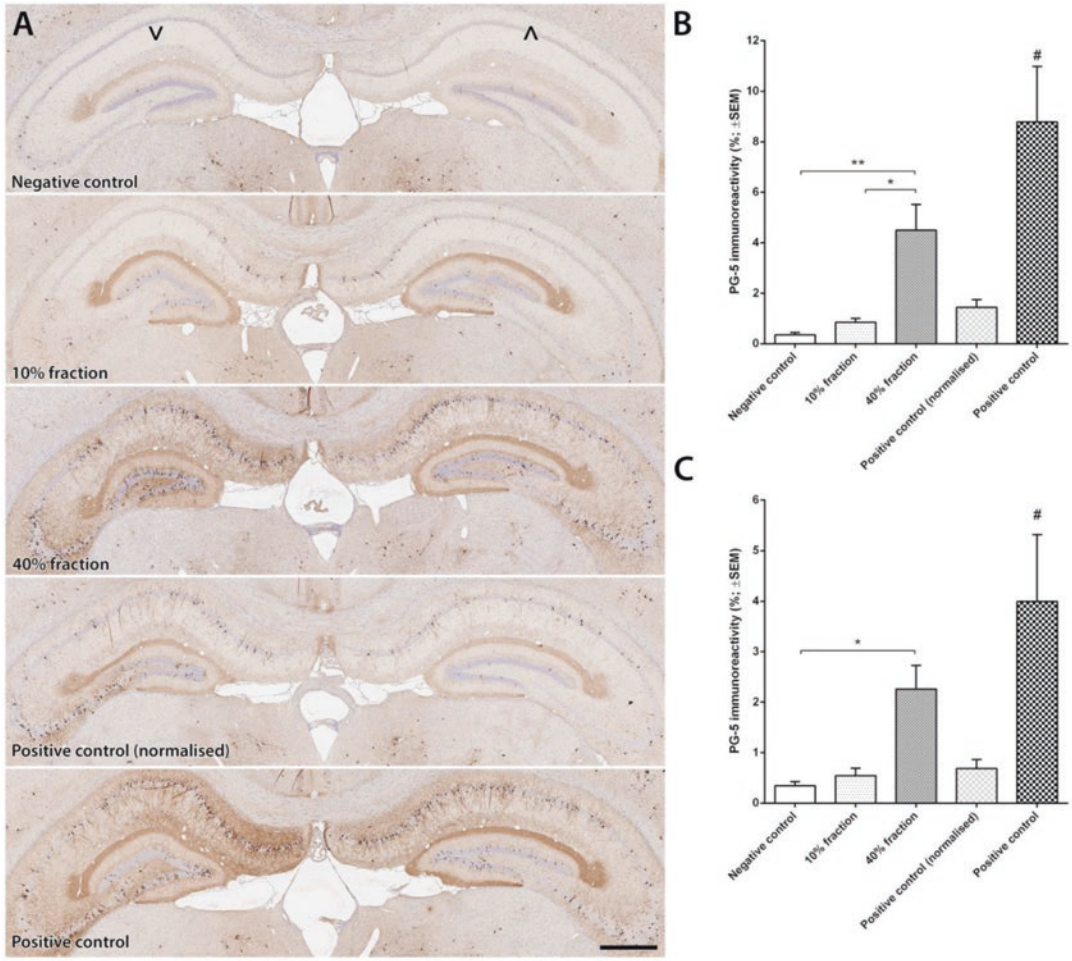

Fig. 1.6 Tau species in the 40% sucrose gradient fractions from the brains of symptomatic mice transgenic for human mutant P301S tau (24.4 weeks) mediate seeding and spreading in brain

(**a**) Unilateral injection of brainstem lysate (positive control) from symptomatic transgenic mice into the hippocampus of asymptomatic transgenic mice led to the accumulation and spread of pathology (detected by antibody PG-5, which recognises tau phosphorylated at S409) in the injected (∨) and contralateral (∧) hippocampus. Mice injected with brainstem lysate of non-transgenic mice (negative control) had minimal pathology. Injection of the 40% sucrose gradient fraction also showed substantial accumulation and spread of pathology (40% fraction), whereas mice injected with the 10% sucrose gradient fraction (10% fraction) were indistinguishable from those injected with brainstem lysate from non-transgenic mice. Injection of brainstem lysate [positive control (normalised)] that had been normalised for total tau did not show robust tau aggregate propagation. (**b**) In the ipsilateral hippocampus, PG-5 immunoreactivity was highest for the positive control, followed by the 40% sucrose fraction. No significant increase in signal was observed for the normalised positive control or the 10% fraction (relative to the negative control). (**c**) In the contralateral hippocampus, a similar, but overall milder, pattern of tau pathology was observed compared to the injecteded side. The Scale bar must be given in micrometers. $*p < 0.05$, $**p < 0.01$, $\sharp p < 0.01$ for all groupwise comparisons

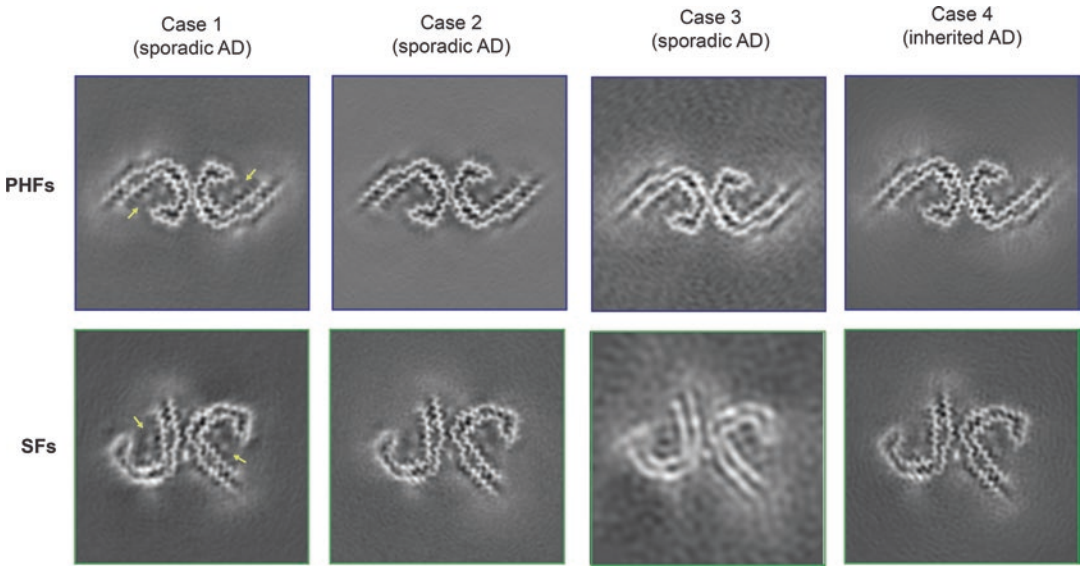

Fig. 1.7 Cryo-EM structures of paired helical filaments (PHFs) and straight filaments (SFs) from the frontal cortex of four AD cases. All structures show identical pairs of C-shaped protofilaments and the same inter-protofilament packing in PHFs and SFs. Cases 1, 2 and 3 had sporadic AD, whereas case 4 had inherited AD (mutation V717F in the amyloid precursor protein gene). All cases had a majority of PHFs and a minority of SFs. Yellow arrows point to the extra densities, which were present in PHFs and SFs from all four cases, bordering the solvent-exposed side-chains of R349 and K375, and of H362 and K369

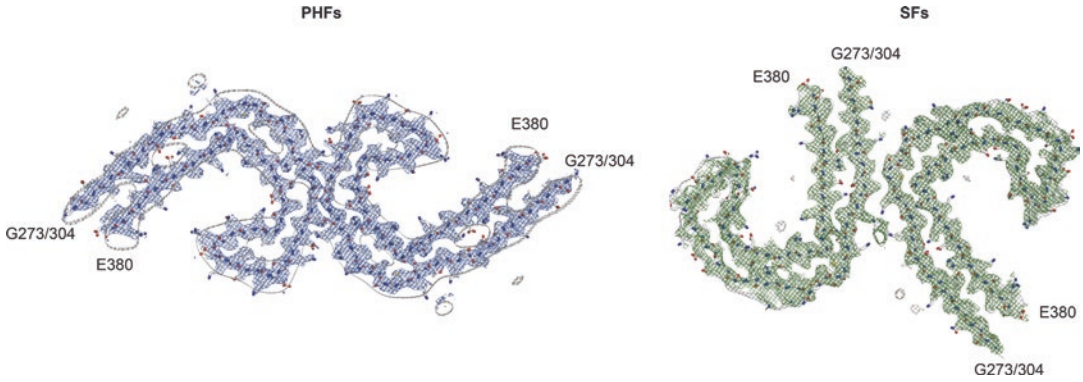

Fig. 1.8 Cryo-EM densities and atomic models of paired helical filaments (PHFs) and straight filaments (SFs) from the frontal cortex of one AD case
PHFs and SFs were resolved to 3.2 Å and 3.3 Å, respectively. Sharpened, high-resolution maps are shown in blue (PHFs) and green (SFs). Unsharpened 4.5 Å low-pass filtered densities are shown in grey. The models comprise G273-E380 of 3R tau and G304-E380 of 4R tau. The protofilaments of PHFs and SFs are identical, but differ in inter-protofilament packing (ultrastructural polymorphs)

by the association of two NPF protofilaments at their distal tips, where they form tight contacts through van der Waals interactions. Each protofilament comprises nine β-strands, which are arranged into four cross-β packing stacks and are connected by turns and arcs (Fig. 1.11). R1 provides two β-strands and R3 and R4 three β-strands each. These stacks pack together in a hairpin-like fashion: β1 against β8, β2 against β7, β3 against β6 and β4 against β5. The final strand, β9, is formed from the ten amino acids after R4 and packs against the opposite side of β8.

Three regions of less well-resolved density bordering the solvent-exposed faces of β4, β5 and

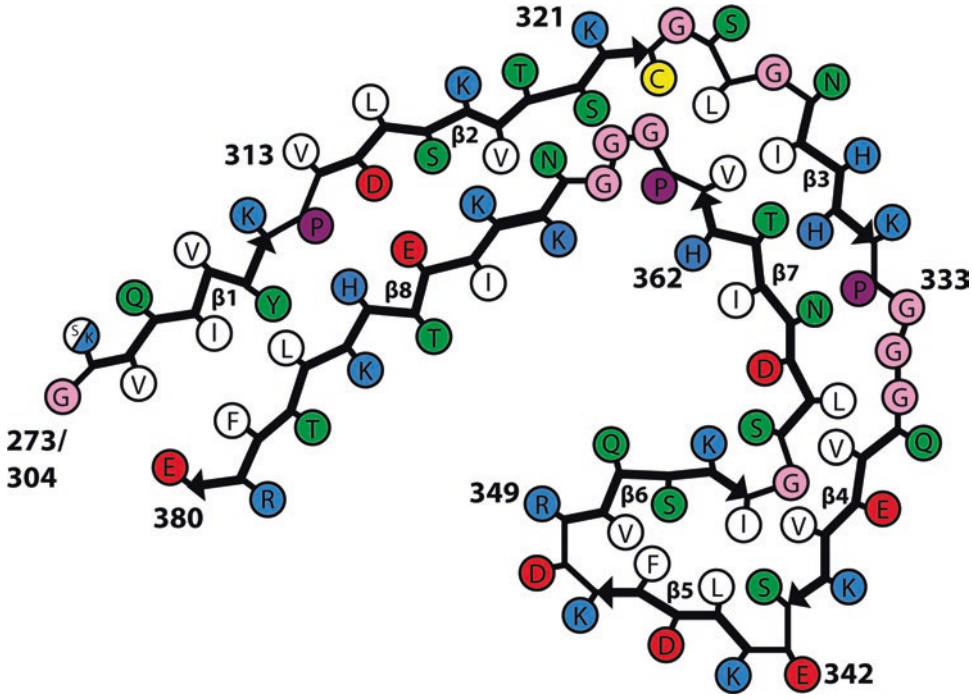

Fig. 1.9 Schematic view of the tau protofilament core of AD. The observed eight β-strand regions (β1-β8) are shown as arrows. Each filament consists of two protofilaments

β9 are apparent in both NPFs and WPFs. They may represent less ordered, heterogeneous and/or transiently occupied structures. The density bordering β4 is similarly located, but more extended, than that found to interact with the side-chains of K317, T319 and K321 in AD filaments.

It was not previously known why only 3R tau is present in Pick body filaments and why S262 is not phosphorylated. Our results suggest that despite sequence homology, the structure formed by K254-K274 of R1 is inaccessible to the corresponding residues from R2 (S275-S305). Moreover, because of steric constraints, the filament structure precludes phosphorylation of S262.

Conclusion

Biochemistry, molecular biology and human genetics have shown that the ordered assembly of tau is at the heart of a large number of neurodegenerative diseases, including AD, tangle-only dementia, CTE, PiD, PSP, CBD and AGD. They may be different clinical diseases, because of the existence of distinct conformers of assembled tau. Assembly leads to propagation of pathology and neurodegeneration, which are characteristic of all tauopathies. It remains to be seen if the same or different molecular species of assembled tau account for propagation and neurodegeneration. Small filaments are the major species of assembled tau responsible for the propagation of pathology.

Cryo-EM of filaments from human brain has established that distinct conformers of aggregated tau are characteristic of AD and PiD [91]. Even though both types of filament share residues G273-F378 of 3R tau, their structures are very different (Fig. 1.12). Whereas PHFs and SFs of AD are made of two identical C-shaped protofilaments that each comprises eight β-strands and a combined β-sheet/β-helix structure, NPFs of PiD are made of a single elongated protofilament comprising nine β-strands and stacks of β-sheets. WPFs consist of two NPFs joined through their

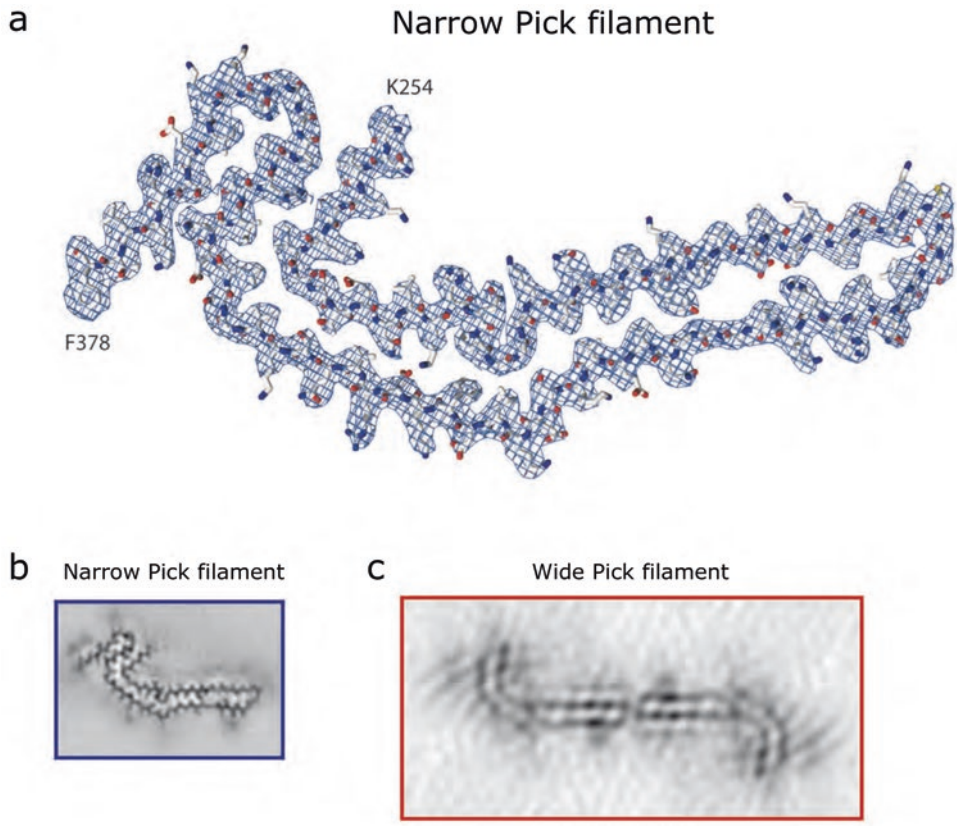

Fig. 1.10 (**a**) Sharpened, high-resolution cryo-EM map of narrow Pick filaments with the atomic model of the Pick fold overlaid (3.2 Å resolution). Narrow (93%) and wide (7%) Pick filaments are characteristic of PiD. Unsharpened cryo-EM densities of a narrow (**b**) and wide (**c**) Pick filament. Wide Pick filaments comprise two narrow filaments that are joined at their distal tips

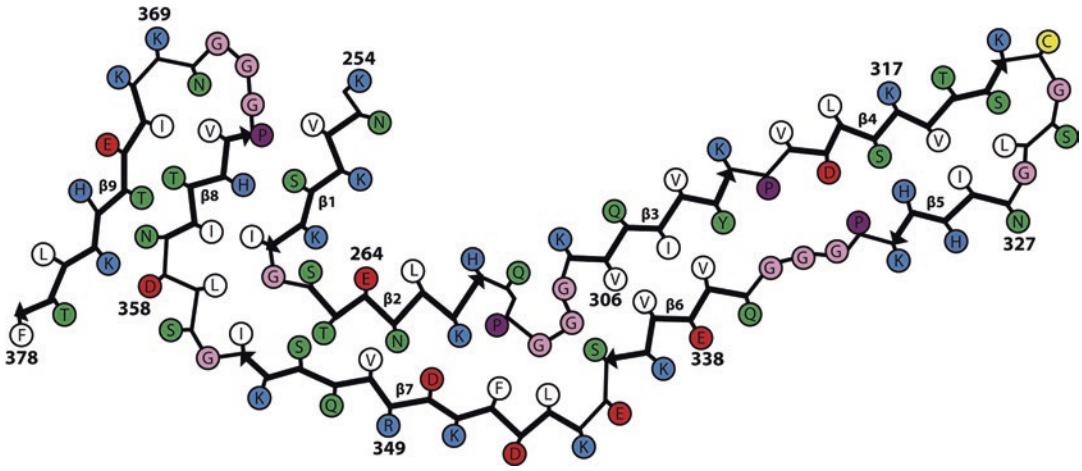

Fig. 1.11 Schematic view of the tau protofilament core of PiD. The observed nine β-strands (β1-β9) are shown as arrows. Narrow Pick filaments are made of one and wide Pick filaments of two protofilaments

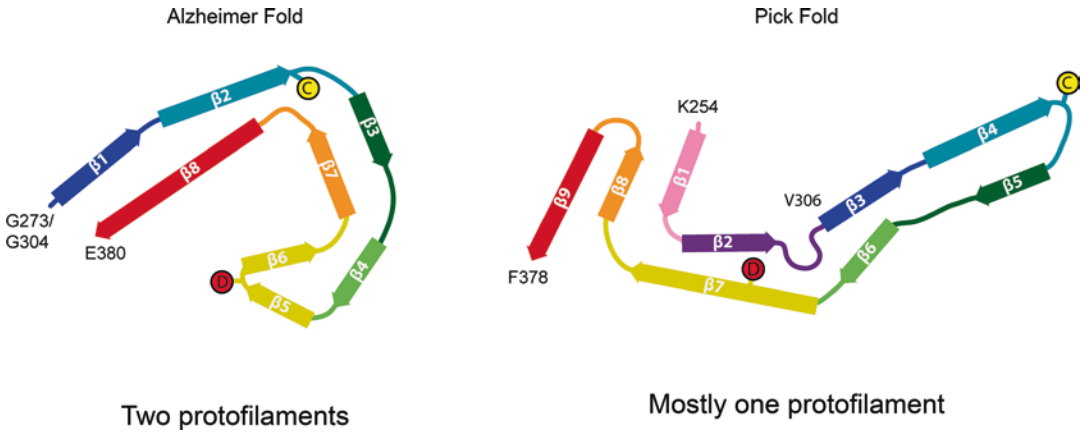

Fig. 1.12 Comparison of Alzheimer and Pick tau filament folds depicted as single rungs
Paired helical and straight tau filaments of AD consist of two identical C-shaped protofilaments that differ in inter-protofilament packing (ultrastructural polymorphs). More than 90% of tau filaments of PiD are narrow filaments that consist of a single J-shaped protofilament. Wide Pick filaments consist of two narrow filaments packed against each other. C322 (yellow) and D348 (red) are highlighted. C322 is on the inside in the Alzheimer fold and the outside in the Pick fold, whereas D348 is on the outside in the Alzheimer fold and the inside in the Pick fold

distal tips. Cryo-EM studies of tau filaments from additional sporadic and inherited cases of AD, as well as negative-stain immunoelectron microscopy of tau filaments from multiple cases of AD and PiD, indicated that the cores of tau filaments from each disease case contain the same sequences. It therefore seems that the structures of tau filaments are distinct between diseases, but identical in different individuals with AD or PiD. It appears likely that additional folds of assembled tau remain to be discovered in other tauopathies.

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2 Structure of NFT: Biochemical Approach

Masato Hasegawa

Introduction

Neurofibrillary tangles (NFTs) and senile plaques (SPs) are the neuropathological hallmarks of Alzheimer's disease (AD). SPs are extracellular aggregates composed of amyloid β (Aβ) peptides, which are cleaved from amyloid β protein precursor (APP). NFTs are intraneuronal pathological structures, which are mainly composed of microtubule-associated protein tau. Before these constituent proteins were found, there were many discussions about which of them is related to the pathogenesis and progression of AD, how they are related each other, and so on. But, after the discovery of mutations in the APP gene in familial forms of AD in 1990, the "amyloid hypothesis" was proposed and many researchers focused on the mechanisms of Aβ production and aggregation, and their regulation. However, clinical trials targeting Aβ with Aβ vaccines have so far had no effect on tau pathologies or little effect on clinical symptoms, even though Aβ deposits were significantly reduced. These results suggest that aggregation of Aβ is not an appropriate target for AD therapy, and also that the Aβ hypothesis should be reconsidered. Conversely, tau pathologies in NFTs and their spread in brains of patients have been found to closely correlate with disease symptoms and progression of AD. In addition, recent findings on prion-like spreading of intracellular abnormal proteins support the idea that the formation and propagation of abnormal tau protein pathologies are the central events leading to neurodegeneration in AD. This chapter describes biochemical and immunochemical approaches used to study pathological tau in AD and other related disorders.

Tau Protein

Tau is a heat-stable microtubule-associated protein that promotes microtubule assembly and stability [1]. It is highly expressed in neurons and is normally located in axons. In adult human brain, six tau isoforms (352–441 amino acid residues) are generated by mRNA alternative splicing from the *MAPT* gene on chromosome 17 [2]. Expression of tau isoforms is regulated in the developmental stages. Namely, the shortest isoform is expressed in fetal brain, and multiple isoforms are gradually expressed during development by insertion of exons 2, 3 and 10; these isoforms have apparent molecular weights of 48–67 kDa [3]. Among them, three-repeat (3R) tau and four-repeat (4R) tau are produced by the exclusion and inclusion of exon 10, respectively. The expression ratio of 3R tau and 4R tau is almost 1: 1 in adult human brain. This ratio is

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important, because mutations that influence the expression ratio of 3R tau and 4R tau induce tauopathies exhibiting tau inclusions composed of the increased tau isoforms [4]. Tau expression in rodents is slightly different from that in human, in that 3R tau is completely replaced by 4R tau in the adult stage. The difference in the splicing of exon10 between the species that determines the 3R tau and 4R tau ratios appears to be due to differences in the splicing enhancer and suppressor mRNA sequences and stem loop structures in exon 10 and intron 10 [5]. Tau is a phosphoprotein and phosphorylation decreases its microtubule-polymerizing ability. Therefore, it is suggested that phosphorylation may regulate microtubule dynamics.

Tau in AD Brain

Tau is structurally less-ordered, and is classified as a natively unfolded protein. Normal tau protein is highly soluble and is mostly recovered in buffer-soluble fractions after ultracentrifugation of brain homogenates. It can be relatively easily purified by boiling treatment and subsequent ion-exchange column chromatography [6] or by perchloric acid (PCA) extraction [7]. However, in AD, the abnormal proteins are accumulated as NFTs in neuronal cell bodies and neuropil threads (NTs) in their processes. Ultrastructurally, they are present as bundles of unique filamentous structures, paired helical filaments (PHFs) and straight filaments (SFs) in NFTs and NTs [8]. In order to purify NFTs, Selkoe et al. investigated their solubility in various buffers, solvents and denaturants, and reported that NFTs are highly insoluble in protein denaturants such as SDS, urea, reducing agent and guanidine [9]. They applied this unusual property to prepare NFTs from AD brains; they partially purified the NFTs as SDS-insoluble tangles, and raised antibodies to the tangles [10]. Ihara, who had worked with Selkoe's group at Harvard University, further investigated the SDS-insoluble tangles together with Kondo after his return to Tokyo. He solubilized the tangles in formic acid and analyzing the peptides generated by digestion with lysyl-endopeptidase, showing that they are composed of the carboxyl-third of tau and ubiquitin [11, 12]. Meanwhile, the presence of SDS-soluble tau in AD brains had been reported, after tau was found to be a component of NFTs (SDS-soluble tau is easy to analyze by SDS-PAGE and immunoblotting). Greenberg and Davis reported a preparation method of PHFs from AD brains using Sarkosyl [13]. Lee's group also purified SDS-soluble, Sarkosyl-insoluble PHFs by means of a similar protocol and demonstrated that hyperphosphorylated tau is the major component of PHFs [14]. Since then, many tau researchers have been working on SDS-soluble, Sarkosyl-insoluble tau, because the SDS-soluble PHFs may represent an early stage of PHFs (NFTs). It is also advantageous that they are more treatable and easy to analyze. Thus, AD brains may contain both SDS-soluble, relatively young PHFs and SDS-insoluble old PHFs (NFTs) that are mainly composed of full-length phosphorylated tau and ubiquitinated carboxyl-third of tau, respectively, because tau accumulation continues for over 10 years from AD onset to death [15].

Solubility of NFTs and PHFs

The solubility of NFTs can be analyzed on slide glasses by conventional Congo red staining or immunohistochemistry of smears of AD brain homogenates with or without various agents. As shown in Fig. 2.1, numerous NFTs and NTs can be seen in AD brain homogenate in Tris-saline (TS), as visualized with anti-tau antibodies such as pS396 or AT8. The numbers of NFTs and NTs are not decreased by addition of 1% Triton-X100 (TX: a noionic detergent), but most of the NFTs disappear upon treatment with ionic detergents such as 1% Sarkosyl or SDS (Fig. 2.1). Immunoblot analysis of the pellets after 15 K rpm centrifugation of the homogenates confirmed that pS396-positive tau in the 15 K-ppt decreased after treatment with Sarkosyl or SDS (Fig. 2.1b) (AT8 failed to detect tau because of the low concentration). However, Sarkosyl-treated tau in the

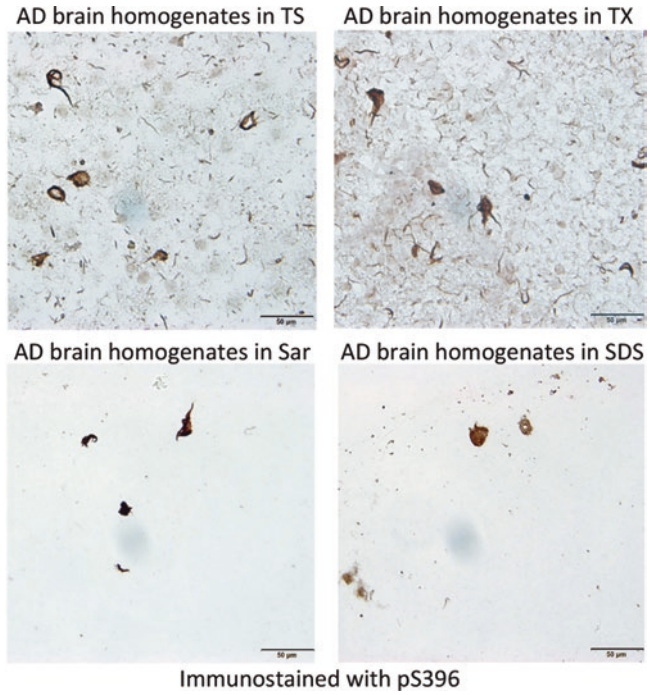

Fig. 2.1 Immunohistochemistry of AD brain homogenates smeared on slide glasses in extraction buffer with or without Triton-X100 (TX), Sarkosyl (Sar) or SDS

supernatant was precipitated after ultracentrifugation at 50 K rpm and was detected with both AT8 and pS396, whereas SDS-treated tau was mostly recovered in the supernatant (Fig. 2.2). These results suggest that NFTs are dissociated to filamentous components by Sarkosyl, but still have structure, whereas most NFTs are solubilized by SDS and recovered in the 50 K-sup. The presence of PHFs and SFs can be confirmed by immuno-EM observation of the Sarkosyl-insoluble fractions of AD. Thus, the abnormal tau forming these filaments can be enriched from Sarkosyl-treated brain homogenates by means of differential centrifugation [16]. This is the reason why many researchers use Sarkosyl extraction protocols for preparation of pathological tau proteins from brains of patients and cellular and animal models. The purification, characterization and analysis of abnormal tau prepared from AD brains have established that abnormally phosphorylated tau is the major component of PHFs and SFs in NFTs.

Biochemical Approaches to PHF-Tau in AD Brains

In order to clarify the molecular mechanisms of tau aggregation and NFTs formation in AD, we first took an immunochemial approach in Ihara's lab at Tokyo Metropolitan Institute of Gerontology. At that time, there was no powerful method to analyze abnormal tau directly, and it was difficult to purify tau from AD brains. So, we prepared Sarkosyl-insoluble fractions from AD brains and used them to immunize BALB/c mice, then screened clones for production of antibodies that stained NFTs. Fortunately, several monoclonals that strongly stained NFTs were obtained. All these antibodies recognized tau in fetal or juvenile brains, but hardly detected the tau in adult brains. We then analyzed the epitopes of two typical clones (M4 and C5) in fetal tau and PHF-tau. When fetal tau was treated with *E. coli* alkaline phosphatase at 37 °C, a mobility shift of the tau band was observed and immunoreactivity to C5

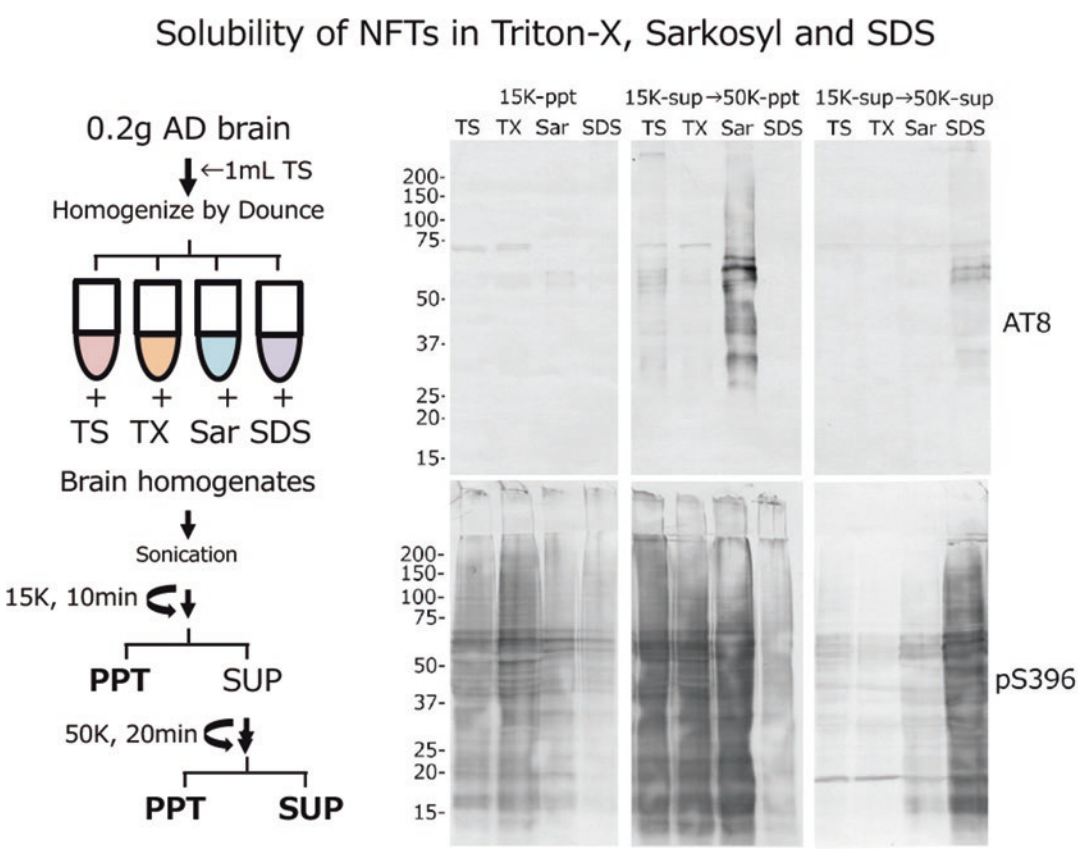

Fig. 2.2 Immunoblot analyses of tau in AD brain. Brain homogenates were sonicated briefly and centrifuged at 15 K-rpm for 10 min. The supernatants were ultracentrifuged at 50 K-rpm for 20 min. The pellets and supernatant were solubilized in SDS sample buffer, applied to gel, and blotted with AT8 and pS396

was completely abolished, whereas the M4 immunoreactivity was unchanged, and was only lost after alkaline phosphatase treatment at 67 °C. These results suggested that the monoclonals recognize distinct phosphorylated epitopes of tau [17]. Then, we tried to map the epitopes in fetal tau and PHF-tau. However, this proved difficult, and the immunoreactivities often disappeared after protease digestion and/or separation by reverse-phase HPLC. Therefore, we tested various chemical cleavage methods such as CNBr treatment and enzymatic digestions with lysylendopeptidase, trypsin and other enzymes, together with peptide sequencing, and finally identified the recognition sites of the C5 and M4 antibodies as residues 386–406 and 198–250, respectively [17]. During these studies, I learned a lot about protein-chemical methods, and also thought it might be faster and more comprehensive if I could directly analyze the phosphorylation sites and other abnormalities of PHF-tau compared with normal tau. So, I started a direct approach to PHF-tau, in which all the digested peptides of purified PHF-tau and normal tau with or without dephosphorylation were analyzed using a protein sequencer and mass spectrometry. This was about 1990, when mass spectrometry was just being introduced for analysis of peptides and proteins, and I spent about a year in Dr. Takio's lab at RIKEN to study the techniques and analyze the PHF-tau.

Protein-Chemical Approach to PHF-Tau

We succeeded in purifying the AD abnormal tau (PHF-tau) from Sarkosyl-insoluble fraction by solubilization with 6 M guanidine-HCl, gel filtration and reverse-phase HPLC. The purified insoluble and soluble tau from AD brains with or without alkaline phosphatase treatment were digested with lysyl endopeptidase, the peptide maps were compared, and all the fractions were analyzed by protein sequencing and mass spectrometric analysis. In the initial analysis with ion-spray mass spectrometry, we identified three phosphorylation sites (Thr231, Ser235 and Ser262) and two extensively phosphorylated regions located in the amino- and carboxyl-terminal portions of the microtubule-binding domain [18]. Thr231 and Ser235 were fully phosphorylated in PHF-tau, but Ser262 was only partially phosphorylated, because unphosphorylated Ser262 peptide was detected in a larger amount than the phosphorylated peptide. We also found that two asparagine residues (Asn167 and Asn279) are deamidated. In addition, both 3R tau and 4R tau specific peptides were identified in PHF-tau at a similar ratio to that seen in normal tau [17]. These results suggested that both 3R and 4R tau isoforms are accumulated in AD, but in abnormally phosphorylated and deamidated states. At the same time, Goedert et al. reported that all six brain tau isoforms are deposited in AD brain in hyper-phosphorylated forms [19].

As described above, mass spectrometry is a very powerful technique to identify peptides with molecular mass of 500~3000, but is less effective for heavily phosphorylated peptides, because it is difficult to ionize these highly negatively charged peptides. Consequently, we struggled to determine the phosphorylation sites in these heavily phosphorylated regions. So, we introduced a method to determine the phosphorylation sites by beta elimination of phosphopeptides followed by protein sequencing. We first analyzed tau in fetal and adult rat brains, which are less highly phosphorylated than PHF-tau, and determined ~10 phosphorylation sites in fetal and adult normal rat brains; we also established how phosphorylation at these sites changes during development [20]. In collaboration with Dr. Morishima, we also investigated PHF-tau by protein sequencing and mass spectrometric analyses of peptides digested with immobilized trypsin and some other proteases, and determined ~20 abnormal phosphorylation sites. Furthermore, we generated several antibodies to these novel phosphorylation sites and confirmed that the sites are really phosphorylated in tau in NFTs of AD brains [21]. Half of the phosphorylation sites identified in the abnormal tau in AD brains are proline-directed, but half are non-proline-directed, suggesting that multiple kinases must be involved in the phosphorylation. During the course of analysis of PHF-tau, we also noticed that ubiquitinated smeared tau can be separated from non-ubiquitinated tau. Then, we digested the samples, separated the peptides by HPLC, and compared the peptide maps to identify the targets of ubiquitin. As expected, we identified several ubiquitinated tau peptides and a Lys-48-ubiquitinated multi-ubiquitin peptide, demonstrating that the ubiquitin target protein in NFTs is tau [15]. Since only a small proportion of the ubiquitin formed multi-ubiquitin chains, it seemed that most of the ubiquitin in the tau occurred as a monoubiquitinated form. In addition, there was ubiquitin-negative tau and smears, and the tau was much less processed, strongly suggesting that tau accumulation and processing precede ubiquitination.

Attempts to Recapitulate the Abnormal Phosphorylation of Tau

These immunochemical and protein-chemical approaches to tau in NFTs demonstrated that the major biochemical difference between PHF-tau and normal tau was the phosphorylation. Therefore, we attempted to recapitulate the phosphorylation of tau in vitro. Recombinant tau was phosphorylated with various kinases, such as Cdk5, MAPK, GSK3, CaMK and PKA, in both single and sequential multiple passes, and the outcome was monitored with various phosphorylation-dependent anti-tau antibodies.

However, no heavily phosphorylated state similar to that in PHF-tau was reproduced in the in vitro experiments with recombinant tau (unpublished data). We also tested tau phosphorylation with rat brain extracts in the presence of phosphatases inhibitors such as okadaic acid, NaF, etc. The brain extracts actually phosphorylated tau very well and a big mobility shift of the tau band was observed in the presence of inhibitors; however, the phosphorylation state was not the same as that of PHF-tau, and tau aggregation or fibril formation was never detected. Most tau researchers were also trying to recapitulate the abnormal tau phosphorylation and find the responsible kinases or phosphatases. At that time, Matsuo et al. reported that normal soluble tau in biopsy brain samples is highly phosphorylated, but is rapidly dephosphorylated in the postmortem period [22]. This raised the question of why PHF-tau remains hyperphosphorylated after a long (~10 h) postmortem delay. To address this, we investigated dephosphorylation of fetal and PHF-tau (Sarkosyl-insoluble pellets prepared from AD brain) by several protein phosphatases in vitro. Protein phosphatases 1 and 2A and calcineurin could dephosphorylate fetal-tau, whereas intact PHF-tau without guanidine-HCl denaturation was hardly dephosphorylated by these protein phosphatases. However, after denaturing treatments with guanidine-HCl or formic acid, the PHF-tau was easily dephosphorylated by these protein phosphatases [23]. The results strongly indicate that PHF-tau deposited in NFTs and NTs in AD brain is resistant to dephosphorylation, because the tau is present in filamentous structures as PHFs in NFTs. Matsuo's paper thus revealed the limitations of the analysis of soluble proteins in postmortem brain tissues, and showed that caution is needed in interpreting data obtained from postmortem samples. However, phosphorylation was still seen as central in the formation of pathological tau by AD researchers, because PHF-tau is much more highly phosphorylated than biopsy tau or fetal tau. Then, heparin was reported as a factor that accelerates tau phosphorylation [24]. Heparin is a negatively charged glycosaminoglycan, and binds to tau through positively charged regions such as the microtubule-binding region. It induces conformational changes of tau, as a result of which enzymes such as kinases can access the substrate more easily. Like heparin, tubulin, a physiological partner of tau, can also bind to tau and promote phosphorylation. When I started work in Goedert's lab in MRC in 1995, Goedert and Jakes were investigating the effect of heparin on in vitro tau phosphorylation. We noticed a high-molecular-weight band on gel in the presence of heparin, and Crowther looked at the sample by electron microscopy. Surprisingly, PHF-like tau filaments were seen in the sample, and after various experiments it was found that heparin and negatively charged glycosaminoglycans induce tau fibril formation [25, 26]. These results suggest that tau can assemble into PHF-like filaments without phosphorylation.

Pathological Tau Proteins in Various Neurological Diseases

Tau pathologies are seen not only in AD, but also in various other dementing neurological diseases, such as Pick's disease (PiD), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD) (Fig. 2.3). For this reason, it was thought that tau lesions might be a final common pathway of neurodegeneration, rather than the cause. However, mutations in the tau gene (*MAPT*) were discovered in familial forms of dementias with tau pathologies in 1998 [27, 28, 29], demonstrating that abnormality of tau is sufficient to cause disease. Although the clinicopathological features of dementia caused by *MAPT* mutations are diverse [30], there are strong correlations between the positions (or effects) of mutations and tau pathologies, suggesting that neurodegeneration occurs via tau accumulation. Mutations outside exon 10 have been shown to affect tau conformation, cause tau dysfunction, and promote tau aggregation, resulting in both 3R and 4R tau pathology. The intronic mutations in intron 10 and many of the mutations in exon 10 affect tau splicing, changing the ratio of 3R tau and 4R tau,

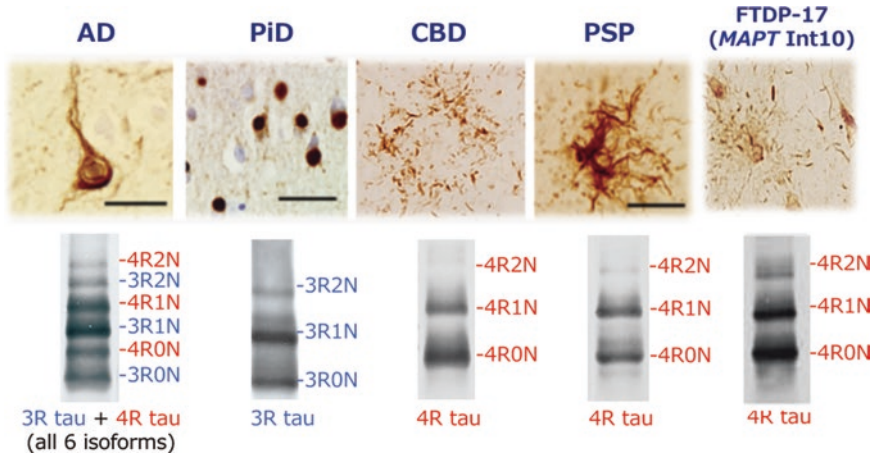

Fig. 2.3 Characteristic tau inclusions in AD, PiD, CBD, PSP and FTDP-17 with intron 10 + 16 mutation, and full-length tau bands (isoforms) in these tauopathies detected by immunoblot analysis after complete dephosphorylation. All six tau isoforms are accumulated in AD, whereas only 3R tau isoforms are deposited in PiD, and only 4R tau isoforms are accumulated in PSP, CBD and FTDP-17 with intron 10 mutation

and resulting in accumulation of increased or affected tau isoforms. These findings clearly demonstrated that tau abnormalities cause accumulation of tau and neurodegeneration. However, most of these tauopathies are sporadic, and no significant changes in the ratio of 3R/4R tau isoforms were detected [31]. Furthermore, the morphologies of tau inclusions, their distributions, and their biochemical features are different among the diseases, suggesting that the molecular mechanisms may be different among the diseases and also different from the *MAPT* mutation cases. So, why tau pathologies are so diverse and different between the diseases? One reason is that tau isoforms accumulated in these tauopathy brains are different in the different diseases. In AD, all six brain tau isoforms (3R tau and 4R tau isoforms) are deposited in neuronal cells as NFTs and NTs. In PiD, only 3R tau isoforms are deposited as Pick's bodies and related inclusions, while in PSP and CBD only 4R tau isoforms are accumulated in neurons and glial cells, with some characteristic structures [16, 32] (Fig. 2.3). However, both CBD and PSP are 4R tauopathies, but they are neuropathologically distinguished, suggesting that the other difference causes these tau phenotypes. Interestingly, the banding patterns of C-terminal fragments (CTFs) of the insoluble tau were shown to be different between PSP and CBD [33], suggesting that the difference in the CTFs banding pattern may reflect the structural differences of tau filaments in PSP and CBD. In fact, ultrastructure of the pathological tau filaments was shown to be morphologically different between the diseases [34]. To address this issue, we investigated the pathological tau and the protease-resistant fragments in these various tauopathies including AD, PiD, PSP, CBD and FTDP-17 with intronic mutations. We found that the trypsin-resistant core units of the tau filaments were composed of different tau repeat regions located between residues 243–406, indicating that the conformations of the cores are disease-specific [16] (Fig. 2.4). These patterns are reminiscent of the proteinase-K-resistant bands in human prion disorders, and suggest that tauopathies may be caused by accumulation of toxic tau 'prions' in the brain.

Thus, the phenotypical differences in the tau pathologies can be classified biochemically according to the banding patterns of C-terminal fragments and protease-resistant tau, which represent the abnormal structures of tau aggregates [16, 33] (Fig. 2.4).

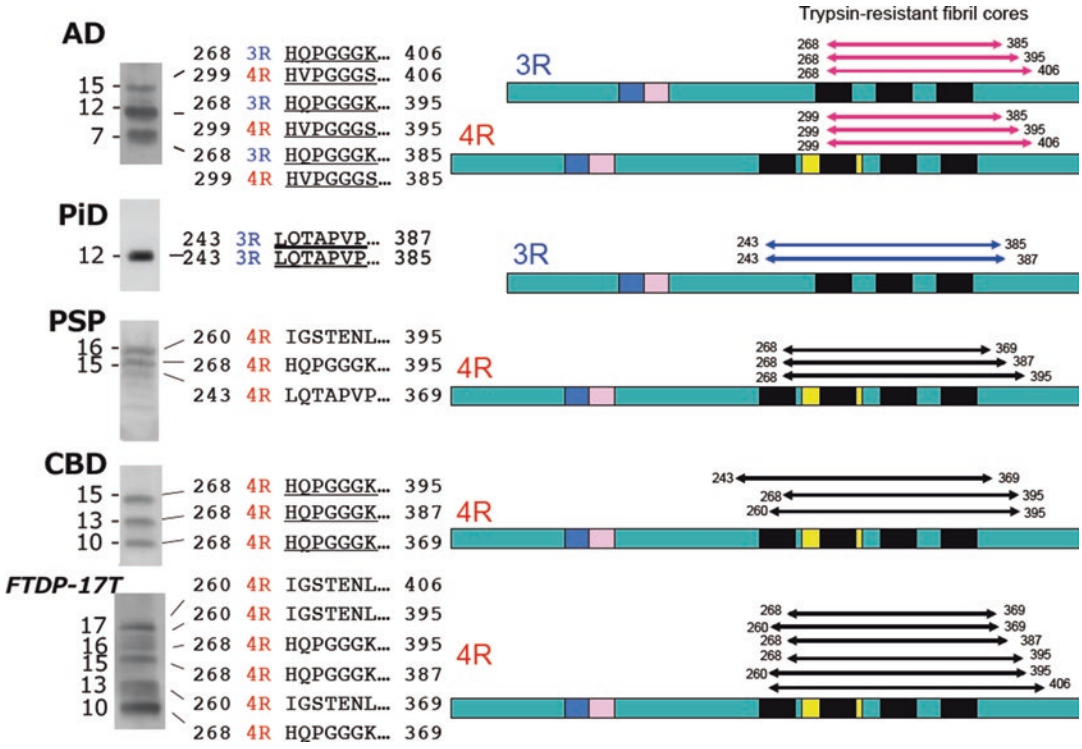

Fig. 2.4 Summary of trypsin-resistant regions of pathological tau from AD, PiD, PSP, CBD and FTDP-17 with intron 10 mutation. The tau fragments identified as trypsin-resistant cores in these tauopathies are indicated by solid lines. AD-tau cores are distinct from the others in terms of both the isoforms and regions. The cores are localized to start from the middle of the 1st repeat of 3R tau and the 2nd repeat of 4R tau, which are different from those of the other tauopathies

Significance of Tau Phosphorylation

The significance of tau phosphorylation for the aggregation or assembly of tau into filaments still remains controversial. However, biochemical analyses of various kinds of pathological tau in tauopathy brains, including PiD, CBD, PSP, FTDP-17, suggest that phosphorylation of tau may occur after aggregation or filament formation, because most of the phosphorylation sites are located outside the core regions of the filaments. It should be emphasized that, exceptionally, Ser262 is not phosphorylated in tau in PiD [35], which is a 3R tauopathy. In PiD, tau residues 243–387 form the filament cores, suggesting that Ser262 is buried in the core regions and cannot be accessed by kinases [16]. In other tauopathies including AD, the Ser262 residue is located outside the core regions of the filaments, so it can be partially phosphorylated. Furthermore, Ser356, which is phosphorylated by the same kinases that phosphorylate Ser262, has never been detected as a phosphorylated residue in the analyses of the pathological tau in theses tauopathies [16]. This is in good agreement with the fact that Ser356 is located in the trypsin-resistant cores of all these tau filaments. Recent Cryo-EM studies of tau filaments prepared from brains of patients confirmed that Ser356 is located in the cross-beta structures of tau filaments from AD and PiD [36, 37], and that Ser262 is located in a position that cannot be accessed by kinases in PiD (Fig. 2.4).

Molecular Mechanisms of Tau Aggregation and NFT Formation

As described above and shown in Fig. 2.5, both 3R and 4R tau proteins accumulate in AD patient's brain as amyloid-like filamentous structures that show physicochemical properties of β-sheet structure, such as Congo red or thioflavin staining. In PiD, only 3R tau isoforms assemble into filaments. In PSP and CBD, only 4R tau isoforms assemble into filaments but the core regions of these aggregates are different, forming different tau filaments. As a mechanism of amyloid fibril formation, seed-dependent aggregation has been proposed and is now widely accepted. A small aggregation nucleus is formed first and then normal proteins bind to this seed nucleus and assemble into filaments by converting the normal protein to an abnormal form on the seed as a template. As illustrated in Figs. 2.5 and 2.6, PHFs and SFs can be formed by coupling of 3R tau and 4R tau in neurons expressing both 3R and 4R tau isoforms, for reasons, such as aging, infection, injury, inflammation, or some other factors or events. The abnormal tau proteins expand by prion-like conversion. The filamentous tau is very stable and the abnormal protein aggregates cannot be degraded by the attacks of kinases, chaperones or proteases; further, phosphatases cannot dephosphorylate the aggregates. The resulting protein aggregates may have chronic toxic effects on proteasome, lysosome and autophagy systems in the cells (Fig. 2.6). Furthermore, the protein seeds may propagate from cell to cell and spread throughout the brains through both known and unknown mechanisms. It should be emphasized that considerable amounts of pathological tau have already accumulated by the time symptoms appeared. Therefore, it is important to block the formation of abnormal tau and the spreading or cell-to-cell propagation of abnormal tau protein for therapy of AD and tauopathies.

Conclusions

Immunochemical and protein-chemical approaches have revealed that hyperphosphorylated and partially ubiquitinated tau is the major component of NFTs, one of the neuropathological hallmarks of AD and related neurological disorders. The most important feature of the pathological tau is its abnormal conformation,

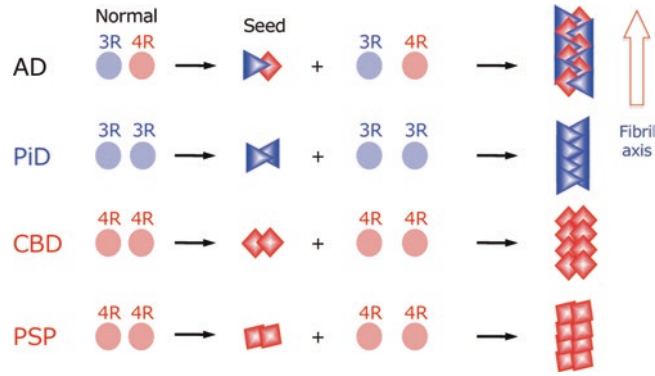

Fig. 2.5 Schematic illustration of possible tau assemblies in AD, PiD, CBD and PSP. The blue and red round shapes illustrate the normal 3R tau and 4R tau isoforms, respectively. The blue triangular and red tetragonal shapes illustrate the filament core domains composed of 3-repeat tau isoforms and 4-repeat tau isoforms, respectively. These filament core domains may act as the seeds for the seed-dependent prion-like conversion and abnormal tau filament formation

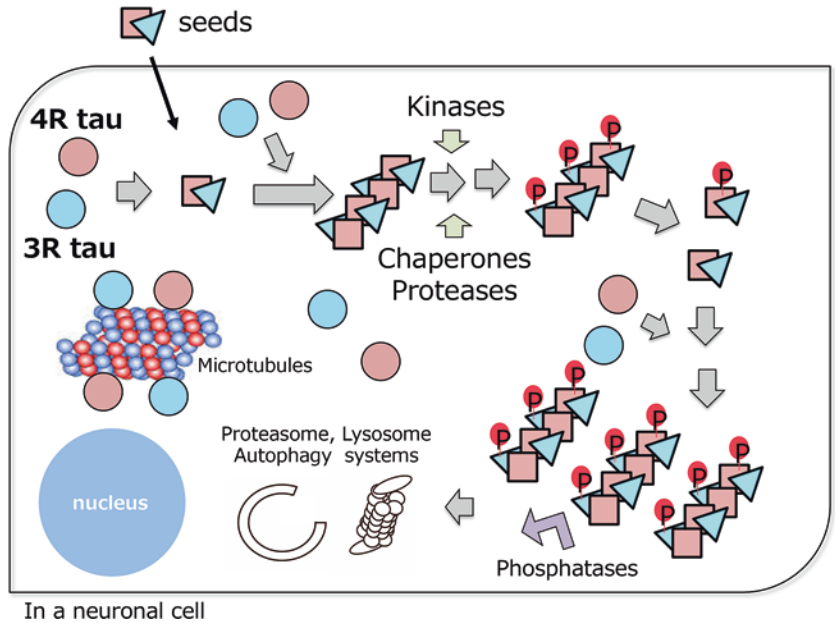

Fig. 2.6 Schematic illustration of tau aggregation in a neuronal cell in an AD brain. Both 3R and 4R tau assemble into filamentous structures by interacting with the seed. Kinases, chaperones and proteases are unable to degrade or restore the proteins. The aggregated filaments may be toxic to various intracellular functions, such as the proteasome, lysosome and autophagy systems, resulting in induction of cell death. Phosphatases do not dephosphorylate residues in these filaments because of the structural stability of the filaments

which enables it to accumulate as amyloid-like filamentous structures. The abnormal tau has prion-like properties, i.e., it converts normal tau into filamentous form by working as a template seed, and can propagate from cell to cell. Importantly, accumulated tau isoforms and the abnormal structures of tau proteins are different among different tauopathies. Clear structural differences can be detected in the protease-resistant core regions of the tau filaments. Most of the post-translational modifications found in the pathological tau in brains of patients may be the results of responses by intracellular defense systems to the abnormal proteins, but may not be causative for aggregation. Proteases and phosphatases may be unable to degrade or dephosphorylate the filamentous tau, which explains why hyperphosphorylated tau can be detected in the brains of patients even after long postmortem intervals. Recent progress in Cryo-EM and its application to amyloid-like pathological protein filaments should provide new insights into the pathogenesis and progression of AD and other neurodegenerative diseases.

Method: take 2 μL of each sample of brain homogenate, smear on slide glass, and stain with pS396 (or AT8)

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3 Nuclear Magnetic Resonance Spectroscopy Insights into Tau Structure in Solution: Impact of Post-translational Modifications

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Introduction

The neuronal Tau protein belongs to the microtubule-associated protein 2 (MAP 2)/Tau subfamily and is mainly described as a cytosolic protein, localized in axons [5, 6] and involved in the regulation of tubulin polymerization and microtubule stability [47]. This simplified view has been enriched by multiple studies demonstrating roles of Tau in many additional biological processes in various localizations including nucleus, dendrites and extracellular medium [20, 25, 44, 46]. Six Tau isoforms are present in human brain (ranging in length from 352 to 441 amino acid residues), resulting from alternative splicing [17, 19]. Tau is composed of two main domains (Fig. 3.1). In the projection domain (Met^{1}-Tyr^{197}) (residue numbering as in the longest human isoform of Tau) an N-terminal extension (with 0, 1, or 2 inserts abbreviated as 0 N, 1 N, 2 N) and the first part of a proline-rich region (residues 165–197) are distinguished. The assembly domain (Ser^{198}-Leu^{441}) consists of the second part of the proline-rich region (annotated PRR), three (Tau3R) or four (Tau4R) microtubule-binding repeats (MTBR, numbered R1 to R4) [9, 18], and a C-terminal extension. MTBR repeats R1 to R4 (31 or 32 residues) have similar sequences. Most NMR studies are performed either using the longest Tau isoform, splicing variant Tau2N4R with 441 amino-acid residues, or the shortest isoform, Tau2N3R with 352 amino-acid residues.

As a disordered protein Tau has no stable two- or three-dimensional structures and behaves as an ensemble of dynamic conformers. Yet, Tau is described as undergoing conformational changes that lead to pathological forms and ultimately to its aggregation. However, the nature of the initial conformational change(s) has remained elusive. Since NMR spectroscopy is well-suited to study highly dynamic systems, such as intrinsically disordered proteins in general, its use has increased in the field over the last 15 years. This has come along with improved resolution and sensitivity offered by higher magnetic fields and cryogenically cooled NMR probeheads. Yet, NMR has limitations, the major one being that the protein under study needs to be isotopically-labelled (with ^{15}N and ^{13}C stable isotopes) since not all atomic nuclei are magnetically active and

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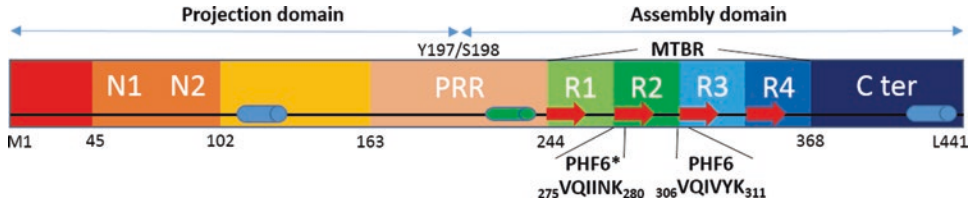

Fig. 3.1 Domains of Tau protein. N1 and N2 correspond to 2 regions included, or not, depending on the splicing variants. PRR: proline-rich region and MTBR: microtubule-binding region. The MTBR consists of four partially repeated sequences named R1 to R4. The nuclei of Tau aggregation, the PHF6∗ and PHF6 hexapeptides, are indicated. Some elements of secondary structure are represented, as red arrows for β-strands, blue cylinders for α-helices and green cylinder for polyproline II helix

detectable by NMR. An important consequence is that NMR research mainly concerns recombinant proteins, and cannot address native proteins extracted from tissues. The other major limitation is the low intrinsic sensitivity of NMR, which reinforces the notion that NMR is best suited for studying proteins produced in recombinant systems.

Tau: A Disordered Protein with Local Secondary Structure

For analysis of protein NMR spectra, resonance assignment has to be performed. That is, the amino acid residue in the protein that gives rise to a resonance in the spectrum has to be identified, such that information about its chemical environment and secondary structure can be extracted (Fig. 3.2a). In early work, performed on a medium-field NMR spectrometer, a limited number of resonances of a two-dimensional (2D) ^{1}H, ^{15}N spectrum of Tau were assigned (Fig. 3.3) [30, 40]. This was not an easy task for the Tau protein, since it is a large protein for NMR with considerable amino-acid sequence degeneracy – with Gly, Ser, Pro and Lys residues each constituting 10% of its amino-acid sequence. The ^{1}H, ^{15}N 2D spectrum of Tau is typical of a disordered protein, with narrow dispersion of resonances in the amide proton dimension, confirming the absence of a global fold. In addition, the resonances in spectra of peptides corresponding to short stretches of Tau sequence match the corresponding resonances in the full-length Tau protein. An amino acid in the peptide thus experiences the same environment and conformational sampling as in the full-length protein, underlining the absence of stable secondary or tertiary structure in Tau [40]. Finally, the ^{13}C chemical shift values of alpha- and beta-carbons (CA, CB) of a selected number of resonances match the random coil chemical shift values found in databases, confirming the disordered nature.

Later work has allowed extensive assignment of Tau resonances, and with it came more detailed information on Tau conformation in solution [34]. These data confirm that no rigid secondary structure elements are present in Tau. However, a few transient elements of secondary structure, restricted to short sequence stretches, were found. Small β-strand tendency was observed in the repeat region of Tau (R1 to R4), with an occupancy estimated around 10–25%, the highest value being found for the PHF6* and PHF6, VQIINK and VQIVYK hexapeptides located in the R2 and R3 repeat, respectively. These peptides thus adopt a β-strand conformation in about 10–25% of the conformers. Alternatively, an individual conformer can be considered to adopt a β-strand conformation 10–25% of the time. This result is of interest since it shows that this structural tendency can be linked to functional consequences, as the PHF peptides are known to be nuclei of aggregation and can self-assemble into β-sheet aggregates [45]. Short stretches of helical tendency were found in the N-terminal (114–123) and C-terminal (428–437) regions (Fig. 3.1). This local structuration is confirmed based on a reported ensemble of Tau conformers calculated from a large number of various NMR parameters combined with small-angle X-ray

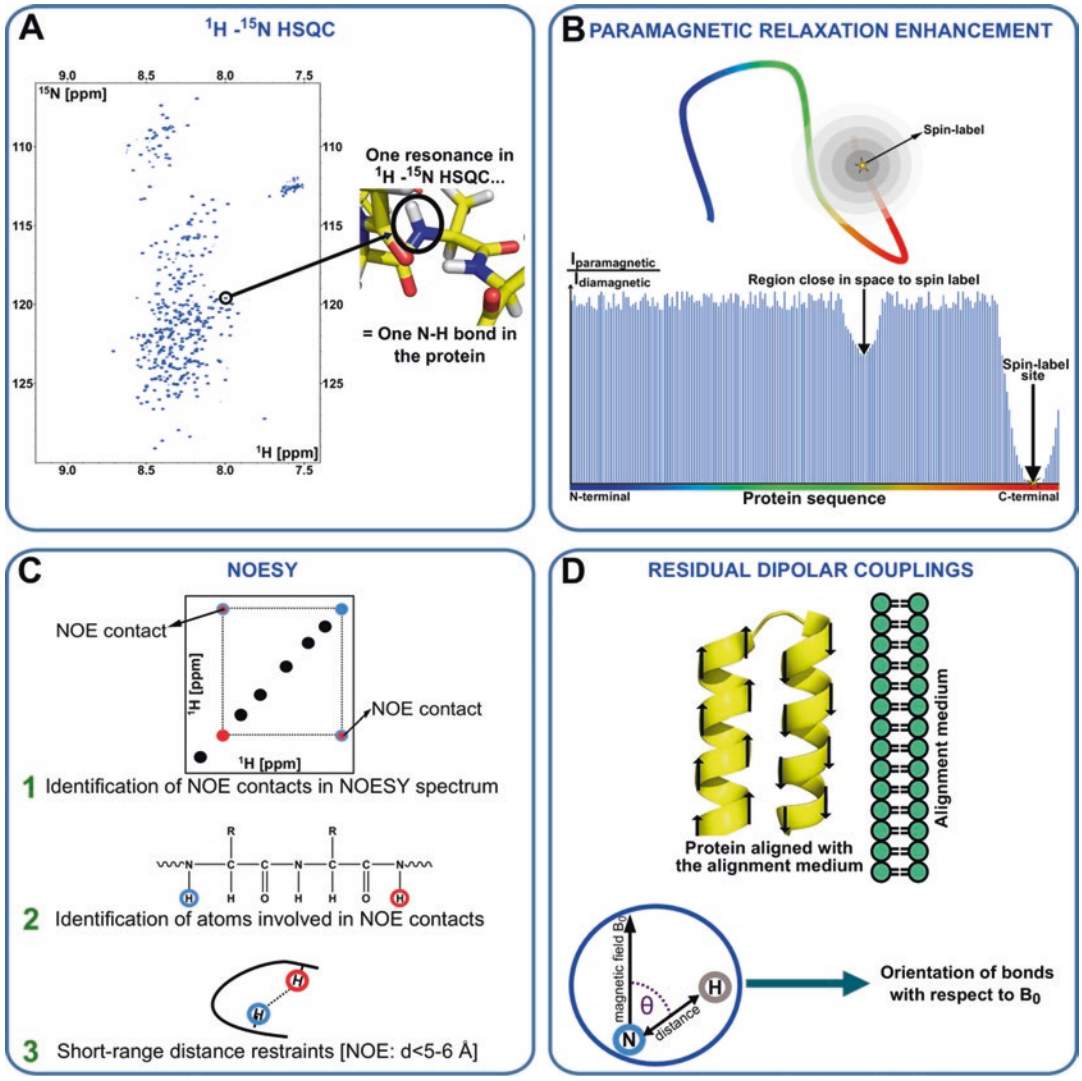

Fig. 3.2 Key NMR techniques used to study the structure of Tau. (**a**) The ^{1}H-^{15}N HSQC spectrum provides residue-specific information, since each resonance in this spectrum corresponds to an N-H bond of an amino-acid residue of the protein. (**b**) Paramagnetic relaxation enhancement (PRE) is used to define the global structure of Tau. A paramagnetic probe is covalently bond to the side-chain of a Cys residue of the protein and induces transverse relaxation of atomic nuclei spatially close to the probe. An HSQC is recorded on a paramagnetic and a diamagnetic sample and the intensities of each corresponding signals are compared. The resonances corresponding to atomic nuclei that are close in space to the probe (up to 25–40 Å) have a decreased intensity in the paramagnetic sample compared to the diamagnetic control experiment. (**c**) NOESY spectra are recorded to obtain NOEs, which provide short-range spatial information (up to 5–6 Å). When an NOE is observed between two atoms, it indicates that the two atoms involved are less than 5–6 Å away from each other. (**d**) Residual Dipolar Couplings (RDCs) can be measured when an alignment medium is added to the NMR tube, which will induce a partial preferential alignment of the protein. This method provides information on orientation of individual bonds and inter-nuclear vectors in the protein

scattering data [37]. Molecular ensemble approaches consist in *in-silico* selection of an ensemble of molecular structures, among a very large number of pre-generated conformers, which fits the experimental data better than the purely random statistical coil model (Fig. 3.4). In

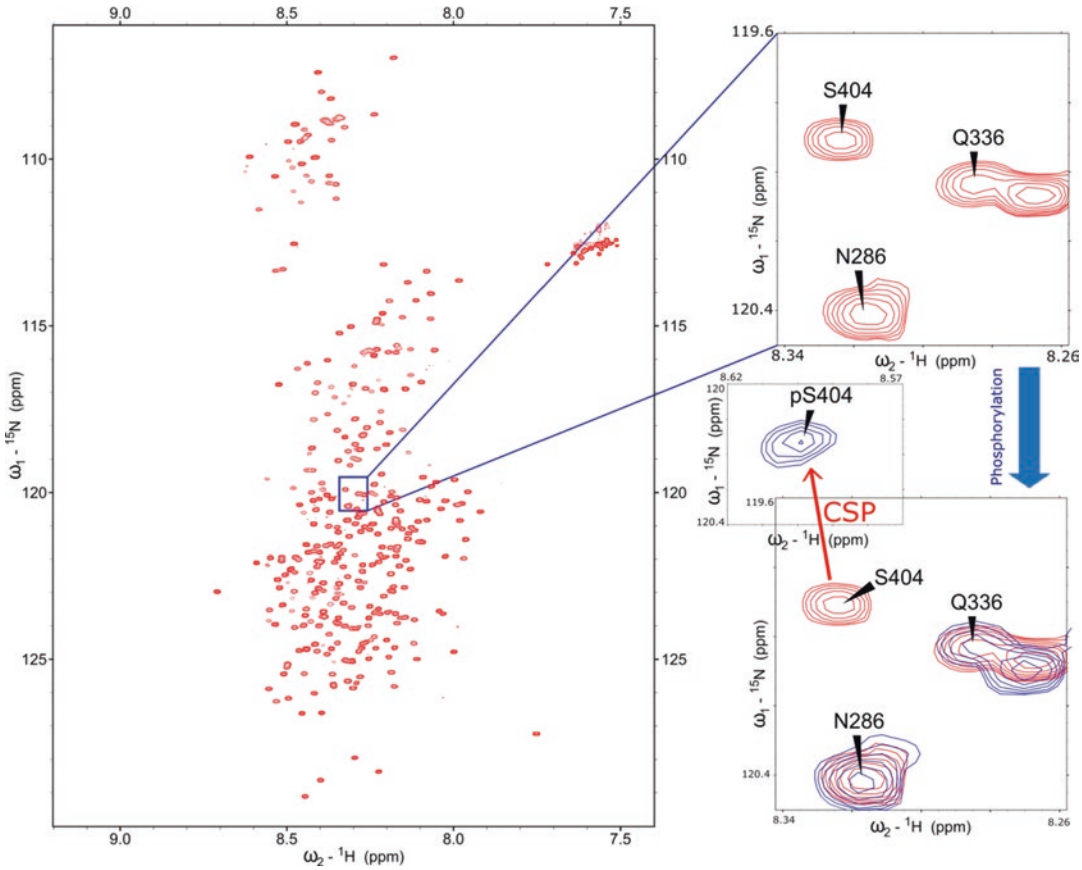

Fig. 3.3 Heteronuclear Single Quantum Correlation Spectrum or HSQC of Tau. The ^{1}H-^{15}N HSQC (in red) provides a fingerprint of Tau in a given chemical environment. Each resonance corresponds to an N-H bond and can be linked to a specific amino acid residue in Tau sequence (see labelled resonances for Asn286, Gln336, Ser404). Upon phosphorylation of a Ser/Thr residue of the protein (for example N-H Ser404 resonance at ^{1}H 8.32 ppm, ^{15}N 119.3 ppm), the corresponding spectrum (overlayed in blue) will be modified specifically for the resonances corresponding to the affected amino-acid residues (see N-H phospho-Ser404 at ^{1}H 8.60 ppm, ^{15}N 120.2 ppm). The chemical shift values (in ^{1}H and/or ^{15}N dimension) of these resonances will be modified (CSP, chemical shift perturbation, compare Ser404 and pSer404 chemical shift values) and/or their intensities

addition this approach detects several stretches located in the PRR and MTBR of Tau that have a tendency to adopt a polyproline II helical conformation, an extended structure typical of IDPs [37]. Although mainly associated with proline-rich sequences, this local structure can be adopted by other amino-acid residues, as observed in Tau for a number of short sequences. In proline-rich regions, such as the sequence of residues 212–230, the population of polyproline II helical conformation exceeds that found in the statistical coil model by up to 20%, and in some regions of the PRR, that particular conformation is estimated to reach a population of as much as 60% [37]. In some cases, polyproline II helices can represent binding sites for a number of protein partners. Peptide 216–223 for example, adopting the polyproline II helical conformation (Fig. 3.1), was later found to be within the interaction site of the SH3 domain of the BIN1 protein [43]. Here again, a conformational property can translate to a functional aspect, although the link is not always obvious. Turn conformations were described within the repeats, based on NMR residual dipolar couplings (RDCs, Fig. 3.2d), which provide orientation of inter-nuclear vectors

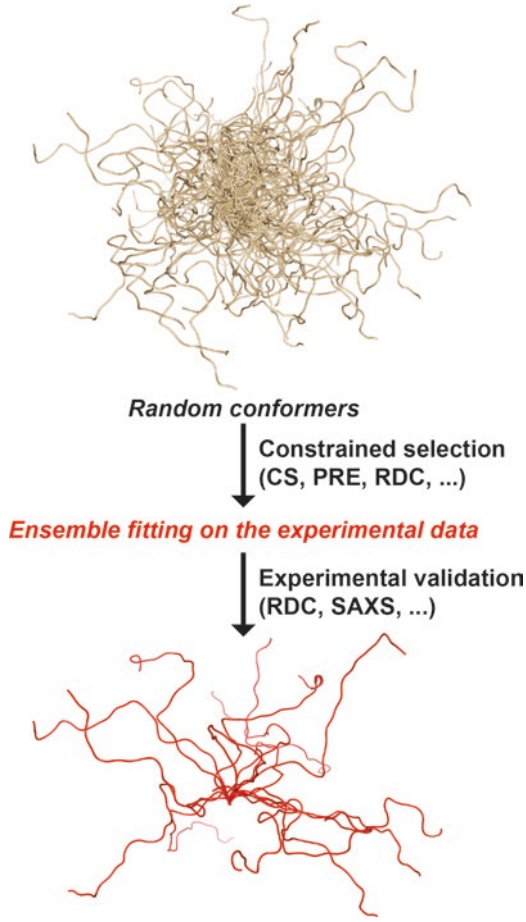

Fig. 3.4 Principle of ensemble calculation for intrinsically disordered proteins. A large number of conformers are first generated, a sub-ensemble is next selected which fits part of the experimental data better than the initial pool of conformers. The ensemble of conformations is lastly validated using another subset of experimental data, independent from the one used in the selection step

in the protein, and molecular dynamics simulations. Four turns were found, one in each repeat, with the amino-acid sequences DLKN, DLSN, DLSK, and DKFD in R1 to R4 [33]. Comparison with the recent electron microscopy (EM) structure of aggregates of Tau, corresponding to paired helical filaments (PHFs) or straight filaments (SF) extracted from Alzheimer's disease (AD) brain tissue [14], offers a view of these short sequences within the native protomer. R1 and R2 repeats are not embedded in the core of the protomer in the EM structure, composed mainly of the R3 and R4 repeats. Most of the Tau sequence (80%) in the fibril adopts a β-strand conformation, forming an overall C-shaped fold. The β-strands are interrupted by β-breaking prolines Pro312, Pro332, Pro364, β-turn glycines Gly323, Gly355 or β -arc residues Glu342 and Asp348, the latter being part of the DKFD motif of R4. The motif DLSK in R3 is however part of β-strand β2. These turn tendencies observed for the MTBR of Tau thus partially reflect the conformation of these sequences in the fibril. However, it was proposed that alternatively, their presence could prevent the aggregation, as β-strand breakers [33].

A Disordered Protein: Tau's Global Fold

Another property of the disordered Tau protein that was characterized is its global fold. Indeed, Tau does not behave as a rod but as a flexible polymer that adopts a preferential fold, although devoid of a hydrophobic core and thus mainly driven by electrostatic interactions [34]. Thus, that fold is dynamic and conformational exchange occurs. To characterize the global fold of a disordered protein, paramagnetic probes are used which are attached to cysteines, with Cys291 and Cys322 being the two native cysteines of Tau. Alternatively, these native cysteines have to be mutated into alanines and a single Cys residue introduced at a point of interest in the sequence. The paramagnetic probes will enhance relaxation of atomic nuclei in their spatial proximity, up to a distance of 25–40 Å, which allows to define regions close in space to the paramagnetic centre (Fig. 3.2b). Applied to Tau, the paramagnetic relaxation enhancement (PRE) experiments show several contacts between regions distant in the sequence. A transient contact between the N-terminal region and the central region (PRR and R1 to R3 repeats) is supported by PRE data [34]. This long-range interaction was confirmed based on a reported ensemble of Tau conformers calculated from a large number of various NMR parameters (including PREs) combined with small-angle X-ray scattering data [37]. The C-terminal domain transiently contacts the N-terminal domain and the repeat domain R3-R4

of Tau [34]. These results converge to the reported global fold based on FRET (fluorescence resonance energy transfer) distances, described as a paperclip shape [21].

Impact of Phosphorylation on Tau Structure

The (average) conformation of Tau, already difficult to characterize for this large disordered protein, becomes even more complex to describe due to the multiple post-translational modifications (PTMs) that are possible in this protein. In particular, phosphorylation of Ser and Thr residues has attracted much interest, since AD progression is characterized by an increased level of phosphorylation and, PHFs are composed of hyperphosphorylated Tau. As phosphate groups introduce charges and can engage in hydrogen bonds, they have the potential to impact the local and global structure of Tau. A first step in the characterization of potential changes of Tau conformation due to phosphorylation was the analytical capacity of NMR spectroscopy to identify phosphorylated residues in a given sample of Tau (Fig. 3.3). Initial studies were conducted with Tau protein phosphorylated *in vitro* by recombinant PKA kinase, followed by identification of all modified serine residues in the sample [28]. This point is of importance since NMR is able to link specific phosphorylations in Tau sequence with the corresponding structural and/or functional output. A first attempt to link specific phosphorylations with local structural impact was made on a Tau fragment phosphorylated *in vitro* with recombinant CDK2/CycA3 kinase [39]. This proline-directed kinase was found to modify the Tau(208–324) fragment, containing the PRR and part of the MTBR (R1-R2 and part of R3), on several sites within the PRR, corresponding to residues T231/S235 (the epitope of the AT180 antibody) as well as Thr212 and Thr217. The CDK-phosphorylated fragment remains globally disordered, but a local structuration can be detected, corresponding to a helical tendency for about 10 amino-acid residues at the C-terminus of the pThr231/pSer235 phosphorylation sites. Phosphorylation of pThr212/pThr217 has no such effect, showing that the stabilization effect is sequence specific. The conformations of phosphorylated Tau(225–246) peptides were additionally evaluated by molecular ensemble calculations [38] based on (i) distance restraints obtained from NOEs (nuclear Overhauser enhancements), NMR signals arising from the close proximity of two protons in space (Fig. 3.2c) and (ii) orientational restraints calculated from RDCs for N-H bonds (Fig. 3.2d). The resulting ensembles confirm the transient helix presence, which does not dependent on phosphorylation of Thr231 but rather of Ser235, and is further stabilized by additional phosphorylation of Ser237 and Ser238 [38]. The enhanced stabilization effect could be due to the formation of salt-bridges between the side-chains of pSer237/Lys234 and pSer238/Arg242. In addition, based on these models of the phospho-Tau(225–246) peptide, the distances between the phosphates pThr231/pSer235 and the nitrogens in the basic groups of Arg230/Lys234, respectively, are shorter than 4.5 Å. This distance is compatible with formation of salt-bridges between the side-chains of the phosphorylated residues and the side-chains of the directly preceding basic residues Arg230 or Lys234.

Interestingly, phosphorylation by CDK2/CycA3 has a functional effect, since the Tau(208–324) fragment loses its capacity to polymerize tubulin into microtubules (MTs) once phosphorylated. When full-length Tau is phosphorylated by the CDK2/CycA3 kinase *in vitro*, phosphorylation at Ser202/Ser205 and Thr231/Ser235 sites are identified by NMR (and weak phosphorylation at Thr212/Thr217). These phosphorylations do not significantly affect binding to MTs [2]. Nevertheless, when at least three phosphates are present in these four positions, Tau loses its capacity to assemble tubulin into MTs. Additional experiments, using the shortest Tau isoform (Tau 0N3R) with residues Thr231 and Ser235 mutated to glutamate residues as phosphorylation mimic, confirm that Glu231/Glu235 by themselves do not abolish the interaction of Tau with MTs [38]. However, NMR signals corresponding to residues in the PRR were less attenuated upon

addition of MTs to the mutated Tau 0N3R than for the wild-type [38]. This might indicate that pseudophosphorylated Tau 0N3R was locally less tightly bound to the MTs. The salt-bridge between pThr231 and Arg230 side-chains is proposed to compete with salt-bridge formation with MTs, participating in the effect of phospho-Thr231 [38]. A direct link between the conformational modification and its functional consequences is difficult to establish. However, this study proved that the effect of Tau phosphorylation is not limited to the introduction of negative charges that may influence its interactions, but that it additionally influences the local structure of Tau, with potential functional significance.

Along the same lines, phosphorylation of residues Ser202/Thr205, associated with the AT8 epitope, was shown to induce formation of a local dynamic turn, based on a combination of NMR data and molecular dynamics simulations [15]. This combination of phosphorylations is of major interest since the AT8 monoclonal antibody is described as targeting the pathological AD-like state of Tau [4] and immunocytochemistry of brain slices using the AT8 allows to evaluate the stages of the disease [8]. The NMR data supporting the formation of a turn conformation consist in NOE contacts, for example between the amide proton of Gly207 and the Hα, Hβ and Hγ of pThr205. The phosphate group of pThr205 contributes to stabilization of the dynamic turn conformation by engaging in a hydrogen bond with the amide proton of Gly207. In the 2D ^{1}H, ^{15}N spectrum of phosphorylated Tau, with pSer202/pThr205 detected, it results in an easily detectable downfield shift of Gly207 resonance in the ^{1}H dimension. Salt-bridges between the pThr205 phosphate and the side-chain of Arg209, as well as between the pSer202 phosphate and the side-chain of Arg211 contribute to an additional stabilization of the turn. An additional phosphorylation on Ser208, a non-proline directed phosphorylation site, disrupts turn formation. Of interest is that the Tau protein is more sensitive to aggregation without this turn conformation [11]. This might thus be the form recognized by the AT8 antibody in immunocytochemistry, since the molecular detail of the interaction of AT8 antibody with its epitope, revealed by a crystallographic study, shows that AT8 best accommodates a triply-phosphorylated sequence [32]. The link between the dynamic turn conformation and the aggregation propensity of Tau is further supported by a mutated Tau protein, with a Gly207 residue replaced by valine. This Tau variant exhibits no turn conformation around the pSer202/pThr205 sites and displays higher aggregation propensity in *in vitro* aggregation assays (without heparin inducer). In this particular case, the link between a small dynamic structural motif, and a (dys)function of Tau can be made. Numerous questions remain as to the mechanism of aggregation protection by the pSer202/pThr205 centred-turn, or whether there might be other phosphorylation patterns with such properties.

Other studies used glutamic acid mutations to mimic phosphorylation. This method of pseudo-phosphorylation may not be ideal to study the impact of phosphorylation on Tau structure, since, for example, the hydrogen-bonding pattern of a phosphate group is not reproduced perfectly. However, a key advantage of glutamic acid mutations is that they result in a homogeneous sample, as opposed to *in vitro* phosphorylation. Pseudo-phosphorylation might thus turn out to be a necessary evil in some cases. For example, glutamic acid mutation of the serine residues in the KxGS motifs found in repeats R1 to R4 of Tau showed a selective conformational effect in repeats R1-R2, based on N-H residual dipolar coupling experiments (Fig. 3.2d). The sign of the RDCs changes for residues ^{265}NLK^{267} upon pseudo-phosphorylation of Ser262, suggesting formation of a turn-like structure [13]. Since phosphorylation of Ser262 is described as protective against aggregation [36], it is proposed that this turn-conformation favours interaction of the ^{259}KIGpS^{262} motif (in R1) with the end of R1 and the beginning of R2, impacting the capacity of the hexapeptide ^{275}VQIINK^{280} in R2 for intermolecular interaction. Pseudophosphorylations at the AT8 (in this study, mutations of Ser199, Ser202 and Thr205 into glutamic acid), AT100 (Thr212Glu and Ser214Glu), and PHF1

(Ser396Glu and Ser404Glu) epitopes were used to evaluate the impact of phosphorylation on global Tau conformation based on PREs combined with an ensemble approach. The data show a reduction of long-range electrostatic interactions between the N-terminal region and the PRR of Tau [3]. However, based on distance measurements from FRET pairs, a Tau protein presenting the same pseudo-phosphorylation mutations was proposed to be more compact than the wild-type, with increased contacts between the N- and C-terminal regions, thus better reproducing the conformation recognized by the conformational antibody MC1 that targets AD-Tau [22]. This apparent contradiction from these very thorough studies serves to emphasize the challenge to capture the elusive conformational changes in a protein as flexible as Tau.

Other Tau PTMs have been studied by NMR [23], although their impact on Tau structure remains to date to be explored. These PTMs play roles in cross-talk with phosphorylation [7, 42].

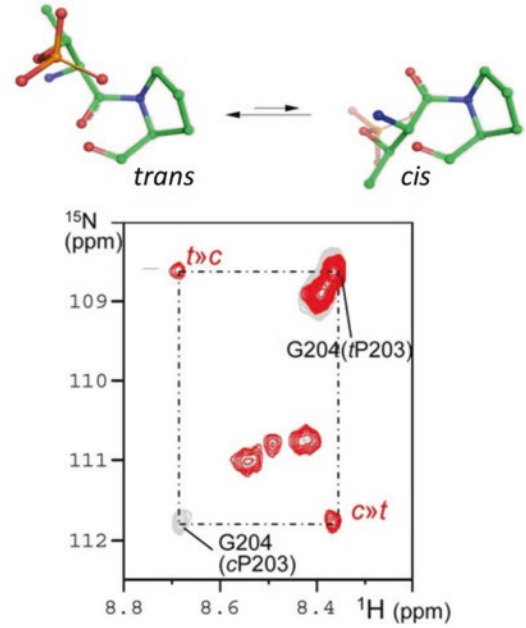

Fig. 3.5 ***cis/trans*** **conformations of pSer-Pro bond** Stick representation of a pSer-Pro dipeptide in *trans* and *cis* conformations. Spontaneous conformational exchange is slow. Detail of the ^{1}H, ^{15}N HSQC of CDK2/CycA3 phosphorylated Tau PRR (in gray), with phosphorylated Ser202 detected, showing the resonance of N-H Gly204. The overlayed EXSY (exchange spectroscopy) spectrum in presence of Pin1 (in red, ratio PRR:Pin1 1:0.1) allows to detect exchange between *cis* and *trans* conformations of the pSer202-Pro203 bond. This exchange, accelerated by the presence of Pin1, can be detected on the NMR time scale as an exchange peak (labelled *c* to *t* and *t* to *c*) between the two resonances of Gly204, used as reporters for the *cis* and *trans* pSer202-Pro203 bond conformations

Tau prolyl *cis*/*trans* isomerization

Tau prolyl *cis*/*trans* isomerization is mediated by prolyl *cis*/*trans* isomerases (PPIases) that accelerate the *cis*/*trans* conversion of the peptidic bond preceding a proline residue (Fig. 3.5). Prolyl *cis*/*trans* isomerases such as Pin1 and FKBP52 have been pointed out as being involved in the Tau pathway in AD [10, 16, 29, 31]. In particular, a *cis*-phospho Tau form, detected by a *cis*-specific antibody, was proposed to represent an early pathogenic form of Tau in AD [26, 35], with the pThr231-Pro232 prolyl bond in the *cis* conformation in AD neurons. This *cis*-phospho Tau cannot promote MTs assembly, is more resistant to dephosphorylation and degradation, and more prone to aggregation [26, 35]. These results have been difficult to reconcile with molecular studies on Tau structure. FKBP52 has been shown to act on Tau by interaction with its MTBR, rather than by its PPIase activity [24]. Pin1, on the other hand, is a particularly interesting PPIase, as it specifically recognizes phosphorylated Tau protein, and potentially its 17 pSer/pThr-Pro phosphorylation sites [31, 41]. PPIase activity and *cis*/*trans* conformation of proline residues in the substrate can both be characterized by NMR: the isoforms are identified by distinct sets of resonances for Xxx-Pro residue pairs in *cis* or *trans* conformations and, the activity is detected as a chemical exchange process during the mixing period of the NMR experiment, once the enzyme is added to accelerate the process (Fig. 3.5). PPIase activity of Pin1 is detected for a number of phosphorylated Tau sites (Fig. 3.5), but not for pThr231-Pro232 [12, 41]. Additionally, when investigating the conformation of each Xxx-Pro bond in the fragment Tau(208–324), only a few were found with a detectable *cis* form, with each

Xxx-Pro bond for over 90% in the *trans* conformation [1]. Phosphorylation by CDK2/CycA3, which phosphorylates four Ser/Thr-Pro motifs in Tau(208–324), does not change this conclusion, with no preferential *cis* conformation for the pThr231-Pro232 bond. From a functional point of view, Pin1 was proposed to restore the capacity of phosphorylated Tau to bind to MTs and restore MTs assembly [31]. This model was however also recently challenged, since Pin1 does not promote *in vitro* formation of MTs by phosphorylated Tau [27]. Although, an involvement of Pin1 in AD is not ruled out, the molecular mechanisms sustaining its role, and in particular its PPiase activity, remain currently elusive.

Conclusions

Although Tau is an intrinsically disordered protein, best characterized by an ensemble of dynamic conformations, some elements of structuration can be defined. Given the dynamic character of disordered proteins, biophysical techniques combined with computational methods have to be used to describe their subtle local and global preferential conformations. These characteristic elements of structuration can be influenced by post-translational modifications. The current challenge remains to link this structuration, or its perturbation, to functional consequences. Much can be gained from such a characterization that may provide a definition for a misfolded Tau protein, which seems at first glance antinomic to its disordered nature.

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Regulation of Tau Homeostasis and Toxicity by Acetylation

4

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Introduction

Tauopathies, including Alzheimer's disease (AD), are neurodegenerative diseases characterized by aggregation of the microtubule-binding protein tau in the brain, which coincides with synapse and neuronal loss [25, 38, 51]. While the majority of tauopathy cases are sporadic, there are over 50 familial tau mutations that cause frontotemporal lobar degeneration with tau inclusions (FTLD-tau), and the field has been shaped by studies of transgenic mice that express these human tau mutations [17, 35, 53, 68, 78, 84, 87, 89].

Tau is normally localized to the axons, and the current prevailing view is that the mis-localization or secretion of aberrant forms is toxic to neurons [64]. Indeed, in Alzheimer's disease (AD), tau accumulates in dendrites, a process associated with accumulation of amyloid β [42]. Importantly, tau accumulation may not always result in cell death; studies of tauopathy mouse models indicate that toxic tau affects postsynaptic function [35, 39, 41, 76, 80, 88] and triggers neuronal dysfunction underlying cognitive impairments without widespread neuron loss [20, 28, 82, 87]. However, the molecular alterations to the tau protein that trigger mis-localization and the formation of aggregates are incompletely understood. Here, we describe recent research highlighting the role that post-translational modifications (PTMs), particularly acetylation, may play in establishing the toxicity of tau, and how this may shape future therapeutic efforts.

Posttranslational Modifications of Tau

The nascent tau peptide is subject to extensive modification, including proteolytic cleavage, phosphorylation [55], ubiquitination [14, 62], glycosylation, methylation [23, 79], and acetylation [10, 60, 63, 80]. How these differently modified forms contribute to the pathogenesis of tauopathies is an area of intense investigation. Phosphorylated tau was first described in tau isolated from the brains of AD patients [32]; it has subsequently been shown that neurofibrillary tangles contain highly phosphorylated tau, and that these form in parallel with the manifestation of cognitive impairments in AD [2, 29]. In support of a pathogenic role of hyperphosphoryla-

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tion of the protein, expression of a tau mutant that mimics the phosphorylation of 14 residues in cultured hippocampal neurons resulted mislocalization of the protein to dendritic spines, and reduced the number of AMPA-type glutamate receptors (AMPAR) at synapses [35]. Cleaved forms of tau may also contribute to synaptoxicity, as blocking caspase-2-mediated cleavage of the protein restores synaptic transmission and rescues memory deficits in mice expressing the P301L tau mutation [90].

Acetylation of the N-terminus of tau was described in early studies of the protein, but as this modification was observed on protein isolated from both diseased and healthy brains, it was initially not considered a pathogenic feature of tauopathies [32]. More than a decade later, it was discovered that tau is acetylated on various lysine residues, and that tau acetylation was elevated in the brains of patients with tauopathy [60], a finding that has since been independently validated in multiple studies of human cohorts as well as mouse models of neurodegeneration [10, 27, 59, 74, 80]. As described in more detail below, mechanistic investigations have revealed that acetylation of tau blocks degradation of the protein [60], inhibits tau microtubule binding [10, 74], and promotes aggregation [10, 59]. To investigate the role of specific acetylated residues, lysine- > glutamine (KQ) substitutions can be made to mimic the structure and charge of acetylated lysine [34, 49, 70, 83]. This approach has helped reveal the role of acetylated tau in neurodegeneration [59], cytoskeletal dysregulation [74], and synaptic dysfunction [80] related to AD pathogenesis.

Tau Acetylation in Neurodegenerative Disease

More than 20 lysines of tau are subject to acetylation [60], of which several appear to be of particular pathological significance. Mass spectrometry identified acetylation of tau K174 as an early change in AD brains [59], and acetylation at two additional residues, K274 and K281, is found in the brains of patients who exhibit memory loss [80] (Fig. 4.1). Acetylation at K274 and K281 is also elevated in the brains of the hAPP-J20 mice [80], a model of AD that expresses high levels of hAPP and amyloid beta

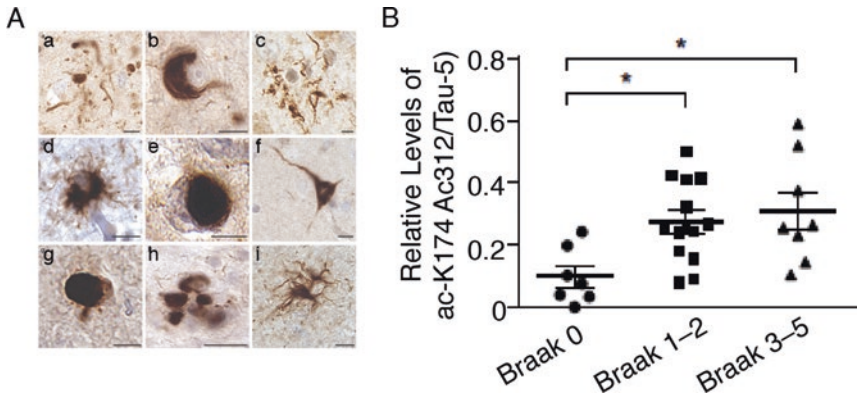

Fig. 4.1 Acetylated tau is enriched in tauopathy brains. (**a**) High magnification view of acetylated-tau inclusions located in inferior temporal cortex (*a–c* and *e–i*) and midbrain (*d*), after immunostaining with an anti-ac-tau antibody. *a* neuritic plaque (Alzheimer's disease – case 3). *b* neurofibrillary tangle (Alzheimer's disease – case 3). *c* astrocytic plaque (corticobasal degeneration – case 9). *d* globous tangles in oculormotor nucleus (progressive supranuclear palsy – case 21). *e* Pick's body (Pick's disease – case 18). *f* intracytoplasmatic neuronal inclusion (chronic traumatic encephalopathy – case 8). *g* intracytoplasmatic neuronal inclusion (FTLP-17 – case 11). *h* globular glial inclusions (White matter tauopathy with globular glial inclusion – case 22). *i* glial inclusion (atypical tauopathy – case 7). Scale bar represents 10 μm. Originally described in Grinberg et al. Acta Neuropathol. 2013 (35). (**b**). Levels of ac-K174 were significantly higher at early and late Braak stages than at Braak stage 0; n = 7 (Braak 0), n = 13 (Braak 1–2), n = 8 (Braak 3–5). ∗p < 0.05, one-way ANOVA, Tukey-Kramer post hoc analyses. Originally described in Min et al. Nat. Med. 2015 (34)

(Aβ). A link between Aβ and tau acetylation in neurodegeneration is also supported by the finding that treating cultured neurons with Aβ oligomers increases tau acetylation [60]. A more recent study showed that site-specific acetylation at K280, which was found only in AD brains [10], significantly enhances the aggregation rate of tau and impairs microtubule assembly [30].

Hyperacetylation of tau in disease may be related to modulation of the acetyltransferase p300, which is upregulated in the brains of AD patients [3, 85] and in neuronal models of AD [50]. Tau also has intrinsic auto-acetylation capability [9], which has been linked to subsequent proteolytic cleavage and the production of tau fragments [8]. Tau deacetylation is believed to be mediated largely by sirtuin 1 [60, 61]. There is also evidence that HDAC6 can deacetylate tau, although the target lysines may be different from those regulated by sirtuin 1 [7, 12]. The levels of sirtuin 1 decrease in the brain during AD progression [40, 52], and treating cultured neurons with Aβ reduces the expression of sirtuin 1 [46], perpetuating accumulation of acetylated tau. Taken together, these findings indicate that enhanced acetylation and reduced deacetylation likely both contribute to the pathological elevation of acetylated tau.

The regulation and functional consequence of each acetylation site is a major point of interest in the field. Notably, K274 and K281 both occur in the microtubule-binding domain of tau, where many of the familial FTLD-tau mutations are located, and acetylation of K274 and K281 can reduce the interaction between tau and microtubules [10, 74]. Acetylation at K274 is present in neurofibrillary tangles and neurotic plaques in AD, as well as many other tauopathies [27, 74]. Acetylation at K274 is also enhanced in late stage AD (Braak stages 5–6), compared to early onset disease (Braak stages 0–2) [74]. In contrast, although acetylation of K281 is enhanced in the brains of patients with mild dementia compared to non-demented cases [80], modification of this residue not appear to correlate with Braak stage [74]. One interpretation of these findings is that acetylation of K281 plays a role in the early stages of cognitive decline, while acetylation of K274 occurs later in disease progression. Alternative splicing of the tau transcript also affects the acetylation status; while the full-length protein has four microtubule-binding repeats (4R), the alternatively spliced version has only three (3R), and lacks the K281 site. Neurofibrillary tangles in AD brains contain both 3R and 4R tau [18], indicating the potential that acetylation at K274 and K281 contribute to pathology independently.

The Effect of Acetylation on Toxic Tau Species

The mechanism by which tau acetylation induces pathogenesis may involve regulation of toxic forms of the protein, such as hyperphosphorylated protein. However, the relationship between tau acetylation and phosphorylation may be both residue- and context-specific. In transgenic mice expressing an acetylation mimetic version of human tau K274 and K281 (tauKQ), the levels of phosphorylation at serine 202 are reduced, while phosphorylation at other residues associated with tau pathology are unchanged [74, 80]. Expression of K174Q in mice has no effect on tau phosphorylation [59], while acetylation at amino acids 259/353 appears to block phosphorylation [12]. Interestingly, in a *Drosophila* model, expression of the acetyl-mimetic K280Q resulted in increased levels of tau phosphorylation [26].

Another potential mechanism by which tau acetylation could modulate tau toxicity is by altering the formation of caspase-cleaved tau fragments, which have been implicated in pathological tau accumulation in the brain [24]. However, tauKQ mice, which do display features of tauopathy, lack tau fragments [80], indicating that acetylation at K274 and K281 is sufficient to drive synaptic and cognitive deficits without creating tau fragments.

Tau acetylation may also affect the formation of tau oligomers and aggregates, which are thought to play a role in the pathogenesis of AD [45, 54] and which are sufficient to impair memory and cause synaptic defects in mice [44]. Acetylation of tau lysines blocks those

residues from being targeted for ubiquitination, slowing the rate of protein turnover and leading to accumulation [60]. Pathological tau, recognized by the conformation-specific MC1 antibody, is detected in the brains of tauKQ mice [80], supporting a role for acetylation in the formation of this species. MC1 positive tau has been implicated in the spread of tau between neurons in aged transgenic mice [48], and its localization in both presynaptic and postsynaptic compartments may indicate that tau oligomers can be spread across synapses [77]. Whether acetylated tau propagates from cell to cell in the brain is unknown, and understanding if this property underlies its toxicity is an area of active research.

The Effect of Acetylation on Tau Localization and Synaptic Plasticity

Although tau is normally localized to axons, acetylated tau appears in the somatodendritic compartment [74], raising the question of where the acetylation takes place and how acetylated tau translocates. The acetyltransferase p300 is primarily active in the nucleus, and tau has also been detected in that compartment [47] and thus may be acetylated there, although p300 has also been shown to act in the cytoplasm [71, 72]. The cytoskeletal network within the axon initial segment (AIS) is thought to play a role in partitioning the cell and retaining tau; interestingly, tau acetylation at K274 and K281 results in destabilization of the AIS [74], which likely contributes to the translocation of the protein from the axons.

In extracts from the brains of AD patients, tau acetylated at K274 and K281 is found in the postsynaptic fraction [80], raising the possibility that acetylated tau is transmitted from presynaptic to postsynaptic sites. Evidence from transgenic models seems to confirm that tau can be propagated from entorhinal cortical neurons into the hippocampus [16, 31, 48], recapitulating the spread of neurofibrillary tangles during human AD progression [5]. In addition, neurons secrete tau in response to enhanced network activity [66, 86], and this secretion is elevated in the setting of AD [75]. It is possible that acetylated tau is preferentially secreted, which would contribute to the propagation of tau at synapses.

Tau interacts directly with filamentous actin (F-actin), and can induce the organization of actin filaments [15, 22]. Synaptic activity can promote microtubule polymerization from the dendrite into F-actin rich spines [58, 73], which affect the strength of glutamatergic transmission [1]. This reorganization is required for the recruitment of AMPARs and the maintenance of long-term potentiation [21, 43]. However, acetylated tau seems to have an antagonistic effect on the cytoskeleton. Acetylation of K281 impairs microtubule stabilization [81], and it has been shown that acetylation at an additional site, K321, also inhibits filament formation, at least in part by preventing phosphorylation of the neighboring residue S324 [7]. In tauKQ mice, the interaction between tau and F-actin is weakened, and activity-dependent polymerization of actin is impaired [80]. However, in this model, neither basal actin polymerization nor glutamatergic transmission are perturbed [80]. Furthermore, other proteins involved in the regulation of AMPARs during plasticity [36] may be affected by interaction with acetylated tau. One of these is the postsynaptic protein KIBRA, which in humans has been linked to memory performance [65] and to the risk for late-onset AD [6, 13, 67]. In the tauKQ model, the level of KIBRA in spines was reduced, but overexpression of KIBRA in tauKQ hippocampal neurons restored the recruitment of AMPARs to synapses [80]. Taken together, these studies provide strong support for a model in which acetylated tau disrupts F-actin organization to specifically disrupt synaptic plasticity.

Notably, the effects of tau phosphorylation on actin filaments are quite distinct from the effects of acetylation. In a *Drosophila* model, phosphomimetic mutations in tau increase F-actin assembly [22]. This may be related in part to differential localization: while acetylated tau is present in dendritic spines [80], phosphorylated tau interacts with actin bundles in the soma [22].

Acetylated Tau Promotes Memory Loss

Patients in the early stages of AD display impairments in episodic memory [4, 11]. A crucial aspect of forming episodic memories is the ability to distinguish between similar experiences, an event that is thought to occur in the granule cells of the dentate gyrus, a part of the hippocampus [57]. In tauKQ mice, long-term potentiation is inhibited in granule cells, and the mice exhibit difficulty in differentiating between experiences [80], indicating that tau acetylation may block episodic memory formation. Tau acetylation has also been implicated in disrupting the formation of spatial memories. For example, tauKQ mice are able to learn the location of a hidden platform in the Morris water maze, but then cannot retain that memory [80]. A similar deficit is observed in mice expressing K174Q tau [59]. Taken together, these findings support a model in which the tau acetylation at multiple residues impairs the encoding of memories in the hippocampus.

Targeting Tau Acetylation as Potential Therapeutic Strategy

Given the abundant evidence that acetylation of tau is a critical element of the pathogenesis of tauopathies, strategies to reduce tau acetylation represent an attractive approach to combat these devastating diseases. Among the potential targets is sirtuin 1, which deacetylates tau *in vivo* [60]. Indeed, overexpression of sirtuin 1 reduces the spread of pathogenic tau in a mouse model of tauopathy [61]. Sirtuin 1 activators, therefore, could be a promising method to reduce tau acetylation and prevent the progression of neurodegeneration. In addition, the sirtuin 1 activator resveratrol is already being explored as a therapeutic for AD given its anti-amyloidogenic properties [56, 69].

An alternative would be to inhibit the activity of p300, the acetyltransferase that targets tau. In support of this approach, it has been shown that treatment with salsalate, which inhibits p300 acetyltransferase activity, blocks tau acetylation and restores memory deficits in FTLD-tau mice [59]. Remarkably, salsalate did not protect against neurodegeneration in mice expressing K174Q [59], strong evidence that the effect of the drug in that model is mediated by blocking acetylation of tau. In a separate study, treatment with the related drug sodium salicylate restored Aβ-induced synaptic and memory deficits in rats [19]. Furthermore, salsalate has anti-inflammatory effects [37], activates adenosine monophosphate-activated protein kinase (AMPK), and increases autophagy [33], and thus may have multiple beneficial effects in neurodegenerative disease. Further exploring the potential of targeting this mechanism to treat tauopathies is an exciting area for future research.

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Tau Clearance Mechanisms

5

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The Proteasome

The catalytic core particle (CP) of the proteasome is a barrel-shaped 700 kD multimeric cellular structure that has three proteolytic activities; chymotryptic-like, tryptic-like, and caspase-like [1, 2]. The CP, also known as the 20S proteasome, has two outer rings made of multiple α subunits and two inner rings made of β subunits. The inner β subunits (β1, β2 and β5) are catalytically active, while the outer α subunits are proteolytically inactive but play a role in defining the size of the pore which allows client proteins inside the structure [3]. The 20S proteasome can be uncapped, singly capped or doubly capped with the 19S regulatory particle (RP) (or in some cases the 11S RP) to form the 26S proteasome. The RPs facilitate the recognition and deubiquitylation of polyubiquitylated client proteins, and mediate the unfolding and targeting of the proteins into the core of the 20S proteasome [2].

The 20S proteasome is able to directly degrade natively unfolded proteins or intrinsically disordered proteins (IDPs) through an ATP- and ubiquitin-independent process. Further, there is increasing evidence that the 20S proteasome represents a prominent protein degradation route in mammalian cells. The majority of proteasomes in mammalian cells are uncapped 20S proteasomes and there are multiple mechanisms by which the proteolytic capacity of the 20S proteasome is regulated [4]. For example, it has been suggested that 20S proteasomes are predominantly in a "closed gate" conformation, but they open intermittently to accept substrates, and the mere presence of substrates induces a shift towards the open conformation allowing substrate entrance into the proteolytic core [5, 6]. Another level of regulation may involve "nanny" proteins that can interact with IDPs and protect them from degradation by the 20S proteasomes [7]. However, further research needs to be done to fully delineate the functioning of 20S proteasomes in cells and how specific substrates are targeted to them for degradation.

The 26S proteasome primarily degrades proteins that have been polyubiquitylated with a ubiquitin chain of at least four molecules. The process of ubiquitylation is ATP dependent and requires the action of three enzymes. The E1 enzyme forms a thio-ester bond with ubiquitin at the expense of ATP, then the activated ubiquitin is transferred from E1 to E2. Finally, the E3 enzyme catalyzes the transfer of the ubiquitin from the E2 enzyme, forming an isopeptide bond between the

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ubiquitin carboxyl terminus and a primary amino group of a client protein (mono-ubiquitylation/ubiquitin chain initiation) or another ubiquitin molecule (ubiquitin chain elongation) [8]. In vertebrates, there are only two E1 enzymes, Uba1 and Uba6, with Uba1 being the most abundant. In contrast, there are approximately 50 E2 enzymes in humans, and more than 600 putative E3 enzymes have been identified in the human genome [8, 9].

Immunoproteasomes are another class of proteasomes and are, in essence, the 20S proteasome with inducible catalytic subunits: LMP2, MECL-1, and LMP7, instead of β1, β2, and β5. These inducible catalytic subunits are usually expressed in response to pro-inflammatory signals [10, 11]. Immunoproteasomes play a key role in the immune response, and have well-defined functions in antigen-presenting cells [10]. However, there is a growing awareness that immunoproteasomes can modulate neurodegenerative processes. For example, when LMP7 knockout mice were crossed with APP/PS1 mice (which express human transgenes for APP with the Swedish mutation (K595N/M596L) and PSEN1 containing an L166P mutation, both under the control of the Thy1 promoter) there was an altered cytokine response in microglia and an improvement in the cognitive deficits in the offspring compared to the APP/PS1 mice with LMP7 [11]. However, given that the immunoproteasome appears to predominantly affect the functioning of immune cells, its role in directly mediating tau clearance is not clear.

Tau Degradation by the 20S Proteasome

Tau is one of the largest IDPs [12], and there are data suggesting that tau, like other IDPs, is degraded by the 20S proteasome. An early study showed that treatment of human neuroblastoma SH-SY5Y cells with the proteasome inhibitor lactacystin, significantly attenuated tau degradation. Additionally, in an in vitro system tau was degraded by the 20S proteasome in the absence of ubiquitylation [13]. Ensuing studies using several different cell models have substantiated this initial finding that tau can be degraded by the 20S proteasome in a ubiquitin independent manner [14, 15]. Indeed, using a variety of different approaches (ATP depletion, ubiquitylation-deficient cells, knockdown of a 19S proteasomal regulator subunit, as well as in vitro ubiquitylation studies) it was demonstrated that ubiquitylation was not required for tau degradation by the proteasome [15]. Further, recent studies have shown that small molecules which enhance the activity or levels of the 20S proteasome resulted in increased clearance of tau in various clonal cell models [5, 16]. Given the fact that the 20S proteasome efficiently degrades IDPs and the fact that tau is an IDP, it is likely that in a physiological setting tau is degraded by the proteasome in a ubiquitin-independent manner (Fig. 5.1). However, when tau undergoes pathological modifications such as phosphorylation and truncation, which occurs in Alzheimer's disease (AD) and other neurodegenerative disorders, it may become less disordered and take on a more structured and perhaps "preaggregation" conformation [17–20]. In this altered conformation/structure tau, may not be as efficiently degraded by the 20S proteasome; hence, other degradative mechanisms may need to be engaged to facilitate tau's clearance.

Tau Degradation by the 26S Proteasome

Data indicate that tau is degraded by the proteasome; however the involvement of the 26S proteasome in degrading tau in neurons has not been fully elucidated. Numerous studies have shown that in clonal cells, treatment with proteasome inhibitors (e.g., MG132, lactacystin, or epoxomicin) increase tau levels or slow tau degradation [13–15, 21]. Since these inhibitors target proteasome activity in general, this approach does not allow a determination of whether the 20S or 26S proteasome or both are involved in clearing tau. In vitro tau can be ubiquitylated [14, 21, 22], and early work suggested that tau

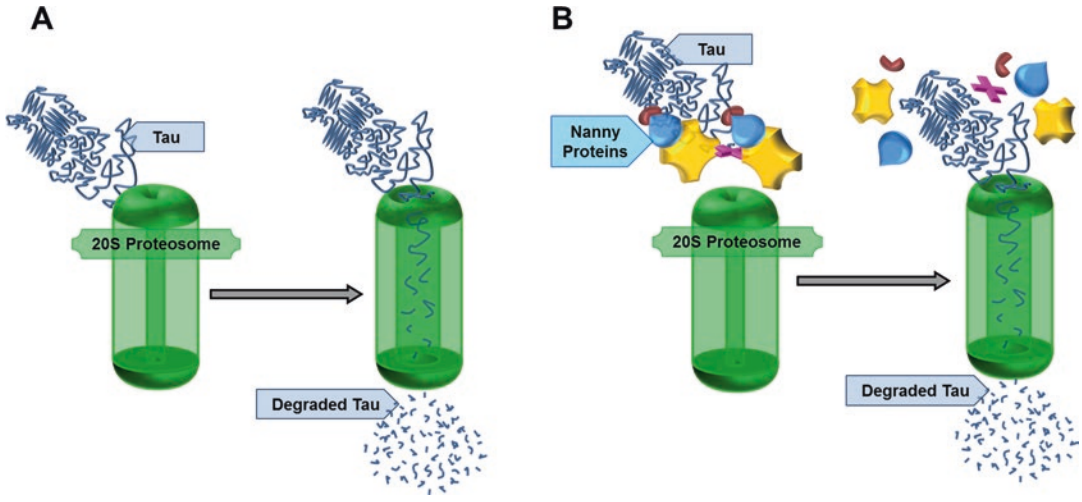

Fig. 5.1 Degradation of tau by the 20S proteasome. (**a**) Tau is a intrinsically disordered protein (IDP) and therefore is likely degraded by the 20S proteasome in an ATP- and ubiquitin-independent manner [4]. It has been suggested that 20S proteasomes are predominantly in a "closed gate" conformation but open intermittently to accept substrates, including tau, and the presence of tau (or other substrates) facilitates the open conformation and thus entrance of tau into the proteolytic core [5, 6]. (**b**) The degradation of tau by the 20S proteasome may also be regulated by "nanny" proteins that interact with IDPs and protect them from degradation [7]

could be ubiquitylated in situ using a HEK cell model [21]. However, in an immortalized mouse cortical cell line this did not appear to be the case [14]. Nonetheless, mass spectrometric analyses of wild type mouse tau revealed numerous sites were ubiquitylated by inference from the detection of GlyGly-modified lysine (K) residues due to trypsin cleavage of ubiquitin [23]. In addition, it is difficult to define the contribution of the proteasome, 20S or 26S, to the turnover of tau in neurons. This is due to the fact that treatment of cultured primary neurons with proteasome inhibitors results in compensatory activation of the autophagy pathway [24] with a subsequent increase in tau turnover [25, 26]. Thus, the contribution of ubiquitin modifications to tau turnover by the 26S proteasome in a physiological setting has not been unequivocally delineated. As indicated above, tau that has been pathologically modified in AD or other tauopathies may take on an altered conformation and thus no longer be efficiently degraded by the 20S proteasome. In this case, tau may be ubiquitylated and turned over by the 26S proteasome. Paired helical filament (PHF) tau isolated from AD brain, which is hyperphosphorylated, is also polyubiquitylated with K48, K11 and K6 linkages. In this study, it was suggested that modification at K6 inhibits ubiquitin-dependent degradation by 26S proteasome and thus could contribute to the formation of neurofibrillary tangles (NFTs) [27]. In addition, the ubiquitin proteasome system (UPS), including the activities of the proteasomal proteases, has been reported to be impaired in AD brain [28]. This could be due in part to the presence of abnormally modified and/or oligomerized tau species (Fig. 5.2). For example, impairment of the 26S proteasome has been reported in the rTg4510 mouse model where P301L tau (a mutant tau species which causes frontotemporal lobar degeneration (FTLD)) is robustly overexpressed [29]. In another study, it was shown that the deubiquitinase Otub1 removes K48-linked ubiquitin from P301S tau (which is another FTLD mutant). Furthermore, overexpression of Otub1 results in increased levels of tau phosphorylated at S202/T205 (AT8 antibody epitope), as well as increased tau oligomerization and seeding both in primary neurons from tau P301S mice (PS19 line) and in vivo

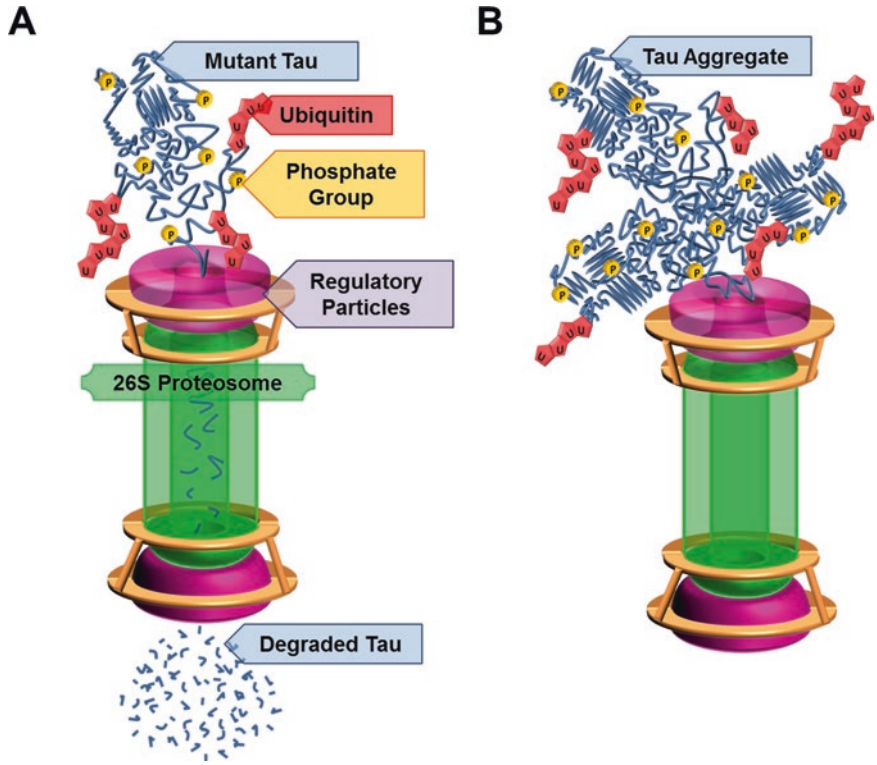

Fig. 5.2 Degradation of tau by the 26S proteasome. (**a**) Studies indicate that FTLD mutant tau species, such as P301S tau, are ubiquitylated and degraded by the 26S proteasome [30]. (**b**) Aggregated tau is ubiquitylated and is likely not effectively degraded by the 26S proteasome but instead may impair its function [28, 29]

[30]. Interestingly, the P301S mutation in tau results in conformational changes which impact structure and function [31], and these changes may make it differentially susceptible to modification by certain E3 ligases and deubiquitinases compared to wild type tau. Overall, these data indicate that the 26S proteasome plays a role directly or indirectly in the turnover of P301S tau (Fig. 5.2).

PROTACs (PROteolysis TArgeting Chimeras) are heterobiofunctional peptides that bind an E3 ligase and a target protein, and enable ubiquitylation and degradation by the 26S proteasome [32]. Therefore it is possible that PROTACs may be useful in facilitating tau clearance. In a recent study, a PROTAC peptide that binds tau and Keap1, and thus recruits the Keap1-Cul3 ubiquitin ligase complex to tau, was shown to enter cells and induce tau degradation in models where tau is overexpressed [33]. These initial findings are intriguing, but further studies are needed to determine if PROTACs can facilitate tau clearance in vivo.

Autophagy

There are three defined types of autophagy, a process that involves degradation of substrates by the lysosome. Microautophagy involves direct lysosomal or vacuolar engulfment of cytoplasmic cargo. In chaperone-mediated autophagy (CMA), substrate proteins are directly delivered into the lumen of lysosomes through a Hsc70/Lamp2a-mediated process. Macroautophagy (which will be referred to simply as autophagy in the sections below) is the most studied type of autophagy, particularly in the context of AD and other neurodegenerative diseases, and involves the delivery of cargo to lysosomes via a vacuole-based

process. For more complete reviews of autophagy, as well as its role in neurodegenerative diseases, see [34–38]. Given that the majority of studies that have examined autophagic tau clearance have focused on macroautophagy/autophagy, a brief review of this pathway will be presented here.

Autophagy involves the formation and elongation of a double membrane structure called a phagophore which then closes around cargos to form the autophagosome which subsequently fuses with lysosomes. This fusion of an autophagosome and lysosome results in the formation of an autolysosome. Alternatively, the autophagosome can fuse with an endosome to form an amphisome which also then fuses with the lysosome [39]. This process can be non-selective and it is sometimes referred to as bulk autophagy, when portions of the cytosol are engulfed in the process of autophagosome formation. The engulfed contents are then degraded to provide the cell with recycled basic components for energy usually in response to nutrient deprivation. On the other hand, selective autophagy involves the recognition of specific clients by autophagy receptor complexes and targeting to the developing autophagosomes [35, 40]. However, the general scheme of how autolysosomes form and degrade substrates is very similar, if not the same, for both non-selective and selective autophagy [37].

Neuronal autophagy is constitutively active and highly efficient [41]. Since neurons are unique in being post-mitotic and highly asymmetric cells, autophagy is not a homogenous event but rather is differentially regulated in the various compartments of the cell (soma, axon, dendrites, pre- and post-synapse) [42–45]. These regulatory differences are key when considering autophagy-mediated tau turnover because tau, though primarily axonal, also localizes to dendrites, as well as the pre- and post-synaptic compartments [46–48], and the autophagy-mediated clearance of tau likely differs in these various cellular compartments [45, 49, 50]. With this in mind, data from studies in which non-neuronal or clonal cell models are used to examine the regulation of autophagy-mediated tau clearance must be interpreted with caution and should be subsequently validated using primary neuron or in vivo models. As an example of this consideration, nutrient deprivation (starvation) and mTOR inhibition (treatment with rapamycin or torin 1) reliably and reproducibly upregulate autophagy in many non-neuronal models; however, data indicate that this is not always the case in neurons [50–53]. Interestingly, autophagy appears to be more efficient in younger neurons, as the expression levels of proteins that induce autophagy, such as Beclin-1, ATG5 and ATG7, decline with age [41, 54] and this attenuation of autophagy may potentially be a contributing risk factor for the onset of age-related neurodegenerative diseases including AD.

Tau Degradation by Autophagy

Numerous studies have provided compelling evidence that autophagy is a significant contributor in the degradation of tau (Fig. 5.3). A functioning lysosome is essential for completion of autophagy and early reports demonstrated that tau was a substrate of the lysosomal aspartyl protease, cathepsin D. In vitro, at a low pH, cathepsin D cleaved tau, and adding cathepsin D to rat brain homogenates also resulted in tau degradation [55]. Treatment of hippocampal slices with chloroquine, which raises lysosomal pH and is often used to inhibit autophagic flux, increased tau levels, and in particular tau phosphorylated at Ser396/404 (PHF1 epitope) [56]. Lastly, in a fly tauopathy model, ablation of cathepsin D potentiated tau-induced neurotoxicity [57].

Investigations carried out in non-neuronal models have also provided supporting evidence that tau is degraded by autophagy. In human neuroblastoma SH-SY5Y cells overexpressing P301L tau, stimulation of autophagy by serum starvation or rapamycin treatment reduced tau inclusions. This effect was blocked when the autophagy protein ATG5 was deleted [58]. When N2A cells engineered to overexpress a tau construct of only the microtubule binding repeat

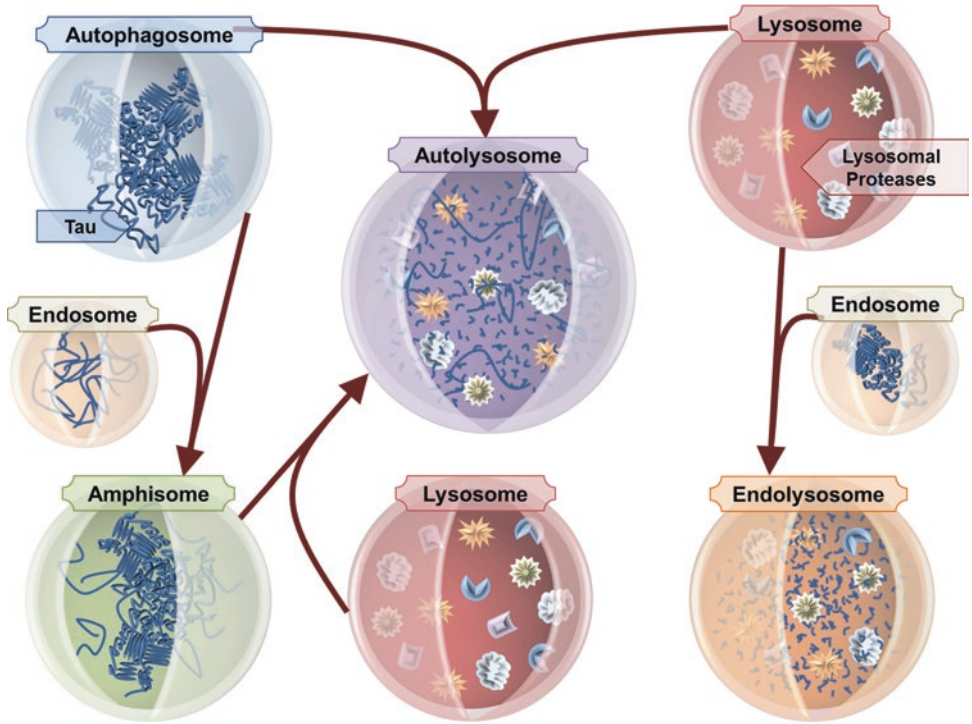

Fig. 5.3 Degradation of tau by autophagy. Tau is likely degraded through different autophagic routes. Autophagy adaptor protein complexes interact with tau targeting it to the autophagosome which fuses with the lysosome to form autolysosomes. Cytosolic, as well as extracellular tau, can also be incorporated into endosomes [87, 88] which then can go on to fuse directly with lysosomes forming endolysosomes, or the endosome can fuse with an autophagosome to form an amphisome prior to fusing with lysosomes [39]

region of tau (referred to as "K18") with the FTLD ΔK280 mutation (which results in increased aggregation) were treated with trehalose, an mTOR-independent autophagy activator, there was a significant reduction in aggregated tau, as well as the levels of the tau construct in both soluble and insoluble fractions [25]. In a human neuroblastoma cell line that inducibly expresses tau, treatment with the autophagy inhibitors chloroquine or 3-methyladenine, resulted in tau accumulation and increases in tau in sarkosyl-insoluble fractions [59]. Lastly, studies in an immortalized cortical cell model in which either full-length tau or tau truncated at D421 (to mimic caspase 3 cleavage which occurs in pathological conditions such as AD) were inducibly expressed, revealed D421 truncated tau was preferentially degraded through the autophagy pathway [14]. Although all these studies were in clonal cell models, they provide data suggesting that autophagy is a mediator of tau clearance.

In vivo and primary neuron models have been used to provide strong support for a role of autophagy in tau clearance. Interestingly, evidence for the role of autophagy in clearing tau in neurons came from studies that were attempting to elucidate the role of the proteasome in tau degradation [25]. In one study, treatment of rat primary neurons with proteasomal inhibitors unexpectedly reduced tau levels. This effect was likely due to a compensatory upregulation of autophagy, as evidenced by increased levels of LC3-II, which is both an indicator of increased autophagy induction, and increased numbers of autophagosomes [25, 26]. In hippocampal slices from JNPL3 mice, which express P301L tau under the control of the mouse prion promoter, treatment with methylene blue to induce autophagy resulted in a decrease of phosphorylated tau

and insoluble tau [60]. In vivo treatment of the JNPL3 mice with methylene blue, as well as treatment of another FTLD mouse model that expresses P301S tau with trehalose, reduced the levels of insoluble tau [60–62]. In the P301S tau model trehalose treatment also significantly reduced tau phosphorylated at T212/S214 (AT100) (however, no other phosphorylation sites were assessed) [61, 62]. Mice with a deletion of autophagy gene ATG7 in their forebrain neurons develop age-dependent neurodegeneration with accumulation of phosphorylated tau inclusions [63]. Taken together these and other studies provide compelling evidence that autophagy plays a key role in mediating tau clearance.

A critically important factor in clearance of tau by selective autophagy is the involvement of autophagy receptors (also referred to as autophagy cargo adaptors), chaperones, and co-chaperones that work in concert to target tau to the autophagy pathway. Autophagy receptors are key components of the selective autophagy pathway as they contain domains that allow them to engage specific cargos. Receptors also have an LC3 interacting region (LIR) for binding LC3 and targeting clients to the developing autophagosome for degradation [40, 64, 65]. Autophagy receptors have domains that allow them to interact with ubiquitylated substrates; however, in some cases, they are also able to interact with non-ubiquitylated clients. For example, the autophagy receptor optineurin mediates the clearance of protein aggregates that were not ubiquitylated [66], and the binding of the autophagy receptor, NDP52, to tau also occurs independent of ubiquitylation [67].

Several different autophagy receptor proteins have been implicated in facilitating the clearance of tau. Deletion of the autophagy receptor p62, in a mouse model resulted in accumulation of hyperphosphorylated tau, and thus, it was suggested that p62 facilitates tau clearance [68]. p62 and LC3 co-stained tau positive inclusions in the brains of mice that overexpress P301S tau (PS19 line) in an ubiquitin-independent manner [55, 62]. In this same mouse model, overexpression of p62 resulted in a decrease in tau pathology [69]. Furthermore, in a clonal cell model, p62 played a role in targeting tau aggregates to autophagy [70]. Interestingly, in the same study, it was found that while p62 bound tau aggregates, it did not interact with the initial "tau seeds". In contrast, these seeds were recognized by NDP52 [70]. Indeed, an earlier study demonstrated that the autophagy receptor NDP52 directly binds tau through its SKICH domain and that, in primary neurons, knocking down NDP52 results in increased levels of phosphorylated tau [67]. It was also shown that NDP52 co-localized with phospho-tau in the brains of APPsw/PS1dE9 transgenic mice (which express mutant APP with the Swedish mutation and PSEN1ΔE9) [71]. NBR1 is another autophagy receptor, and in sporadic inclusion body myositis muscle fiber inclusions containing phosphorylated tau also co-label with an antibody to NBR1 and it was postulated that abnormalities in NBR1 may contribute to the accumulation of phosphorylated tau species [72]. However, the role of NBR1 in mediating tau clearance in neuronal models has not been substantially investigated. The role of optineurin in tau clearance has also not been extensively studied. However, a report in HeLa cells suggested that optineurin may play a role in clearing soluble tau species [69].

Recent studies have provided evidence that autophagic degradation of tau is mediated in part by Nrf2 induction of NDP52 [67]. Intriguingly, Nrf2 also appears to regulate the expression of BAG3 (BCL2-associated athanogene 3) which is induced by proteotoxic stress, is increased during aging [73], and plays a critical role in regulating soluble tau levels [26, 49, 74]. BAG3 is a multidomain protein that plays a key role in regulating autophagy [49, 75, 76], as well as functioning as part of a complex that targets specific clients, including tau, to this degradative pathway [26, 49, 77]. BAG3 binds heat shock proteins and it has been shown that a BAG3-HspB8-Hsp70 complex can bind misfolded protein aggregates and target them for degradation via autophagy [78]. In young primary neurons, BAG3 overexpression induced autophagy which facilitated the clearance of endogenous tau [26]. Interestingly, in mature

neurons BAG3 appears to regulate autophagy differentially depending on the cellular compartment, preferentially contributing to autophagosome-lysosome fusion and phosphorylated tau clearance in dendrites and at the post-synapse. In a recent study it was demonstrated that expression of BAG3 is significantly higher in inhibitory neurons compared to excitatory neurons in control human brain. These findings are significant as in AD, pathological tau species accumulate in excitatory neurons to a greater extent than inhibitory neurons [74]. Overall, these studies indicate that BAG3 is a significant contributor to the autophagic processes that mediate tau clearance.

PICALM/CALM (phosphatidylinositol-binding clathrin assembly protein) has been shown to associate with AD based on genome-wide association studies [79]. In addition to regulating endocytosis, PICALM/CALM is able to mediate different steps of autophagy and facilitates tau clearance. Since PICALM/CALM plays a role in regulating autophagy and tau clearance it can be suggested that this may be why it is a genetic risk factor for AD [80].

Tau Clearance by Chaperone-Mediated Autophagy (CMA) and Microautophagy

Although the majority of studies have focused on clearance of tau by macroautophagy/autophagy, CMA has also been examined. In CMA, substrate proteins are first recognized by the cytosolic chaperone, hsc70 and directly targeted to the lysosome by binding LAMP2a [81]. Most proteins targeted by hsc70 contain a KFERQ targeting motif [82, 83]. Tau has two imperfect KFERQ motifs in the fourth repeat domain (^{336}QVEVK^{340} and ^{347}KDRV^{Q351}), which are recognized by Hsc70 [84, 85]. Using the mouse N2A neuroblastoma cell line with stable expression of full length wild type tau, A152T tau, or P301L tau, it was shown that CMA contributed to the degradation of wild type and A152T tau, but not P301L tau. In this study it was also demonstrated that wild type and A152T tau could be degraded through an endosomal-microautophagy pathway [86]. It was also recently demonstrated that tau can be delivered to lysosome via early endosomes and multivesicular bodies (MVBs), and that this is dependent on Rab35 and the ESCRT system [87]. Further, the uptake of monomeric tau by human neurons can occur through endocytosis and micropinocytosis with the resulting vacuoles likely routing tau to the lysosome [88]. However, the mechanisms involved and the exact fate of tau once it is internalized through these routes have not been fully elucidated.

Summary

Overall, it is clear that tau is degraded by multiple mechanisms and that many factors determine which route is taken. In addition, pathological conditions such as AD, likely have altered degradative pathways and, hence, altered tau clearance. Decreased efficiency of any of these clearance pathways is likely to have detrimental effects on tau turnover, potentially enhancing tau accumulation and pathology. Further studies to elucidate tau clearance mechanisms are likely to provide key insights into the underlying processes that contribute to tau accumulation and its subsequent role in the pathogenesis in AD and other tauopathies.

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6 Mechanisms of Axonal Sorting of Tau and Influence of the Axon Initial Segment on Tau Cell Polarity

Hans Zempel and Eckhard Mandelkow

Tau Is an Axonally Targeted Microtubule Associated Protein in the Central Nervous System

The protein Tau (UniProt: P10636, with isoform F being the largest isoform in the human CNS with 441 residues) is a microtubule-associated protein (MAP). MAPs are proteins that bind to assembled microtubules, and can promote the assembly of tubulin dimers into microtubules. Besides Tau, other MAPs such as STOP/MAP 6 (stable tubule only proteins), MAP 1a and MAP 1b are also present in neuronal axons, while MAP 2 is exclusively present in the somatodendritic compartment.

Tau was originally discovered by Marc Kirschner and colleagues in their quest for microtubule assembly-promoting factors [55]. Tau expression is upregulated during neuronal differentiation together with tubulin [10]. In normally matured healthy neurons in the Central Nervous System (CNS), Tau is predominantly present in neuronal axons and only very little present in dendrites [5, 34]. Human Tau is encoded on chromosome 17q21 [40], and in the human CNS comprises six major alternatively spliced isoforms, with different isoform ratios depending on the developmental stage, cell type, and brain region [1, 11, 51].

The major CNS isoforms differ by the presence or absence of two near N-terminal inserts of 29 residues each (N1, N2), encoded by exons 2 and 3, and by the second of four repeats (R2, 31 residues) in the repeat domain, encoded by exon 10. Domains can be subdivided into (i) the 'assembly domain' in the C-terminal half, comprising the repeat domain plus flanking regions, which supports microtubule assembly, (ii) the 'projection domain', which does not bind to microtubules and projects away from microtubules (N-Terminal half), and (iii) the 'proline-rich domain' in the middle part (aa 150–240), which contains seven PXXP motifs, an interaction motif for binding proteins with SH3 domains (for review see Zempel and Mandelkow [57]. Tau is a highly soluble and natively unfolded protein which normally resists aggregation. However, repeats R2 and R3 contain two hexapeptide motifs with increased propensity for β-structure, which can form the nucleus for amyloid-like

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aggregation [53]. Otherwise the content of secondary structure is unusually low [38].

Sorting of Tau During Neuronal Development

Tau is a commonly used marker for axons and for neuronal cell polarity. Cell polarity is pronounced for most neurons in the CNS, with usually one long axon and several highly branched dendrites, and a corresponding asymmetric distribution of the cytoskeleton and other cellular components. Although most protein synthesis in mature mammalian neurons occurs in the somatodendritic compartment, the axon represents up to 99% of the volume of a neuron. The asymmetry of high axonal volume versus protein synthesis in the soma requires efficient long-haul transport mechanisms, mainly based on microtubules and their motor proteins (kinesin, dynein). The process of cell differentiation, neuritic outgrowth and establishment of neuronal cell polarity can be subdivided into five 'Banker' stages [34]: (i) formation of lamellipodia; (ii) outgrowth of minor processes; (iii) formation and growth of the axon; (iv) growth of the dendrites; and (v) maturation. With increasing maturation, Tau becomes sorted into axons (in contrast to MAP 2, which is restricted to soma and dendrites). The predominance of axonal sorting of Tau may be lost permanently in certain neurons in AD and other tauopathies where Tau accumulates in the somatodendritic compartment ('missorting'), or temporarily in the brains of hibernating animals (for review of missorting of Tau in pathological conditions see Zempel and Mandelkow [57]).

Proposed Sorting Mechanisms of Tau

Several models have been proposed to explain the polarized cellular distribution of Tau. The suggested Tau sorting mechanisms can be broadly divided into mRNA based and preferred translation in axons (mechanisms 1,2), protein turnover based and preferred compartment specific degradation (mechanism 3), and preferential retention and active sorting (mechanisms 4–6; for a graphical depiction of the different mechanisms see Fig. 6.1, for a summary and important references see Table 6.1).

1. **Axonal targeting of Tau mRNA:** In a first attempt to understand the differential localization of Tau and MAP 2, the Ginzburg lab found that in primary rat neurons Tau mRNA

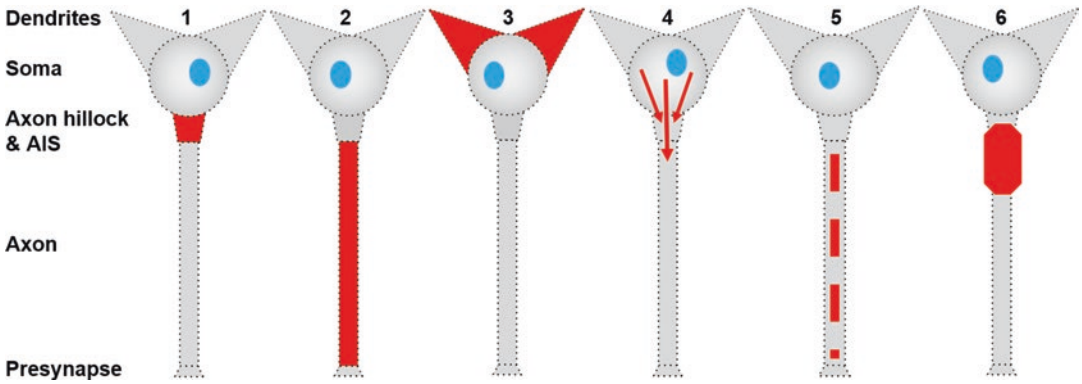

Fig. 6.1 Graphical depiction of proposed sorting mechanisms for axonal targeting of Tau protein. Compartments indicated from top to bottom are: Dendrites, soma, axon hillock/AIS, axon, synapse. Red colors indicate the localization of the presumed active region of the proposed sorting mechanism. *1* Targeting of Tau mRNA into the axon hillock resulting in axonal presence of Tau protein. *2* Preferred axonal translation of ubiquitously present Tau mRNA. *3* Degradation of Tau protein specifically in dendrites, but not in axons. *4* Active axonal sorting of somatically translated Tau protein into the axon by an AIS localized pumping function. *5* Axonal enrichment of Tau protein by specific association of Tau with axonal microtubules. *6* Barrier function of the AIS: The Tau Diffusion Barrier (TDB) restricts Tau diffusion out of the axon

Table 6.1 Overview of currently discussed sorting mechanisms resulting in axonal presence of Tau protein

Sorting mechanism	Supporting arguments	Counter-arguments
1. Axonal targeting of Tau mRNA	Tau mRNA visible in the proximal axon [32]; 3′-UTR of Tau mRNA with axon localization sequence [2]	Tau mRNA also identified in other compartments (soma, proximal dendrites) [3, 27]; Tau protein expressed without 3′-UTR successfully sorted [60], also when microinjected [17]
2. Preferred axonal translation of Tau mRNA	5′-UTR oligopyrimidine tract of Tau mRNA mTOR governed protein synthesis in developing axons [37]	Little evidence of sufficient protein synthesis in mature axons
3. Dendritic degradation of Tau	Blocking protein degradation results in appearance of dendritic Tau, blocking dendritic protein synthesis prevents appearance of dendritic Tau [3]	Expression of Tau with little turnover and/or traceable Tau (pulse-chase) results in redistribution of Tau from the soma into the axon [31, 60]
4. Active axonal sorting of somatically translated Tau	Tau is transported along microtubules into the axon [25]; Blocking microtubule dynamics with nocodazole results in somatic appearance of Tau [31, 59]	Tau preferentially binds to axonal microtubules in fixed cells [22]; taxol inactivation of microtubule dynamics does not result in somatodendritic appearance of Tau [58, 59]
5. Axonal retention of Tau protein by specific association of Tau with axonal microtubules	Tau preferentially binds axonal microtubules in fixed, permeabilized cells [22]; favorable kinase/phosphatase distribution for immobilization of axonal Tau (low KxGS phosphorylation of Tau (immobilizes Tau) in axons [58] due to preferential localization of PP2a in axons [61], and Tau-KxGS kinases in dendrites (MARK, SAD [15, 23]; differential PTMs of microtubules [33, 60]	Hypothesis not sufficiently tested, indirect evidence
6. Barrier function of the AIS: The Tau diffusion barrier (TDB)	TDB retains Tau in the axon [31]; specific Knockdown of AIS components results in impaired function of the TDB [60]; establishment of AIS/TDB in parallel to establishment of sorting of Tau [34, 43]	TDB works both antero- (soma-to-axon) and retrogradely (axon-to-soma) [60]

localizes to the proximal region of the axon. Whereas tubulin mRNA was mainly detectable in the soma, MAP 2 mRNA was present mainly in dendrites [32]. In neuronally differentiated p19 cells, an embryonic carcinoma cell line that retains pluripotency, the same group later identified a cis-acting sequence at the 3′ UTR of Tau mRNA that acts as a zip code in its targeting to the axon, and also for mRNA stabilization. Importantly, swapping this sequence with the dendrite targeting 3′ UTR of MAP 2 resulted in dendritic targeting of Tau mRNA *and* Tau protein, as well as axonal targeting of the normally dendritically targeted MAP 2 mRNA and MAP 2 protein [2]. Other studies showed that a small fraction of Tau mRNA localizes also in the proximal regions of dendrites [3, 27]. Further, injected Tau protein as well as transfected Tau DNA without the 3′ UTR also results in axonal targeting of the injected or transfected Tau [17, 60]. In developing neurons and in neurons from non-mammals axonal protein synthesis appears to be important for several processes such as axonal guidance and outgrowth (see e.g. for *Xenopus laevis*, Cagnetta et al. [7]); however, evidence that axonal protein synthesis in mature mammalian neurons can maintain Tau protein levels is missing.

2. **Preferred axonal translation of Tau:** In young primary neurons, a 5′ UTR of Tau (and CRMP2) regulates axonal translation of Tau mRNA in the growing axon. This process is mTOR dependent, and insertion of this 5′ UTR upstream of the coding sequence of luciferase also resulted in axonal localization of this marker protein when myristoylated to prevent diffusion out of the axon [37]. However, the

evidence for axonal translation in this study is indirect, and the study was limited to young developing neurons. Recently, using microfluidic chambers, dendritic presence and translation of Tau mRNA was detected [3], questioning the generalizability of the study. Direct injection of Tau protein [17], as well as transfection of Tau without the 5′ UTR [60] also results in axonal targeting of Tau, indicating that preferred axonal translation of Tau might only be important in immature neurons.

3. **Degradation of dendritic Tau:** The first protein based mechanism of axonal sorting of Tau was proposed by Hirokawa and colleagues. They observed that after 4 days of injecting Tau or MAP 2 into neurons, Tau remained only in axons, while MAP 2 remained only in the soma and dendrites [17]. More recently, specifically blocking proteasomal or autophagic degradation (with wortmannin and epoximicin) in dendrites resulted in increased presence of dendritic Tau to ~4-fold, while enhancement of proteasome or autophagy activity (with trehalose and rolipram) resulted in decrease of dendritic Tau by~ 3-fold [3]. Compartment-specific degradation of Tau could be explained by differential interactions with degradation pathways (proteasome, autophagy [8, 28, 42, 54] or folding pathways (chaperones), depending on post-translational modifications (phosphorylation) or chaperone components (e.g., C-terminus of Hsc70 interacting protein, CHIP) [9, 26].
4. **Active axonal sorting of somatically translated Tau protein**: Tau is a microtubule associated protein, but can also be transported along microtubules, typically at the rate of slow axonal transport, 0.2–0.4 mm/day [36]. Over short distances, Tau can be transported anterogradely at the typical rates of kinesin (1 μm/s) along microtubules [25]. Microtubules appear to be essential for anterograde sorting, as the microtubule depolymerizer nocodazole results in loss of anterograde sorting [31, 59]. Since the dwell-time of Tau on microtubules is low (sub-second range), the protein can distribute locally by rapid diffusion ("kiss and hop", [20]). There is also evidence that Tau can slide one-dimensionally along microtubules [46], and with predominant anterograde polymerization of microtubules this would enable Tau to propagate into the axon by 1D-diffusion [52]. However, within the axon initial segment stable microtubules are scarce, so that active Tau transport would have to overcome the microtubule gap within the AIS [60]. As nocodazole acts as an inhibitor of polymerization of microtubules, rather than destroying existing microtubules, one could assume that microtubule dynamics might be essential for Tau sorting. Yet in several studies the microtubule stabilizer Taxol, which inhibits microtubule dynamic instability, did not prevent axonal targeting of Tau [31, 58]. Furthermore, Tau protein is able to associate with axonal microtubules even in fixed cells [22] (see also the following mechanisms 5), indicating that active transport mechanisms or processes depending on microtubule dynamics cannot be the only sorting mechanisms.
5. **Preferred axonal retention of Tau due to axonal binding sites**: Axonal stabilization of Tau or retention of Tau in axons could be explained by a high affinity of Tau for axonal microtubules vs. low affinity for dendritic microtubules, as shown with fixed permeabilized cells [22]. Swapping the microtubule binding domain of Tau and MAP 2 resulted in increased axonal targeting of MAP 2 and dendritic targeting of Tau [17], demonstrating specific association of certain microtubule binding domains with compartment specific microtubules. This goes in line with the idea of differential compartment specific post-translational modifications of microtubules [19, 33]. Not only microtubules, but also Tau itself could be differentially modified depending on its localization in axons or in the somatodendritic compartment. This would in turn change Tau's interaction pattern with microtubules and other structures. For example, missorted dendritic Tau is preferentially phosphorylated at the KXGS motifs (Ser262, Ser356), which makes Tau more diffusible and less able to bind to microtubules [45].

Axonal Tau is less phosphorylated at these sites [58], probably because of the preferential localization of PP2a in axons, the main phosphatase responsible for the dephosphorylation of the KXGS motifs [61]. That way, dephosphorylated Tau could be enriched on axonal microtubules. All mechanisms described above have been demonstrated not only in developing immature neurons, but also in terminally polarized neurons, making this mechanism a likely contributor to axonal targeting of Tau protein. However, most of the evidence is indirect, and no specific microtubule modification has yet been identified that particularly attracts Tau.

6. **Restricted Diffusion of Tau due to selective entry at the AIS: The Tau Diffusion Barrier:** In our research we discovered a Tau Diffusion Barrier (TDB) that is located within the Axon Initial Segment (AIS), the proximal region of the axon where action potentials are generated. The TDB serves as a diffusion restrictor for Tau protein, and its efficiency roughly correlates with the gradual establishment of the AIS [31]. This barrier was originally described as a retrograde barrier (preventing axonal Tau from diffusing back into the soma). More recently we found that the TDB can differentially regulate anterograde trafficking of Tau isoforms, and that it can be disrupted by Alzheimer Disease-like stress conditions resulting in somatic accumulation of some Tau isoforms [60].

The TDB Within the AIS Regulates Tau Transit into the Axon

As the TDB is currently the best hypothesis for regulation of axonal entry of Tau protein, and as the TDB precisely colocalizes with the AIS [31, 60], we will in the next section describe possible influences of the AIS on the polarized distribution of Tau.

The Axon Initial Segment (AIS) is localized between the cell body/axon hillock and the axon proper and stretches over 20–50 μm [29]. Structurally, the area between the soma and the AIS is defined by local enrichment of MAP 2 and TRIM46 [13]. The AIS itself is the major unit responsible for generating action potentials and is a crucial player for the establishment and maintenance of neuronal cell polarity [43]. The AIS was initially described to contain only sparse microtubules [41], but was later shown to contain dense bundles of (unstable) microtubules [21]. The different results obtained in these studies can be explained by the different experimental cell fixation conditions.

Since most protein synthesis takes place in the cell body, Tau must transit through the AIS on its way from the cell body to the axon. The AIS is enriched with cytoskeletal scaffolding proteins, notably AnkyrinG and βIV spectrin, which together cluster Nav and Kv channels and link them to the actin cytoskeleton [4]. Apart from generating action potentials, the AIS is also an essential element of neuronal cell polarity [6]. Several studies described the barrier function of the AIS to be based either on lipids, membranes, F-actin or microtubules, depending on the components studied [24, 31, 39, 47, 48, 56]. The internal structure of the AIS and its relationship to barrier functions remain disputed since its discovery by electron microscopy in 1968 by Palay and colleagues, but recent studies revealed a dense, fibrillar/globular submembranous coat containing the classical AIS proteins (e.g., AnkyrinG, βIVspectrin) which covers microtubules bundles, actin rings similar to axonal actin structures, but also actin patches, and enrichment in EB proteins [21, 29, 60].

Efficiency of the Tau Diffusion Barrier Depends on the Composition of the AIS

The TDB is precisely located within the AIS, and functionally depends on particular microtubule dynamics. Thus, microtubule destabilization by nocodazol results in Tau missorting and breakdown of the TDB, whereas microtubule stabilization by taxol prevents Tau missorting, e.g. after Aβ-insult [31, 58]. Microtubules within the AIS are extremely dynamic: In the AIS, mark-

ers of stable microtubules, such as acetylation and polyglutamylation are rare, and microtubules cannot be stained without using molecular densifier polyethylene glycol (PEG) and taxol [21, 60]. EB3-based live-imaging of growing microtubule tips further reveals a hub of EB3-comet spawning within the AIS, consistent with observations of enrichment of EB-proteins in the AIS [30, 60].

Knockdown of important structural AIS components results in partially impaired neuronal cell polarity, e.g. with the appearance of spines at the proximal axonal shaft [16]. We recently knocked down some key components of the AIS and investigated the effects on Tau distribution and the TDB [60]. This revealed that knockdown of structural components of the AIS results in weakening of the TDB. Tau transit through the TDB is facilitated when classical AIS components (e.g. AnkG, EB1) are knocked down via shRNA. All knockdowns also induced increased invasion of the dendritic marker MAP 2 into the proximal part of the axon. This indicates that the classical AIS components contribute to the TDB, but also to the nonspecific filter function of the AIS.

The protein kinase GSK3β, a major player anchored within the AIS [50] is also an important kinase for the phosphorylation of Tau, but does not phosphorylate the KXGS motifs of Tau, which regulate microtubule binding. GSK3β induces accumulation of endogenous and exogenous Tau in the somatodendritic compartment, and thus impairs the normal Tau cellular distribution. This effect is independent of direct phosphorylation of Tau by GSK3β, because even non-phosphorylatable Tau shows the same retrograde propagation from the axon into the soma. This indicates that the interplay of GSK3β with components of the AIS/TDB is essential for the maintenance of proper TDB function and cellular polarity.

Modulation of AIS Plasticity by Tau Expression and Phosphorylation

We described so far that the AIS is instrumental in establishing neuronal cell polarity [43], that certain structural elements (EB1, AnkyrinG) are essential for normal function of the TDB, and particular constellation of microtubule dynamics and f-actin promote the anterograde targeting of Tau [60]. By contrast, few studies have addressed whether Tau also modulates AIS properties. This possibility is suggested by the many "atypical" functions of Tau (besides stabilizing axonal microtubules), such as regulating presynaptic or postsynaptic functions in neurotransmitter shuttling [18, 35], cargo and vesicle release in the presynapse, or stabilizing DNA integrity (for review see Sotiropoulos et al. [49]). A direct modulation of neuronal activity in the axon itself was until recently elusive.

The Gan laboratory showed recently that Tau, when acetylated at certain Lysines (K274 and K281), destabilizes the AIS via reduction of important structural elements of the AIS, namely AnkyrinG and βIV-spectrin, similar to what occurs in the brain of Alzheimer Disease patients. Overexpression of Tau mimicking acetylated Tau also resulted in increased microtubule dynamics in the AIS and impairment of the TDB, both confirmed by live-imaging, resulting in somatic accumulation of Tau [47].

The AIS localization influences the activity and excitability of neurons [12]. Hatch and colleagues showed that Tau phosphorylated at specific sites (12E8 and AT180 epitopes), induced a distal shift of AnkyrinG localization in transfected primary neurons, which also correlated with a decrease in excitability of the cells. The same was true for neurons from young rTg4510 and old pR5 mice, models that show neurodegeneration due to overexpression of FTLD-derived P301L-Tau [14].

It is thus clear that at least certain components (AnkyrnG, EB1, microtubule dynamics) of the AIS influence the TDB and overall Tau sorting into the axon. In overexpression models of pathologically modified Tau (phosphorylation, acetylation), Tau is also able to influence AIS structure and function. Of note, there is also evidence suggesting that antibody mediated reduction of AnkyrnG in healthy aged individuals and in actively vaccinated AD-model mice protects from Alzheimer Disease, indicating that at least structural integrity of the AIS is not a prerequisite for preserved cognitive function during aging

[44]. Future studies will have to show whether Tau can influence AIS properties also when not overexpressed, and whether this change contributes to AIS and cognitive function.

Conclusion

Several mechanisms for preferred axonal targeting of Tau have been proposed, both mRNA and protein based (for a summary see Table 6.1 and Fig. 6.1). Most of these mechanisms have been addressed in separate studies, using cells of different origins and of different developmental (Banker-) stages. While the individual evidence supporting the different mechanisms is strong, no study has addressed the real contribution of each mechanism in each developmental stage. In more mature neurons with full establishment of the AIS the Tau-Diffusion-Barrier within the AIS plays a major role in regulating the transit of Tau through the AIS.

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Part II

Tau Localization and Function

Tau and Axonal Transport Misregulation in Tauopathies

7

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Introduction

Neurons are unique cells because of their morphology and polarity, with the cell body extending two types of processes. These are the dendrites that primarily receive signals from other cells and the surrounding environment and the axons that are long processes connecting neurons to their target cells. The longest neuronal process, the axon, can extend up to a meter in length from the cell body, in the case of some motor and sensory neurons. One of the unique challenges of the neuron is the provision of materials to distant synapses, where communication with target cells takes place. Such provision relies on the trafficking of material to and from the synapse by a complex set of intracellular trafficking events collectively referred to as fast axonal transport (FAT) (reviewed in [6]). Microtubule motor-based FAT along axons is critical to the function and health of neurons, delivering organelles, vesicles, and other cellular materials that ultimately support communication with target cells. FAT is also necessary for returning damaged material to the cell body for recycling, and for delivering neurotrophic signals received at the axon terminal to the cell body, where they can affect gene transcription (reviewed in [67]). The importance of this process is highlighted by examples of neurodegeneration following its disruption. Mutations in the genes encoding proteins involved in transport along the axon (including molecular motors, cytoskeletal tracks, and adaptor proteins) cause several neurodevelopmental and neurodegenerative diseases (reviewed in [65, 79]). The tau protein is among those implicated in affecting FAT in a group of

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neurodegenerative diseases known as tauopathies (reviewed in [42, 45]). In this chapter, we will discuss the relationship between tau and FAT under normal conditions and how disruptions to this process may be a toxic mechanism in multiple neurodegenerative diseases.

Tau Protein and Disease

From its initial discovery in 1975, the tau protein was closely associated with microtubules and is somewhat enriched in axons [5, 105]. It is primarily expressed in the brain with higher levels of mRNA and protein in the cortex and hippocampus than white matter and the cerebellum [95]. The protein exists as six major isoforms in the adult human brain, generated through alternative mRNA splicing. The isoforms differ in their inclusion of 2, 1 or 0 of exons 2 and 3 in the N-terminus and their inclusion of exon 10 which determines whether the protein contains either 4 or 3 microtubule-binding repeat regions (+exon 10 = 4R tau, −exon 10 = 3R tau; Fig. 7.1a). The microtubule-binding regions in the C-terminal half of the protein are positively-charged, while the N-terminus is enriched in acidic amino acids leaving the protein isoforms with a relatively low net charge. Directly upstream of the microtubule-binding domains is a proline-rich region which includes several phosphorylation sites and PXXP motifs that may bind to SH3 domains (Fig. 7.1b). Other functional domains include a phosphatase-activating domain (PAD, discussed in greater detail below) at the extreme N-terminus [40]. Structural studies indicate that tau is a highly dynamic protein, capable of multiple conformations that may underlie its diverse role in multiple cellular functions. The protein contains regions of secondary structure and acquires global conformations such as the "paperclip", which involves the N- and C-termini interacting with each other and the C-terminus interacting with the microtubule-binding repeat regions [38, 69].

Approximately a decade after the discovery of tau, a series of studies demonstrated that tau was the primary component of the hallmark tangle pathology in Alzheimer's disease (AD) and that tau was heavily phosphorylated in these inclusions [29, 30, 107]. Interest in the tau protein has continued to grow as a central role in AD and several other neurodegenerative disorders became apparent. The discovery of inherited mutations within the tau gene that lead to early-onset frontotemporal dementias (FTDs) was the first line of evidence demonstrating that pathological tau is sufficient to cause neurodegenerative disease [35]. In these diseases, tau undergoes a number of pathological post-translational modifications (reviewed in [55, 84]) and forms a variety of morphologically different aggregates that range from small oligomers to much larger filamentous inclusions (reviewed in [46]). Pathological tau is highly modified compared to its normal state with some of the most prominent modifications including increased levels of phosphorylation, changes in overall conformation, and truncation of the protein, among many others [7, 15, 29, 31, 34]. These modified versions of tau accumulate in the somatodendritic and axonal compartments of neurons and often display impaired microtubule binding [8, 12, 28, 82], fueling the notion that reduced stability of the microtubule cytoskeleton represents a critical pathogenic event in tauopathies. However, almost 50 years after its discovery, the exact mechanisms of tau toxicity continue to be debated.

Some potential clues may be found by elucidating tau's normal cellular functions as well as examining the neurodegenerative phenotypes associated with tauopathies. As mentioned above, tau was traditionally linked to microtubule-based functions involving stabilization and dynamics. However, the protein's putative functions have expanded to include regulation of FAT, scaffolding for phosphotransferases, synaptic plasticity, neuronal activity, actin bundling, and mediating interactions between cellular components (reviewed in [45, 103]). These functions may be reliant on the local tau isoform composition as well as specific sets of regulatory post-translational modifications. Thus, many, if not all, of these potential functions of tau are likely affected in disease as the protein undergoes abnormal modification, misfolding and aggregation.

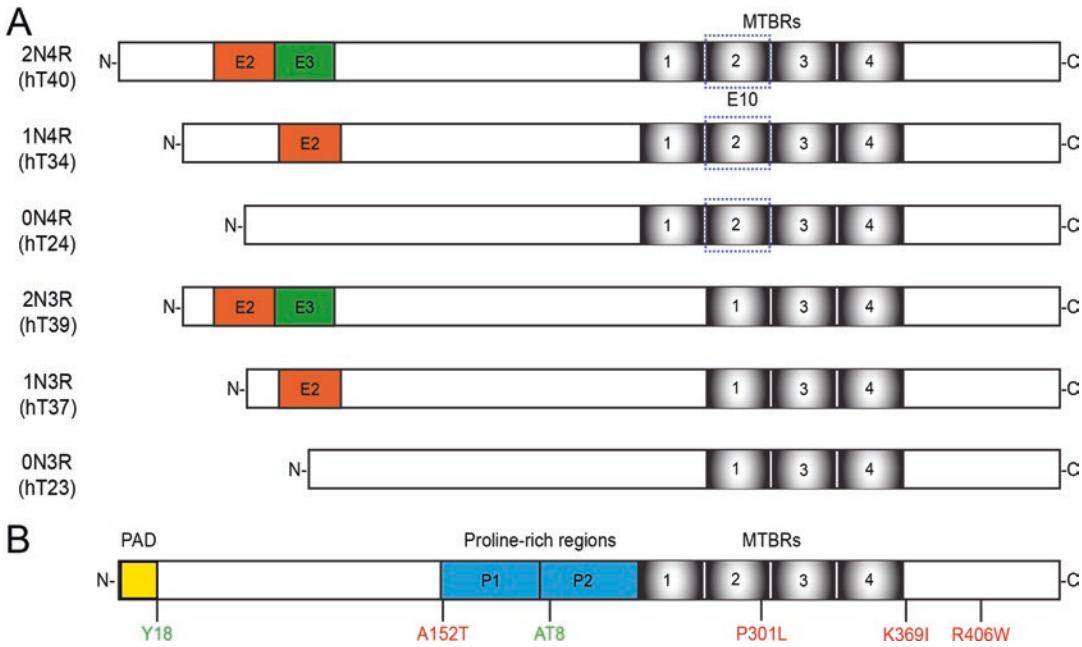

Fig. 7.1 Tau isoforms and selected modifications. (**a**) In the adult human brain tau primarily exists as six isoforms generated through alternative mRNA splicing. The isoforms differ based on the inclusion of exons 2 and 3 in the N-terminus of the protein (2 N, 1 N, or 0 N) and exon 10. Exon 10 contains the second of four potential microtubule-binding repeat regions. Isoforms are referred to as 3R or 4R based on the number of repeat regions they contain. (**b**) Modifications to the protein can affect its function and induce dysfunction in disease. Some selected modifications discussed here include phosphorylation sites (green) at tyrosine 18 and the AT8 sites (serine 202 and threonine 205). FTDP-17 mutations can lead to inherited early-onset frontotemporal dementias (reviewed in [53]). Several of these mutations have been linked to FAT dysfunction including A152T, P301L, K369I, and R406W (red). Functional domains associated with transport include the phosphatase activating domain (yellow), a motif that is conformationally displayed in disease-related forms of tau and linked to changes in signaling pathways that regulate transport. The tau molecule contains many phosphorylation sites throughout the sequence, some of which are found in healthy tissues and others are associated with tau pathology. (Reviewed in [84])

Axonal Transport

Projection neurons affected in tauopathies face a unique challenge in maintaining their health and functional abilities due to their cellular architecture. On one end of a neuron is the somatodendritic compartment, the primary location of protein synthesis and degradation, and on the other end are the synapses where inter-neuronal communication takes place. The transport of material in both directions is therefore vital to the cell and defects in this process contribute to a variety of neurodegenerative disorders (reviewed in [9]).

Transport in the axon occurs along the axonal microtubules [49] while actin microfilaments and neurofilaments have roles that are more structural; although microfilaments are important in short distance movements such as secretion and neurotransmitter release. Two major molecular motor families are responsible for bidirectional transport along microtubules in the axon, kinesins and dyneins (Fig. 7.2). The kinesin superfamily is organized into 14 families, from which 46 kinesins are expressed in the human brain (reviewed in [59]). Conventional kinesin, or kinesin-1, is the most abundant

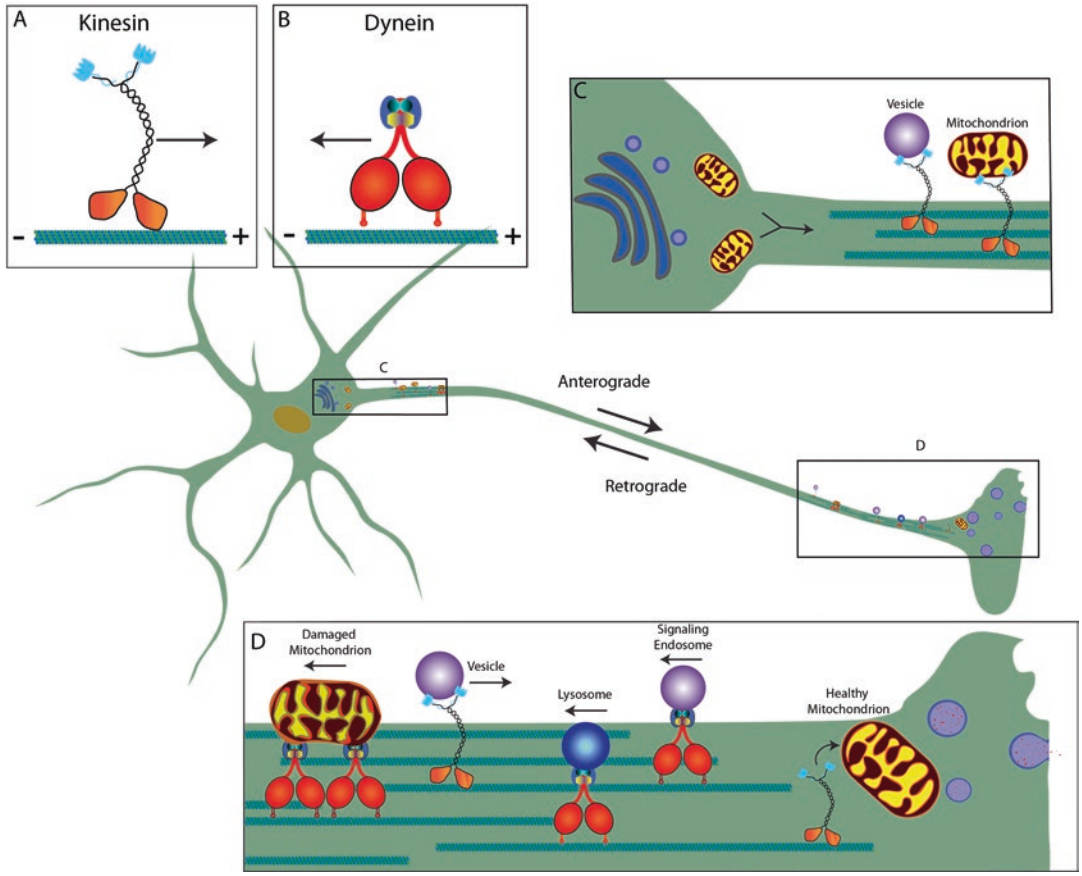

Fig. 7.2 Neurons depend on robust microtubule-based transport in axons. A healthy, functional neuron is dependent on the molecular motor complexes kinesin (**a**) and dynein (**b**), whose roles are to transport material along microtubules in the plus- (anterograde) or minus-end (retrograde) direction, respectively. Materials synthesized in the soma (e.g., cytoskeletal components, mitochondria and membrane-bound organelles) rely on kinesin for their delivery to the correct axonal compartment (**c**). Kinesin-driven anterograde transport is necessary for the delivery of synaptic components, including mitochondria and vesicles, to the axon terminal where they will aid in cell signaling as well as delivery of channels to axon to support propagations of the action potential. Dynein-driven retrograde transport is necessary for the transport of signaling endosomes and material undergoing breakdown and recycling, like damaged mitochondria, multivesicular bodies and lysosomes (**d**) back to the neuronal soma

member of the kinesin superfamily and exists as a heterotetramer composed of two heavy chains (involved in ATP and microtubule binding and movement) and two light chains (involved in cargo binding) that produce plus-end directed movement of cargoes toward the axon terminal [20], a process known as anterograde fast axonal transport (aFAT) (reviewed in [67]). Other members of the kinesin superfamily, kinesin-2, kinesin-3 and kinesin-4 may also be involved in a subset of aFAT [75, 86], but the bulk of trafficking appears to be mediated by conventional kinesin [3, 10, 88]. Cytoplasmic dynein is a large multisubunit (two proteins each of a heavy chain, intermediate chain, light intermediate chain, and three light chains) motor complex responsible for minus-end directed movement, or retrograde FAT (rFAT) (reviewed in [76]). In contrast to the FAT of membrane-bound organelles, other cytoplasmic and cytoskeletal proteins move through slow axonal transport, a process that likely involves kinesin-1 and dynein

but is not fully understood yet ([51, 98] and reviewed in [78]).

Normal FAT is regulated through a complicated system involving the composition of different motor protein complexes and regulatory post-translational modifications of the motor complexes (e.g. phosphorylation) (reviewed in [9, 67]). Several studies have suggested that variation in kinesin-1 motor protein subunits can lead to specificity in the cargoes being transported [20, 90, 92]. Phosphorylation is the best studied post-translational modification in the context of regulating multisubunit motor complexes. Studies in various experimental systems, most notably the isolated squid axoplasm preparation [44, 85], revealed that signaling pathways involving several kinases or phosphatases can tightly regulate FAT by inhibiting binding to microtubules or promoting cargo dissociation [19, 61, 62]. In fact, several regulatory kinase and phosphatase pathways for FAT are disrupted in tauopathies and other neurodegenerative diseases providing another potential link between FAT, loss of axonal connectivity, and neuronal degeneration (reviewed in [42, 65]).

Axonal Degeneration in Tauopathies

There are 27 different tauopathies described to date including Alzheimer's disease (AD), chronic traumatic encephalopathy (CTE), Pick's disease (PiD), progressive supranuclear palsy (PSP), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), and corticobasal degeneration (CBD) (reviewed in [53]). Tauopathies typically display pathological features consistent with a "dying back" pattern of neuronal degeneration. These include dystrophic axons and spheroids, as well as evidence of disrupted FAT (Fig. 7.3) (reviewed in [42, 45]). Accordingly, synaptic dysfunction and loss also occur very early in disease and synaptic loss correlates closely with cognitive deficits in AD and other tauopathies [21]. Studies using post-mortem human tissue samples suggest that tau inclusions appear as neuropil threads or dystrophic neurites early during the progressive accumulation of tau pathology in AD brains [25, 47]. Advanced brain imaging studies confirm degeneration of white matter containing axonal projections during the mild cognitive impairment

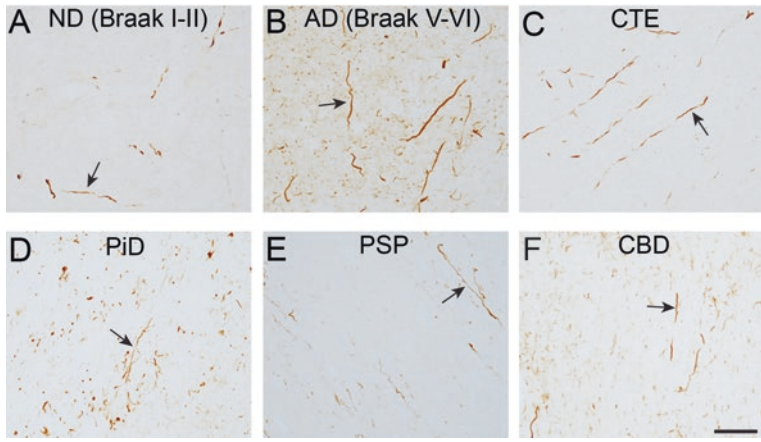

Fig. 7.3 Dystrophic axons containing tau pathology is a prominent feature in multiple tauopathies. The TNT1 antibody detects exposure of the phosphatase activating domain (PAD). (**a**, **b**) TNT1 pathology-containing axons are observed in the subcortical white matter in non-demented aged patients with early stages of tau deposition (**a**; ND (Braak stage I-II)) and robust TNT1 axonal pathology is seen in severe Alzheimer's disease brains (**b**; AD (Braak stage V-VI). C-F) Axonal tau pathology in the subcortical white matter displays PAD exposure (i.e. TNT1 reactivity) in chronic traumatic encephalopathy (**c**; CTE), Pick's disease (**d**; PiD), progressive supranuclear palsy (**e**; PSP) and corticobasal degeneration (**f**; CBD) as well. Scale bar is 50 μm

stage, prior to onset of AD, and continued degeneration in other regions with disease progression. Tau pathology and degeneration are also prominent features of other tauopathies. In PSP, pathological tau is observed in neuropil threads and in subcortical white matter along with dystrophic axons and spheroids indicative of FAT disruptions and degeneration in the same regions [2, 33, 36, 70]. Tau-positive neuropil threads are also observed in CBD and closely associated with white matter degeneration detected by advanced imaging [36, 111]. Pick bodies are the defining feature of PiD but tau pathology can also be detected in axonal terminals along with neuritic threads and axonal spheroids in mossy fibers and cerebellar white matter [74]. Again, this is associated with degeneration of white matter regions that can be quite severe [108]. CTE brains present with neuropil threads and axonal atrophy within subcortical white matter and transported proteins accumulate in axons after traumatic brain injuries [48, 56, 99].

Together, these and other studies demonstrate that the most common tauopathies are characterized by several features that point to a significant role for axonal dysfunction that may be caused by deficits in FAT. Pathological tau modifications and axonal degeneration are closely associated with each other, beginning with the earliest stages of tauopathy pathogenesis. Axonal swellings and accumulation of vesicular organelles also suggest abnormalities in FAT. Collectively, these observations suggest that pathological forms of tau may exert toxic effects through disruption of normal processes in the axon. In fact, as discussed below, evidence from several model systems supports this hypothesis and indicates that regulation of FAT appears to be disturbed by several disease-associated pathological changes to tau.

Tau-Based Effects on Fast Axonal Transport

Tau and Regulatory Signaling Pathways for Fast Axonal Transport

Accumulating evidence clearly implicates a number of kinase and phosphatase signaling pathways in the regulation of motor complex-based transport of organelles along axons. The squid isolated axoplasm model of FAT was instrumental in several significant discoveries of the biology of FAT, including the initial discovery of conventional kinesin, the most abundant member of the kinesin superfamily [11]. In this model, the giant axons are removed and the axoplasm extruded, which allows the study of axon-autonomous molecular events including FAT [44, 85]. Video-enhanced contrast-differential interference contrast microscopy is used to visualize and measure the velocity of membrane-bound organelle cargoes undergoing FAT along microtubules [85]. Over the past several years the squid axoplasm assay also was used to elucidate a number of signaling pathways regulating aFAT and rFAT through different mechanisms. For example, activation of the p38α MAPK pathway results in inhibition of kinesin-based aFAT, while activation of CK2 or JNK3 causes inhibition of both aFAT and rFAT [61, 64, 66, 72]. The squid axoplasm preparation was particularly important in elucidating tau's role in regulating FAT through the PP1-GSK3 signaling pathway. This pathway involves activation of PP1 and subsequent dephosphorylation of Ser9 in GSK3β, an event that results in activation of this kinase (Fig. 7.4) [62]. Active GSK3 regulates aFAT by phosphorylating kinesin light chains and causing release of conventional kinesin holoenzymes from their transported cargo [61, 97]. Importantly, several disease-relevant pathological modifications of tau were shown to disrupt FAT through alterations of this signaling cascade [40, 50].

Initial studies using the squid axoplasm focused on testing effects of tau monomers on FAT, which had been proposed to compete with conventional kinesin for microtubule binding [81]. Introduction of soluble tau monomers at concentrations from 1-25 μM tau had no apparent effect on FAT in either direction, while much lower concentration of a dynein microtubule-binding domain peptide blocked both directions [63]. These studies raised questions about whether tau aggregates would affect FAT. Recombinant wild-type tau aggregates (a mixed population of oligomers and filaments) specifically inhibited aFAT but did not affect rFAT, while monomeric tau had no effect on FAT

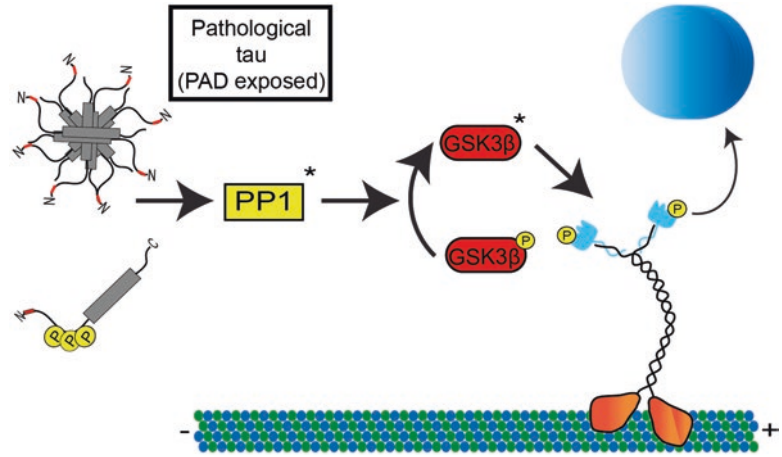

Fig. 7.4 Tau alters kinesin-cargo interactions through modulation of a PP1-GSK3β signaling pathway. Tau contains a phosphatase-activating domain (PAD) within amino acids 2–18 at the extreme N-terminus of tau (shown in red). Under normal conditions this epitope is obscured allowing for normal kinesin-based transport along the microtubules. In disease conditions tau can undergo a variety of modifications including aggregation or specific phosphorylation events that can aberrantly expose the PAD epitope. In the proposed model, these forms of tau can disrupt normal kinesin-based transport by activating protein phosphatase 1 (PP1). This in turn dephosphorylates an inhibitory phosphate on GSK3β in order to activate it. GSK3β then phosphorylates kinesin light chain inducing a release of cargo and disruption of FAT

in either direction [18, 50]. The toxic effect of aggregated tau on aFAT was blocked when tau aggregates were co-perfused into isolated axoplasm with either PP1-specific or GSK3-specific inhibitors demonstrating activation of a PP1-GSK3 pathway as the underlying mechanism [40, 50]. Additional work found that tau-mediated transport toxicity was dependent upon a 17 amino acid motif in the extreme amino terminus of tau (i.e. aa 2–18), which was since termed the phosphatase-activating domain (PAD). Additional experiments showed that the PAD was sufficient to disrupt aFAT through this pathway [40, 50]. Relevant to tauopathies, the PAD epitope was normally sequestered in control tau, but exposed in pathological forms of tau. This was true for both isolated tau and tau aggregates *in vitro* [40] and *in vivo* for all tauopathies examined to date [16, 17, 43]. Collectively, this body of work provided a new framework for understanding tau-mediated toxicity. Specifically, disease-associated tau modifications (e.g. aggregation, specific phosphorylation events, etc.) alter tau structure in a way that leads to conformation-dependent exposure of PAD. Subsequently, aberrant PAD exposure is directly linked to a specific molecular pathway of toxicity (i.e. the PP1-GSK3 pathway) that results in FAT impairment. Tau contains three putative RVxF PP1-interacting domains (including one within PAD) and is able to localize the phosphatase to microtubules, which is consistent with a role for tau in regulating this pathway [24, 52]. The biological activity of the PAD suggests that physiological changes in tau could lead to tightly regulated exposure, which may allow delivery of selected organelles at specific axonal subdomains under normal conditions (reviewed in [67]).

Further studies tested the PAD-dependent mechanism of toxicity using two specific tau modifications that were predicted to facilitate PAD exposure by impairing tau paperclip folding. First, a key set of studies showed that phosphomimicking triple phosphorylation at AT8 sites in tau (i.e. Ser199, Ser202 and Thr205) disrupted the paperclip conformation causing extension of the extreme amino-terminus [39] and subsequently PAD exposure. Consistent with enhanced PAD exposure, when applied to squid axoplasm the phosphomimetic AT8 tau monomers disrupted aFAT [40]. Second, an FTDP-17 mutation was described that causes deletion of exons 6–9 com-

prising the first microtubule-binding region and the proline-rich region, the domain of tau that allows flexibility for the N-terminus to fold onto the C-terminus in the paperclip conformation [77]. The prediction that the monomeric form of this protein would significantly impair aFAT was confirmed in squid axoplasm as well [40]. A role of tau-mediated FAT toxicity in AD was consistent with studies in mammalian neurons, where toxic effects of oligomeric Aβ on FAT were dependent on both tau and GSK3β [102]. Finally, PAD-dependent impairments in synaptic transmission were found in the squid giant synapse [60] and other studies demonstrate oligomeric tau (a multimeric form with increased PAD exposure) is toxic to synaptic function suggesting PAD exposure may disrupt both axonal and synaptic functions in neurons. These studies not only confirmed this novel PAD-mediated mechanism of tau toxicity but also indicated that modifications of monomeric tau can confer toxicity independently of aggregation.

Human tissue studies further confirmed this working model of tau toxicity in multiple tauopathies, demonstrating that tau species shown to impair FAT manifest early during disease progression. For example, the TNT1 and TNT2 antibodies are markers of conformation-dependent PAD exposure (Fig. 7.3) [17, 40]. These antibodies label the earliest forms of tau deposition, pre-tangles, and robustly label neuropil threads early in AD as well as the hallmark pathologies in AD and other tauopathies. Importantly, these antibodies show little to no detection of normal tau in post-mortem human brains or in non-denaturing *in vitro* assays [16–18, 40] (see Fig. 7.3). This pattern of staining is very similar to the robust labeling of pre-tangle inclusions and neuropil threads observed early during disease progression with the AT8 phospho-tau antibody [8, 40] and TOC1, a tau oligomer-specific antibody [18, 71, 104]. The conformation- and disease-specific labeling with these specific tau antibodies suggests that changes in the global conformation of tau leads to exposure of the PAD motif early in disease.

Activation of other pathways by mutant forms of tau have also been proposed. Transgenic mice expressing a K369I FTDP-17 mutation displayed aFAT defects that were proposed to be associated with tau interacting with JIP1 [37]. However, this suggestion was based on overexpression of mutant tau and cannot rule out a role for PAD exposure in the FAT changes. Expression of another FTDP-17 mutant tau, A152T, in *C. elegans* neurons led to abnormal localization of synaptic vesicles that may be due to minor disruptions in aFAT and rFAT independent of any tau aggregation or changes to microtubule binding [14, 73]. These studies do not identify a specific signaling pathway, but the conclusions may be similar to observed impairment of aFAT and rFAT by tau filaments phosphorylated at S422 [93], a disease-specific phosphorylation event that occurs early in pre-tangles and robustly labels neuropil threads in areas of the brain involved in memory and cognition [32, 94]. Such observations suggest that certain modifications in tau may expose PAD and other domain(s) that in turn activate alternative signaling pathways.

Tau May Physically Interfere with Kinesin Binding to Microtubules

Several studies reported that increased levels of tau can alter the behavior of motor proteins, while others have failed to see such effects. Overexpression of fluorescently-tagged tau in primary neurons was reported to inhibit aFAT of amyloid precursor protein (APP) and similar effects on kinesin-based transport of mitochondria were observed in other cell lines [23, 87]. In complementary *in vitro* studies, the presence of tau reduced the processivity of multiple motor proteins but did not affect their overall speed [96]. All of these effects were proposed to occur through tau-based hindrance of kinesin's binding sites [54]. A similar mechanism was proposed based on tau's interference with kinesin activity and dynein reversals on stabilized microtubules [22]. However, other studies have not supported this hypothesis. The kinesin- and dynein-binding sites on microtubules are overlapping, so addition of the dynein microtubule-binding domain effectively blocks both kinesin- and dynein-based motility [63]. In contrast, levels of tau as high as 1

tau per tubulin dimer had no effect on FAT in the squid axoplasm preparation, providing strong evidence against the notion that tau competes with kinesin for microtubule binding [63]. Also, tau binding to microtubules did not alter kinesin speed or run length and only marginally affected microtubule-binding at tau:tubulin concentration ratios much higher than physiological levels [50, 63, 81]. Studies in tau-overexpressing transgenic mice also found that increasing levels of normal tau had no effect on kinesin-based transport *in vivo* [110]. Efforts to explain these discordant results suggested that tau may interact differentially with microtubules under varying conditions. For example, the nucleotide-binding state of microtubules were reported to alter tau effects as tau inhibited kinesin-based transport on GDP-microtubules but not on GTP-microtubules and in an isoform-dependent manner [57]. In this case the shortest 3R tau induced a more severe phenotype than the longest 4R isoform. However, it is difficult to find a physiological role for these effects and the high levels of tau and the distinctive conditions involved are not known to exist in any disease condition. Overall, definitive evidence for direct physical interference of tau with microtubule-based motor proteins *in vivo* is lacking, but more complicated interactions under non-physiological conditions cannot be ruled out.

Tau Isoforms Differentially Affect Fast Axonal Transport

Other studies have also examined isoform-specific effects of tau. Expression of wild-type human 3R tau, but not 4R tau, induced an accumulation of vesicles in the axons of *Drosophila* larva motor neurons [80]. This effect was exacerbated by tau phosphorylation and reversed upon inhibition of GSK3β, potentially implicating it in the mechanistic pathway [68]. In human induced pluripotent stem cell (iPSC) dopaminergic cultures, shRNA-mediated knockdown of only 4R isoforms reduced the average velocity of axonal mitochondria compared to control and total tau knockdown neurons [4]. The shortest 3R tau isoform shortened kinesin run length to a greater degree than the longest 4R isoform in an *in vitro* assay, an effect that was dependent upon the number of motor proteins bound to the beads and was interpreted to be a result of tau reducing kinesin binding to microtubules [100]. Therefore, the local tau isoform composition was suggested to act as a regulatory factor in influencing cargo travel and final destinations within the axon, although a mechanism for creating such differential distributions of tau isoforms in cells remains to be identified.

The functional implications of having different tau isoforms remain unclear and the pathognomonic inclusions of different tauopathies are typically composed of specific isoforms. For example, AD and CTE are a mixture of 4R and 3R inclusions, while PSP and CBD are primarily 4R inclusions and PiD is primarily a 3R inclusion disease [13, 27, 83, 109]. To evaluate the effects of different tau isoforms, preparations of monomeric and aggregated tau were generated for each of the 6 isoforms found in human CNS. Although there were differences in their relative toxicity, aggregated forms of the six tau isoforms similarly impaired aFAT in the squid axoplasm preparation, suggesting the PAD-dependent PP1-GSK3 mechanism of toxicity may be a common element among tauopathies independent of the tau isoforms that comprise the pathology [18]. Further work is required to fully appreciate the normal and pathological functions of each tau isoform.

Other Mechanisms of Fast Axonal Transport Regulation by Tau

Tau may also alter FAT through direct effects on microtubule organization. Tau, in an isoform-specific manner, may act as a microtubule-spacer that simultaneously bundles microtubules and prevents them from overcrowding which could facilitate transport under normal cellular conditions [58]. Microtubule-based effects were also examined by overexpressing or deleting the fly homologue of human tau in *Drosophila melanogaster* neurons [91]. Increasing tau expression levels resulted in increased pause times of vesicles being transported in aFAT and rFAT. This was associated with changes to microtubule den-

sity and axon caliber as well, which may have influenced FAT. Unlike humans, flies do not express redundant MAPs so it is difficult to know how these findings translate to mammalian neurons. Other studies have failed to see a significant effect of tau overexpression on either direction transport in mouse axons [110].

Tau may also mediate effects of other AD-related molecules. In addition to tau pathology, diseases such as AD are characterized by the accumulation of pathological amyloid-β (Aβ) peptides in plaques [106]. Treatment of primary neurons with Aβ oligomers inhibited FAT of mitochondria and TrkA and the effect was dependent upon presence of tau [101]. A cross of $MAPT^{P301L}$ mice, an FTDP-17 tau line, with TgCRND8, a mouse line expressing mutant amyloid precursor protein, resulted in more severe decrease of mitochondrial transport while in the tau line alone aFAT was slightly increased before decreasing with age [1, 26]. Synergistic effects of oligomeric Aβ have been noted in a variety of other studies as well. In particular, GSK3 phosphorylation of kinesin light chains requires a priming phosphorylation event [61], which is efficiently provided via activation of CK2 by oligomeric Aβ [72]. The result of activating both pathways at the same time would exacerbate inhibition of FAT in AD (reviewed in [9]).

Reinforcing the notion of tau playing a role in FAT regulation, a post-translational modification within the PAD was shown to modulate tau's effects. Specifically, phosphorylation at Tyr18 rescued PAD-mediated aFAT impairment in the isolated squid axoplasm model [41]. Further, phospho-Tyr18 co-localized with TNT1 immunoreactive tau inclusions in human disease [41]. Thus, Tyr18 phosphorylation may represent a normal regulatory mechanism that attenuates tau's ability to cause dissociation of kinesin from cargoes for delivery, and in pathological contexts may be neuroprotective and block the aberrant activation the PP1-GSK3 pathway caused by PAD-exposed tau species. Accordingly, surviving neurons at late AD stages display strong immunoreactivity when using an anti-phospho-Tyr18 antibody [41]. Phosphorylation at this site may affect other mechanisms of tau-mediated transport regulation. For example, pseudophosphorylation at Tyr18 rescued inhibition of kinesin-1 motility induced by the shortest 3R isoform [89].

Conclusion

Based upon the evidence presented in this chapter, pathological forms of the tau protein found in AD and other tauopathies may induce toxicity by disrupting FAT. Tau modifications, such as conformational changes and increased phosphorylation, are detected within axons very early in the course of disease progression. In addition, the initial signs of neurodegeneration typically occur with synaptic dysfunction followed by a "dying back" degeneration of axons. Given that pathological tau and neurodegeneration first occur in the axon, it is natural to ask if the protein may exert toxic effects through disruption of a critical cellular process like FAT. In fact, many pathological forms of tau were found to disrupt FAT across multiple model systems ranging from *in vitro* biochemical systems to squid axoplasm and mouse models. Several potential mechanisms of tau toxicity, not mutually exclusive, are proposed and supported experimentally. Given the discovery of biological activity for the PAD, it is reasonable to speculate that tau may help regulate FAT under normal conditions and exert toxicity in disease through a hypermorphic gain-of-function mechanism. Tau's normal function may take the form of modulating signaling pathways that regulate FAT by modulating kinesin-cargo interactions and/or kinesin-microtubule interactions. Further studies will provide a better understanding of tau's normal and pathological roles, potentially providing a specific mechanistic framework for the development of effective therapeutic strategies to treat tauopathies.

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8 Presynaptic Pathophysiology Encoded in Different Domains of Tau – Hyper-Versus Hypoexcitability?

Jochen Martin Decker and Eva-Maria Mandelkow

Introduction

Hyperphosphorylated and aggregated forms of Tau are indicators of progressive neurodegeneration in AD and several tauopathies (Braak stages [1]), but certain forms of Tau can enhance toxicity (e.g. FTDP-17 mutations [2]) and have therefore been used to generate animal models of Tau pathology [3, 4]. Various mechanisms of Tau-induced toxicity have been proposed, operating either in the cytosol (e.g. effects on MT-dependent transport [5], protein degradation [6]), in the nucleus (interferences with DNA, RNA, or nuclear pores, [7, 8]) or at synapses [12, 13]. Loss of hippocampal synapses is an early hallmark of memory loss in AD, and therefore much attention has been paid to possible roles of Tau in pre- and postsynaptic compartments [9–11]. Our recent focus is on electrophysiological effects of two mutations that lie in distinct domains of Tau and have distinct effects on Tau aggregation, strong (ΔK280) or weak (A152T). We find that both affect mainly presynaptic sites, but unexpectedly the effects on physiological parameters are opposite [12, 13].

Results

The repeat domain (RD, repeats 1–4, Fig. 8.1) controls both the microtubule-binding and the aggregation of Tau. This is largely due to two hexapeptide motifs at the beginning of repeats R2 and R3 [33]. The deletion mutation ΔK280 (related to frontotemporal dementia and AD, [2]) is one of several mutations that render Tau more pro-aggregant by enhancing its propensity for β-structure, thus promoting Tau's toxic effects. Conversely, insertions of β-breaking prolines prevent β-structure and thus prevent aggregation and toxicity in transgenic animals [14]. This argues for a close correlation between Tau toxicity and neurodegeneration. By contrast, the mutation A152T (a risk factor for FTD-spectrum disorders, e.g. PSP [15]) is located in the N-terminal "projection" domain which does not bind to microtubules, at the beginning of the "proline-rich" domain. This region interacts with signalling molecules containing SH3 domains (e.g. tyrosine kinase fyn [16]) but is not directly involved in aggregation (Fig. 8.1b). Both mutations cause severe changes of synaptic structures and function in the hippocampus, the "gate to memory". But remarkably the effects of both mutations on synapses point in opposite directions.

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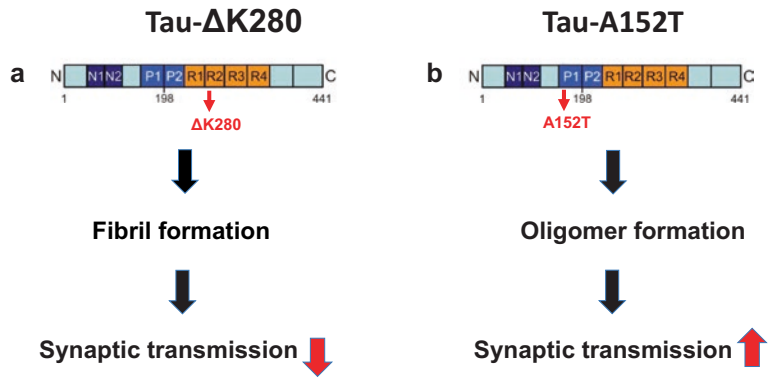

Fig. 8.1 Bar diagrams of human Tau (2N4R), showing domain structure, locations of Tau mutations, and effects on synapses. (**a**) Full length human Tau (441 residues, Uniprot 10,636–8, Tau-F) representing the 2N4R isoform of the protein. The N-terminal projection domain points away from microtubules, the C-terminal microtubule assembly domain is responsible for microtubule binding. The assembly domain contains the repeat domain (R1-R4) and is flanked by proline-rich domains. The repeat domain is responsible for the pathological aggregation of Tau. The mutation ΔK280 is located at the beginning of R2 and leads to pronounced aggregation of Tau. This pathological process causes a reduced basal synaptic transmission in the hippocampus. (**b**) In contrast the mutation A152T is located in the first proline rich domain (P1) towards the N-terminus. This mutation leads to an increased oligomer formation thereby enhancing basal synaptic transmission

The ΔK280 mutation leads to a decrease in presynaptic bouton density in the mossy fiber pathway, the synaptic connection of granule cells from the dentate gyrus to pyramidal cells in area CA3 of the hippocampus [12]. Moreover these presynaptic boutons contain less synaptic vesicles after expressing TauRD$^{\Delta K280}$, and their calcium dynamics is impaired as well. This severe presynaptic phenotype is accompanied by a decrease of dendritic spines at the postsynaptic side [12].

The pathological phenotype shows up as a reduced basal synaptic transmission (Fig. 8.1a) and impaired synaptic plasticity in transgenic mice overexpressing TauRD$^{\Delta K280}$. This pathophysiological picture is similar in mice overexpressing full-length Tau with the ΔK280 mutation, Tau$^{\Delta K280}$ [17].

In contrast the A152T mutation leads to an increase in basal synaptic transmission (Fig. 8.1b) but has no effect on synaptic plasticity [13]. In this model the expression of full-length Tau^{A152T} causes an increase in extracellular glutamate, NR2B-dependent increase in intracellular calcium and finally to neuronal death by excitotoxicity [13].

The pronounced reduction of basal synaptic transmission in mice overexpressing TauRD$^{\Delta K280}$ may point to a general network hypoexcitability, whereas the mutation A152T causes network hyperexcitability and excitotoxicity as demonstrated both *in vitro* and *in vivo* [13, 18]. The fact that Tau knockout mice show a similar impairment of basal synaptic transmission as mice expressing TauRD$^{\Delta K280}$ [12] suggests that Tau may be an important regulator of basal synaptic transmisson in the mossy fiber pathway in a normal physiological environment.

It remains to be elucidated why a mutation located in the N-terminal proline-rich domain has an opposite effect than the one in the repeat domain. It is likely that the mutations have different effects on synapses. In case of the ΔK280 mutation there is reduced release of synaptic vesicles and a gradual loss of hippocampal spines and neurons, whereas in the case of the A152T mutation there is an increase of glutamate release. This is consistent with the observed hyperexcitability and mossy fiber sprouting in this region [13]. In older animals (20 month) expressing A152T the number of dendritic spines is finally

decreased in area CA1 and CA3 when increased cell death becomes dominant [19].

An underlying mechanism of Tau's effect on network activity may be calcium dynamics. In area CA3, where mossy fiber synapses are located, we observed impaired calcium dynamics after high KCl stimulation, using calcium imaging in organotypic hippocampal slices (Fig. 8.2a, b). Slices expressing the ΔK280 mutation showed less calcium influx after depolarization (Fig. 8.2a, c). By contrast, slices expressing the A152T mutation had a pronounced increase in calcium levels after KCl depolarization (Fig. 8.2b, d). The hyperexcitable phenotype (A152T mutation) can

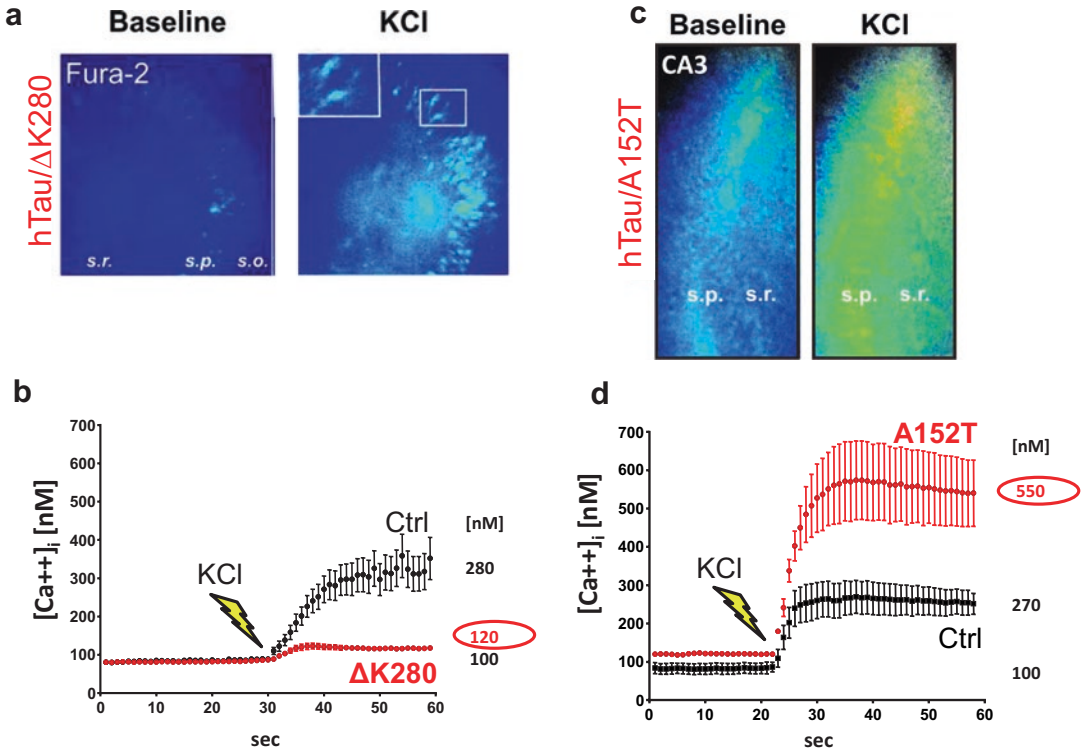

Fig. 8.2 Hypo- and hyperactivity of intracellular calcium dynamics after chemical depolarisation (KCl) due to the overexpression of human TauΔK280 and human TauA152T as observed in organotypic hippocampal slices of transgenic mice. (**a**) Microphotograph showing area CA3 of organotypic hippocampal slices expressing TauΔK280 loaded with Fura2-AM. Ratiometric color-coding shows intracellular low calcium concentrations in dark blue, blue and green regions and high calcium concentrations in yellow, red and white regions. In imaging experiments slices are recorded in unstimulated, basal conditions for 30 seconds. Subsequently a high potassium chloride (KCl) stimulation is applied and membrane depolarization induced. Calcium rises rapidly, reflecting neuronal activity. (**b**) Diagram showing the quantification of experiments described in (a). X-axis denotes time in seconds, the Y-axis shows absolute intracellular calcium concentrations (nM). At baseline conditions the control slices and hTauΔK280 overexpressing slices have similar intracellular calcium concentrations of ~80 nM. After chemical stimulation (KCl) intracellular calcium concentration rises up to 280 nM in control slices whereas slices overexpressing TauΔK280 reach only ~120 nM. (**c**) Microphotograph showing area CA3 of organotypic hippocampal slices expressing Tau-A152T loaded with Fura2-AM. Note that at baseline level neurons have higher calcium concentrations than control slices in (a). After KCl stimulation calcium concentrations rise to high levels (red and green). (**d**) Diagram showing intracellular calcium concentrations over time. Before stimulation calcium concentrations range around 90 nM in control slices. In slices expressing Tau-A152T intracellular calcium is already increased at baseline conditions. However after KCl stimulation calcium rises up to 550 nM

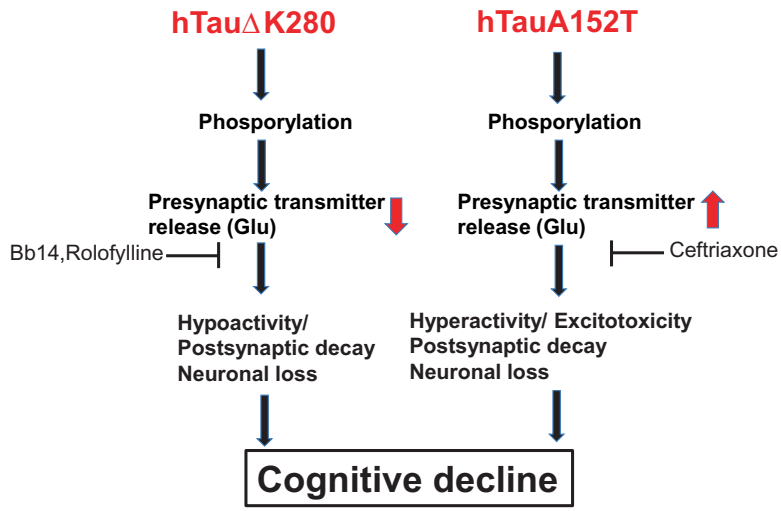

Fig. 8.3 Flow chart of events leading to cognitive decline due to the overexpression of Tau with the mutations ΔK280 or A152T. Initially the overexpression of hTauΔK280 and hTauA152T causes pathological phosphorylation. Thereafter presynaptic release is affected. Presynaptic release can be decreased due to structural and functional impairment of presynapses by hTauΔK280. By contrast, hTauA152T expression enhances presynaptic release leading to glutamate overspill and excitotoxicity. Both mutations finally decrease postsynaptic structures leading to neuronal loss and cognitive decline. These scenarios can be prevented in the case of the ΔK280 mutation by application of aggregation inhibitor (bb14) or by the adenosine A1 receptor antagonist (rolofylline). In the case of the A152T mutation the application of ceftriaxone, memantine, or ifenprodil can prevent excitotoxicity and neuronal loss

be reversed by application of the drug ceftriaxone, which causes an increased glutamate uptake by astrocytes via the glutamate transporter EAAT1/2 (Fig. 8.3). The hypoactivity phenotype (ΔK280 mutation) was ameliorated by the Tau aggregation inhibitor bb14 [20] and by rolofylline, an antagonist of the adenosine A1 receptor (Fig. 8.3 [21]).

Discussion

Much work has been done in recent years to understand Tau's impact on hippocampal neurophysiology [22–24]. The focus was on postsynaptic structures and dysfunction caused by pathological Tau [10, 11]. More recently some studies focussed on the evaluation of pathophysiological features at presynapses during the progression of tauopathies [9, 12, 25]. Some mechanisms of presynaptic pathology affected neurotransmitter release [26], arguing that pathological Tau can change the tethering of synaptic vesicles to actin filaments via synaptogyrin-3, thereby slowing down presynaptic transmitter release [27]. By analogy, other Tau mutations like A152T could increase presynaptic transmitter release and thus network excitability. In both cases, a presynaptic origin of early pathology would be consistent with the axonal localization of Tau [28].

The ΔK280 mutation of Tau has a direct impact on structure and function of presynapses [12], which would be consistent with the effect of this variant on Tau conformation and aggregation. In the case of the A152T mutation an effect on structure cannot be readily explained, but the proximity to the proline-rich domain suggests an influence on signalling pathways. An increase of presynaptic vesicle release (and thus of glutamate) was shown via tetanus toxin application [13] and may well be an initial mechanism of

A152T toxicity (Fig. 8.3). A direct comparison of presynaptic effects of different Tau mutations remains to be studied in the future.

Finally it is important to consider Tau's impact on functional and structural plasticity in the brain. For instance, Tau's regulatory role in hibernating animals is well described [29]. In this case Tau adopts "Alzheimer-like" features (hyperphosphorylation, somatodendritic mislocalization) which have transient physiological roles. Tau-dependent modulation of synaptic plasticity has proven to be important in the regulation of hippocampal LTP/LTD, as demonstrated by mouse models overexpressing human Tau [17, 30] or in Tau knockout models [31]. Indeed Tau was also found to impact Delta/theta network oscillations important for cognition [32]. Changes of intrinsic neuronal excitability [22], pre- and postsynaptic dysfunction and deficits in calcium dynamics/signaling due to the expression of pathological Tau may underlie its role as a modifier of network excitability (Fig. 8.4).

The A152T mutation in Tau may trigger pathophysiological events at an early stage where neuronal malfunction is triggered by oligomer formation and network hyperexcitability/hyperactivity. By contrast, the aggregation-prone ΔK280 mutation may reflect a later stage of tauopathy where aggregated, fibrillary Tau causes network hypoexcitability/hypoactivity. This "later stage" of tauopathy may reflect a kind of "protection mechanism" in order to "calm down" increased network activity which may be triggered by different pathological conditions like Aß, ROS and others.

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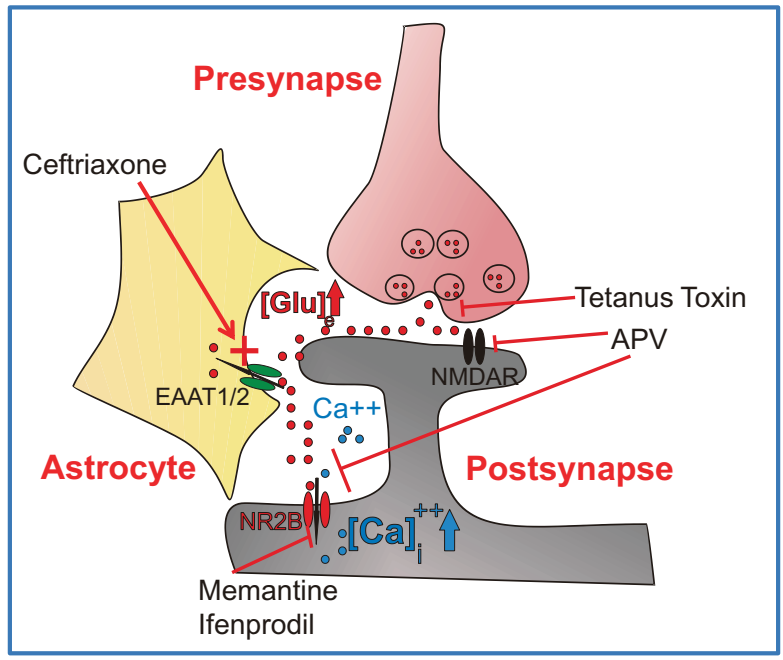

Fig. 8.4 Model of synaptic pathophysiology induced by TauA152T at the tripartite synapse. Expression of Tau-A152T in the presynapse leads to enhanced presynaptic release of glutamate which causes excitotoxicity which is mediated by excess Ca++ influx through extrasynaptic NR2B-containing NMDA receptors. The impairment can be reduced by low concentrations of memantine or ifenprodile (inhibition of NR2B) or by ceftriaxone (upregulation of astrocytic glutamate transporters (EAAT1/2))

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Synaptic Localisation of Tau

9

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Introduction

Chemical synapses are highly specialised structures in which neuronal activity at presynaptic boutons results in the release of neurotransmitters from synaptic vesicles (Fig. 9.1). Neurotransmitter release is stimulated by an increase in intracellular calcium ions caused by depolarisation of presynaptic voltage-gated calcium channels, leading to fusion of synaptic vesicles with the presynaptic plasma membrane [1]. Release of neurotransmitters from synaptic vesicles into the synaptic cleft activates postsynaptic receptors, resulting in the transduction of either excitatory (glutamate) or inhibitory (gamma-aminobutyric acid/glycine) signalling. The postsynaptic density (PSD), an electron-dense region in the receiving neuron, is a specialised scaffolding structure that anchors primarily excitatory, ionotropic and metabotropic glutamate receptors to the postsynaptic membrane of dendritic spines, forming a signalling complex [2]. Ionotropic glutamate receptors are activated by α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), N-methyl-D-aspartate (NMDA), or kainate, with the specific receptor subunit type being both brain region and cell-type specific [3].

Synaptic plasticity enables a rapid response to electrical stimulation resulting in changes in the morphology of synapses. Synaptic plasticity and the formation of dendritic spines are considered the structural correlates of learning and memory [4]. Long-term potentiation (LTP) is induced by recurring synaptic activity that activates NMDA receptors, resulting in increased synaptic strength. In contrast, long-term depression (LTD), whilst also activity-dependent, weakens synaptic activity through phosphorylation of AMPA receptors which can affect their density at the postsynaptic membrane [5]. Thus, functioning synaptic connections are required for the regulated transmission of chemical information between neurons and dysfunctional signalling can lead to deficits in synaptic function and ultimately loss of connectivity between neurons and neurodegeneration.

Synaptic loss is the earliest indication of neuronal malfunction and the best biological correlate of disease progression in Alzheimer's disease and related tauopathies [6–8]. Tau has long been regarded as an axonal microtubule-associated protein [9, 10], but recently it has become apparent that tau is also associated with other neuronal cellular compartments, including the plasma membrane and the synapse [11, 12]. Notably, overexpression of tau in transgenic mice leads to loss of synapses [13], and co-expression of tau

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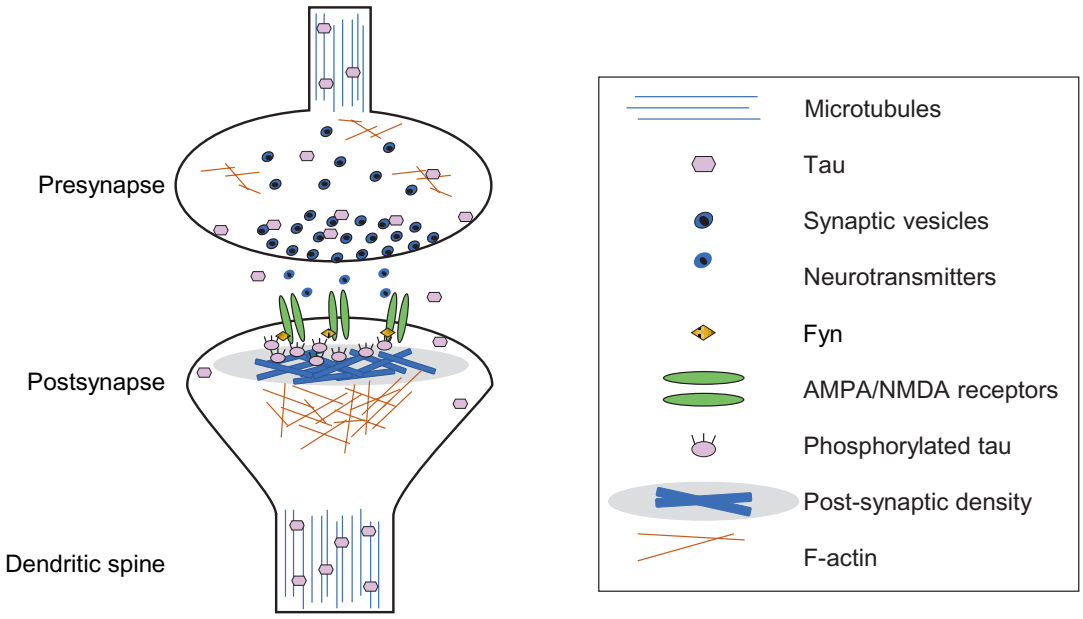

Fig. 9.1 Structural organisation and tau localisation in excitatory synapses. Neuronal activity at the presynaptic terminal results in the calcium-mediated fusion of synaptic vesicles with the presynaptic membrane, releasing neurotransmitters into the synaptic cleft. Interaction of the neurotransmitters with postsynaptic receptors causes membrane depolarisation, increasing the likelihood of action potential propagation in the postsynaptic neuron. Tau binds to microtubules in axons and to synaptic vesicles in the presynaptic compartment. Tau is also present extracellularly, as well as at the postsynaptic terminal and in dendritic spines. Accumulation of phosphorylated and/or acetylated tau, including that associated with fyn, in the postsynapse occurs in the tauopathies leading to detrimental effects on synaptic function

with amyloid beta peptide (Aβ) suppresses neuronal activity [14]. It is important therefore, to gain a better understanding of synaptic dysfunction and neuronal loss in the tauopathies since this will aid elucidation of the molecular mechanisms that lead to tau-induced cognitive dysfunction and will inform the development of strategies to protect synapses.

Synaptic Localisation of Tau

In the axon, tau functions as a cytoskeletal protein with roles in microtubule stabilisation and axonal transport [10], whereas synaptic tau appears to be involved in neuronal signalling and synaptic plasticity [15, 16]. The presence of tau in synapses under physiological conditions has been somewhat controversial, with some studies detecting presynaptic tau in human and animal brain tissue [17] and others failing to detect any overlap of labelling of the dendritic proteins, drebrin and microtubule-associated protein 2, with endogenous mouse tau using highly specific tau antibodies [18]. However, since reducing tau expression results in loss of synapses [16], this suggests a critical role for tau in maintaining synaptic integrity. Under pathological conditions, such as in human tauopathy and when tau is overexpressed in animal models of disease, the presence of tau at synapses is more apparent, suggesting a role for tau in disease pathogenesis [12, 15, 18–22]. Synaptic tau has been demonstrated in cell and animal models that overexpress tauopathy-associated tau mutations, including in cultured hippocampal neurons [19], in rodents, and in *Drosophila* [17, 23, 24]. Importantly, tau spread from presynaptic to postsynaptic sites precedes synapse and neuronal loss in transgenic mice expressing mutant human tau [25]. Mislocalisation of tau to dendrites is a neuropathological feature of Alzheimer's disease brain

that occurs early in disease pathogenesis, possibly even pre-clinically and prior to tau aggregation [26, 27]. Both presynaptic and postsynaptic compartments have been identified as sites of tau accumulation in Alzheimer's disease [20, 28–30] and the appearance of synaptic tau correlates with the onset of cognitive decline [31, 32].

Human brain preparations enriched in synaptic vesicles from control and Alzheimer's disease contain tau, with disease-associated phosphorylated tau detectable only in Alzheimer brain [33]; the abundance of phosphorylated tau oligomers at synapses is particularly associated with dementia [34]. Accumulation of phosphorylated tau results in neuronal injury that is mediated by damage to synapses, which increases with disease progression in the tauopathies [30, 35]. Whilst the molecular mechanisms underlying synaptic loss are incompletely understood, there are several potential routes through which pathological forms of tau might prove to be toxic to neurons either directly or indirectly, and at both presynaptic and postsynaptic sites.

Tau Binds to Synaptic Vesicles

The observation that axonal dystrophy occurs in advance of the appearance of both neurofibrillary tangles and amyloid plaques has led to the proposal that loss of presynaptic terminals leads to a retrograde "dying back" of neurons in Alzheimer's disease [32, 36]. The presence of soluble (monomeric or oligomeric), pathological tau species in the presynaptic compartment has been linked to disease pathogenesis in Alzheimer's disease [35] and to synaptic vesicle release deficits in fly and rat neurons [24]. Several studies have demonstrated the association of tau with presynaptic proteins, including synaptophysin, synapsin 1, synaptotagmin, synaptogyrin-3, syntaxin-1B, α-synuclein, and β-synuclein, all of which co-immunoprecipitate with tau [37]. More recently, the synaptic vesicle transmembrane protein synaptogyrin-3 has been identified as a key tau interactor and a regulator of tau-induced synaptic dysfunction [33].

Tau clusters synaptic vesicles by interacting with both F-actin and synaptogyrin-3, which binds directly to the amino terminal region of tau, reducing the efficiency of synaptic vesicle release [24, 33]. Notably, synaptogyrin-3 preferentially binds 0N tau isoforms rather than 1N or 2N-containing tau isoforms, which is compatible with the identification of residues 1–111 (numbering of the 0N4R human tau isoform used in that study), which is equivalent to residues 1–44 and 103–169 in the longest CNS isoform of human tau of (2N4R tau, 441 amino acids) [24, 37]. The tau-induced clustering of synaptic vesicles at the presynaptic terminal, mediated by synaptogyrin-3, impacts on neurotransmitter release [33]. These data suggest that the amino terminal region of tau has an important influence on neuronal function in the presynaptic compartment by reducing the mobility of synaptic vesicles and release of neurotransmitters, resulting in synaptic toxicity.

Post-Translational Modifications of Tau Affect Synaptic Function

Tau is subject to a wide variety of post-translational modifications, several of which are altered in the tauopathies, and these modifications can have deleterious effects on the neuronal localisation and function of tau [38]. For example, tau becomes increasingly phosphorylated during the development and progression of tauopathy. Indeed, one of the key neuropathological features of the tauopathies is the development of neurofibrillary tau inclusions comprised of highly phosphorylated tau fibrils, the appearance of which likely leads to neuronal dysfunction and loss of cognitive ability as disease progresses [38].

Although tau phosphorylation is a physiological process, aberrantly increased tau phosphorylation may be a forerunner of tau-induced neurofibrillary degeneration. Changes in tau phosphorylation have significant impacts on tau function, including microtubule binding and axonal transport, as well as bundling presynaptic actin filaments, which is reduced by increased tau

phosphorylation [39]. Phosphorylation-mimicking tau mutants expressed in cultured neurons reduce the number of AMPA receptors at synapses [19]. Furthermore, increased tau phosphorylation results in tau mislocalisation to postsynaptic sites in Alzheimer's disease brain and in transgenic mice overexpressing P301S tau, a mutation that causes frontotemporal dementia [21, 30]. A potential mechanism that may be responsible for the observed increase in phosphorylated tau in the tauopathies comes from studies of the association of tau with the tyrosine kinase fyn [15, 40–43]. Src homology-3 (SH3) domains in fyn bind to Pro-X-X-Pro motifs in the proline-rich region of tau [41–43] thereby directing tau to the postsynaptic density [15, 44]. Importantly, the interaction of fyn and tau, and hence tau localisation is regulated by tau phosphorylation [41, 42, 45–47], suggesting that this may be a component of the molecular mechanism leading to increased postsynaptic localisation of phosphorylated tau. Dendritic tau likely mediates the toxicity of Aβ, the major component of amyloid plaques in Alzheimer's disease, since neurons cultured from tau knockout mice are resistant to Aβ toxicity [42, 44]. Importantly, translocation of tau to the postsynaptic density of excitatory synapses is driven by Aβ, which could be a key event inducing synaptotoxicity in dementia [48].

Acetylation of tau also blocks activity-induced actin polymerisation, through a mechanism involving the postsynaptic memory-associated KIdney/BRAin protein (KIBRA), which is encoded by the WWC1 gene [49]. Notably genetic variation in WWC1 is associated with the development of late-onset dementia [50] and KIBRA protein is reduced in Alzheimer's disease brain, whereas tau acetylation is increased [51]. The finding that increased tau acetylation reduces KIBRA and impairs LTP by impeding actin polymerisation and thereby affecting postsynaptic membrane localisation of AMPA receptors, adds to the evidence implicating the importance of tau in regulating synaptic plasticity [49].

Structural Consequences of Aggregated Tau

Oligomeric and fibrillar tau but not monomeric tau, have detrimental effects at synapses because these tau species have been shown to be capable of diffusing laterally through membranes and forming clusters that are proposed to selectively increase calcium-impermeable GluA2 AMPA receptors and to reduce sodium-potassium ATPase [52]. Notably, this clustering of tau is enhanced by the presynaptic protein α-synuclein, which is frequently found in association with tau deposits in the tauopathies [53]. However, others have shown a reduction in GluR1 AMPA receptors in neurons cultured from P301L tau transgenic mice [19, 54] and directly in P301L tau mouse brain [55]. Moreover, soluble tau can induce synaptic loss in mouse brain [56], suggesting that multiple mechanisms may be involved in tau-induced disruption of synapses. It is plausible therefore, that exposure to tau induces mild structural changes at the synapse and the accumulation of tau gradually progresses to result in significant functional abnormalities at the synapse [55, 57].

Oligomeric and fibrillar forms of tau can also be generated by proteolytic cleavage, and a large number of tau fragments have been identified in tauopathy brain [10]. Reduced clustering of AMPA receptors has also been noted in mice expressing a mutant form of tau (tauΔ314), that corresponds to caspase-cleaved tau [58]. Thus, deposition and mislocalisation of tau at postsynaptic sites could damage neurons by interfering with their ability to regulate synaptic membrane depolarisation and by interfering with the membrane organisation and function of AMPA receptors, resulting in cognitive impairment in the tauopathies.

Functional Impacts of Synaptic Localisation of Tau

Impact of Tau on Neuronal Activity and Neuronal Circuitry

Tau appears to have a role in modulating synaptic activity since exposure of neurons to tau inhibits hippocampal LTP in rat hippocampal synapses [59]. Similarly, inhibition of LTP by extracts of Alzheimer's disease brain could be prevented by co-injection with an antibody to tau, indicating the tau dependence of LTP inhibition in this model [59]. However, tau has also been reported to suppress cortical activity in P301L tau mice [14] and when P301L tau is targeted to the entorhinal cortex, one of the earliest sites of tau pathology in Alzheimer's disease [60], indicating a negative impact of tau on neuronal connectivity.

There is increasing evidence of a close relationship between tau and epileptic activity, with an increased prevalence of seizures in Alzheimer's disease [61]. Several tau mutations that increase the risk of developing frontotemporal dementia are associated with seizures and abnormal network activity, some of which can be ameliorated with anti-epileptic drugs, such as levetiracetam [62]. Seizures are also linked to cognitive impairment and tau pathology in temporal lobe epilepsy [63], suggesting the possibility of some commonalities in the mechanisms underlying epilepsy and dementia, that could be related to tau deposition.

Impact on Neuronal Tau Release

Increased neuronal activity induces tau secretion from neurons [64, 65] and therefore, epileptic activity could enhance the spread of tau in the tauopathies [66]. The precise region of the neuron from which tau is released has not yet been established but one possibility is that tau could dock at or near the synaptic membrane prior to release. This view is supported by the finding of equivalent amounts of tau in the soluble and detergent-soluble fractions of synaptic preparations from human brain that release tau in response to potassium-induced depolarisation [20]. Notably, in a mouse model of Alzheimer's disease (3xTg-AD mice) in which exogenous tau is both mutated and highly phosphorylated, potassium chloride-induced depolarisation was unable to induce tau release from organotypic brain slices [67]. These findings suggest that tau mutations and/or increased phosphorylation as found in human tauopathy brain, could potentially obstruct the elevated release of tau in response to neuronal activity.

Conclusions

Tau is present in multiple intracellular domains within neurons, including being associated with the microtubule cytoskeleton and plasma membrane, as well as being present in a cytosolic pool and as a secreted protein. These findings have led to increased scrutiny of the role of tau in these differing locations, including at synapses, where tau may either directly or indirectly affect the response of neurons to changes in neuronal activity (Fig. 9.1). During the development of tauopathy, phosphorylated tau accumulates in postsynaptic regions, although it is not yet clear whether this is a cause or consequence of disease, or precisely how this mis-localisation might damage neurons, leading to loss of synaptic dysfunction and neuronal connectivity. Synaptic tau interacts with both presynaptic and postsynaptic components and the strength of these associations may be modulated by disease-relevant post-translational modifications of tau. Thus, increased tau phosphorylation, acetylation and/or the occurrence of pathogenic mutations in tau, can all lead to its mislocalisation at both presynaptic and postsynaptic sites, potentially contributing to disease progression in the tauopathies. Understanding the role of tau at the synapse and the impact on neuronal viability and function may provide new avenues for therapeutic intervention in the tauopathies.

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The Role of Tau in the Post-synapse

10

Philip Regan and Kwangwook Cho

Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's disease
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
GSK-3	Glycogen synthase kinase-3
LTD	Long-term depression
LTP	Long-term potentiation
NMDAR	N-methyl-D-aspartate receptor
pTau	Phosphorylated Tau
Tau	Microtubule associated protein tau

Introduction

Tau mostly localises in the axon and regulates axonal transport of cargo along microtubules. Aberrant hyperphosphorylation of tau causes detachment of tau from microtubules and precipitates neurofibrillary tangles (NFTs), a pathological hallmark of Alzheimer's disease (AD) consisting of insoluble and aggregated protein tau [1]. To date, the majority of tau biology research has focused on the processes of tau aggregation and its subsequent toxicity in neurons. Recently, the post-synaptic localisation of tau has been documented, suggesting that tau has physiological functions not only in the axon but as a synaptic protein [2–4]. Synaptic tau, however, remains a debatable subject and its precise synaptic function is currently uncertain.

Studies of tau knockout mice have provided significant but inconclusive input into our understanding of the synaptic role of tau. Several physiologically 'normal' phenotypes of tau knockout mice have been reported, including normal cognitive function in spatial learning tasks (see review, Ke et al. [5]). In contrast, some studies have described tau knockout mice with aging-dependent memory deficits [6], impaired contextual fear conditioning, yet enhanced spatial learning [7] and deficits in spatial reversal learning [4]. Mixed effects of tau deletion on synaptic plasticity are also evident, with reports of impairments to long-term potentiation (LTP) of synaptic transmission [6, 7] that are not evident in another study, in which specific impairments to long-term depression (LTD) are observed [3]. To add to this confusing picture, tau deletion alters dendritic architecture and spine density in some studies [8, 9] but not others [10, 11], the latter being more consistent with reports of unaltered

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basal synapse function following tau deletion [3, 6, 7, 12]. What is clear from the sum of these studies is that tau must play a complex role at the synapse, differentially affecting specific forms of synaptic plasticity that may support specific cognitive functions.

To delineate the role of tau in synaptic plasticity, Kimura et al. [3] extended their *in vivo* findings to an *in vitro* model in which confounding compensatory and pre-synaptic variables were reduced. Corroborating their *in vivo* data, tau shRNA transfection prevented the induction of LTD, while LTP was unperturbed. Replacement of endogenous tau with human tau rescued the LTD phenotype, supporting a necessary role for tau in the hippocampal expression of an NMDAR-dependent form of LTD. Several studies have now linked spatial reversal learning, a form of behavioural flexibility, to the molecular mechanisms of hippocampal NMDAR-LTD [13–15]. The requirement for tau in hippocampal LTD is therefore quite striking in the context of the selective deficits in spatial reversal learning that have been observed in tau knockout mice [4].

These findings, together with the alterations to synapse function that are consistently observed in experimental models of tauopathies [11, 16, 17] and the requirement for tau during BDNF-driven synaptic plasticity [8], clearly highlight the numerous pathophysiological implications for tau's function at the synapse. Uncovering the precise nature of this function is a necessary milestone for tau biology research.

Localization of Tau: Axon vs. Dendrite

Tau is generally considered an axonally segregated protein [18]. Indeed, some recent research agrees with this notion [19], supporting early findings that tau is only present in dendrites and synapses either during neuronal development or under pathological conditions [20, 21]. Nevertheless, overwhelming evidence that tau exists in dendrites and pre- and post-synaptic structures of healthy mature neurons must now also be considered [3, 4] (Fig. 10.1).

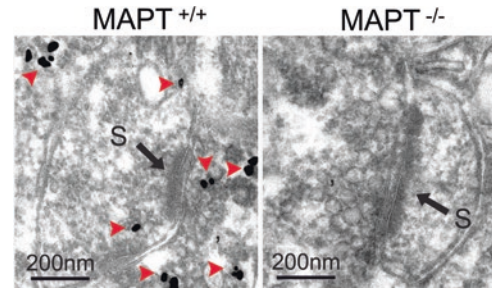

Fig. 10.1 Using immunogold electron microscopy (EM) and labelling tau antibody (JM, rabbit polyclonal anti-tau antibody), tau is found in both the pre and postsynaptic compartment of hippocampal tissue obtained from MAPT$^{+/+}$ (left panel; 4-month-old) but there is no tau signal (JM anti-tau antibody) in MAPT$^{-/-}$ (right panel; 4-month-old) mice. Arrow (S) shows postsynaptic density and arrowheads indicate tau. (Figures are taken and adapted from Kimura et al. (2014), *Phil. Trans. R. Soc. B 369: 20130144* [3])

Translation of synaptic protein tau may occur locally within dendrites [22, 23], or phosphorylated isoforms of tau may overcome an axonal diffusion barrier allowing diffusion into the somatodendritic compartment [24]. Alternatively, tau released from pre-synaptic terminals may be trans-synaptically internalized into post-synaptic regions following neuronal activity [25–27]. Spreading of tau via synaptic contacts might contribute to both physiological and pathological tau-mediated consequences in neuronal function [28, 29]. In this respect, neuronal activity and external stimuli appear to be critical regulators of tau subcellular location. For example, increased neuronal activity or treatment with amyloid-beta (Aβ) or glucocorticoids can increase the dendritic and synaptic localization of tau [30, 31].

How Does Tau Affect Synaptic Function?

The post-synaptic mechanisms by which tau can affect synapse function are, by the nature of accumulating evidence, either complex or difficult to resolve. Except for muscarinic acetylcholine receptors (mAChRs) [32, 33], tau is not known to bind directly to any synaptic receptors and is therefore likely affecting synapse function

through interactions with signalling pathways, of which some possibilities are outlined below.

Tau has been demonstrated to bind to the Src family kinase Fyn, and one hypothesis is that this binding is necessary for the synaptic localization of Fyn [2]. Indeed, Fyn fails to localize to the synapse in tau-depleted neurons [2, 9]. Tau, Fyn, PSD-95 and NMDARs are predicted to form a protein complex at the synapse [34], wherein Fyn-mediated tyrosine phosphorylation of NMDAR GluN2B subunits facilitates synaptic NMDAR function, with both physiological and pathological implications [2, 35]. Thus, tau and Fyn may work in tandem at the synapse to regulate a post-synaptic scaffolding complex that links GluN2B containing NMDARs to intracellular signaling cascades that affect synapse function. Whether tau-Fyn binding is truly necessary for these effects is disputed [36] and their inherent physiological relevance is difficult to reconcile with observations of unaltered basal NMDAR function in tau knockout synapses [3].

Tau-shRNA transfected neurons display impaired AMPAR removal from the synapse following LTD-inducing stimuli [3, 37], and mounting evidence supports a role for tau in the regulation of an AMPAR internalization mechanism involving protein interacting with protein kinase C (PICK1). Tau depletion impairs the interaction between the AMPAR subunit GluA2 and PICK1 [4] and the presence of tau enhances GluA2-PICK1 interaction [38]. Furthermore, Tau-shRNA transfected neurons have higher levels of intra-dendritic GluA2 [37], consistent with impaired AMPAR trafficking at extrasynaptic sites where PICK1 primarily operates [39]. Given that PICK1-driven AMPAR internalization is a key post-synaptic step in the manifestation of LTD [40], these findings are consistent with the LTD deficits observed upon tau depletion. However, PICK1-GluA2 interactions do appear to have complex effects on AMPAR trafficking, including synaptic AMPAR delivery [39] and intracellular AMPAR retention [41], which may go some way to explaining the mixed reports of the effects of tau depletion upon synaptic plasticity. A direct interaction between tau and PICK1 has not been reported, and therefore the precise manner by which tau may regulate PICK1-GluA2 interactions is currently unclear (Fig. 10.2).

Though no direct evidence currently exists to support it, tau regulation of the cytoskeleton may have important implications for the regulation of synapse function. By binding to microtubules and regulating their turnover, tau may modulate the dynamic movement of microtubules into and out of dendritic spines – an important facet of LTD [42]. Similarly, through direct binding to actin [43] or regulation of microtubule entry into spines, tau could thus affect the cross-talk between the microtubule and actin cytoskeletal networks [44] – the latter being an important regulator of synapse function and AMPAR trafficking [45, 46]. Clearly, further work is required to

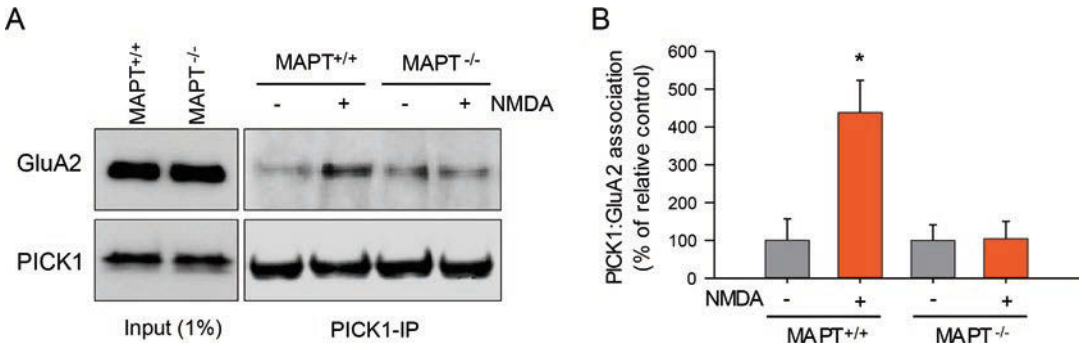

Fig. 10.2 NMDA treatment (25 μM for 3 min) increases GluA2-PICK1 association in hippocampal slices of wild-type mice ($MAPT^{+/+}$) but there is no effect in tau knockout mice ($MAPT^{-/-}$). (**a**) Representative example of western blot data. (**b**) Pooled data, all bars represent the mean ± SEM ($*p < 0.05$). (Figures are taken and adapted from Regan et al. (2015), *J Neurosci 35, 4804–4812* [4])

establish which mechanism(s) enable tau to exert its effects on synapse function.

Since the identification of tau within presynaptic regions [3, 47], there is a notion that its regulation may have an important role in presynaptic function. For instance, the accumulation of pathological tau reduces synaptic vesicles in the mossy fibers and dysregulates basal synaptic transmission, and certain pathological forms of tau interact with synapse vesicles to impair presynaptic function [48, 49]. The synaptic vesicle is an essential part of synaptic transmission [50], making it plausible that the presynaptic role of tau indirectly affects postsynaptic function associated with learning and memory processes [51]. However, there is no evidence of differences in presynaptic-mediated transmission in wild type and tau knockout mice ($MAPT^{-/-}$) [3]. This suggests that normal, or physiological, forms of tau have no role in presynaptic-mediated transmission, but pathological forms of tau dysregulate presynaptic function. Therefore, the regulation of tau in presynaptic and postsynaptic regions may be essential for both the physiological and pathophysiological weakening of synaptic transmission.

Phosphorylated Tau at the Synapse and Functional Consequences

Tau can be phosphorylated by multiple enzymes and 45 sites of phosphorylation have been identified (Y18, S46, S68, S69, T71, S113, T123, T153, T175, T181, S184, S185, S191, Y197, S198, S199, S202, T205, S208, S210, T212, S214, T217, T231, S235, S237, S238, S258, S262, S289, S356, Y394, S396, S400, T403, S404, S409, S412, S413, T414, S416, S422, T427, S433, S435) (see review, Hanger et al. [52]). Multiple kinases can regulate the status of tau phosphorylation and, thus, tau function. Certain phosphorylation sites of tau are closely associated with neuropathology, including neurofibrillary tangles [53] and aberrant hyperphosphorylation is implicated in numerous forms of tauopathy [54]. Specifically, GSK-3 mediated pTau has been implicated in synapse weakening in Alzheimer's disease [55, 56].

The activity of one of the most prominent tau kinases, GSK-3, is strongly influenced by patterns of neuronal activity that induce synaptic plasticity, and in turn, is a key driver of plasticity induction. For example, an LTP-inducing stimulus inhibits GSK-3, while its activity is critically up-regulated during LTD [57]. This correlates with findings that LTD-inducing stimuli cause phosphorylation of tau at GSK-3 substrate residues in an NMDAR-dependent manner [3, 4, 34], raising the possibility that LTD is associated with pTau downstream of GSK-3 activity. Similarly, depotentiation of LTP involves hyperphosphorylation of tau at residues downstream of ERK1/2 activation [58]. These data indicate that the phosphorylation status of tau is dynamically modified by neuronal and synaptic stimuli. Could pTau therefore be key mechanistic step in plasticity processes?

Mutagenesis studies, in which, phosphorylation-prone tau residues are mutated to prevent their phosphorylation (serine to alanine mutation), have gone some way to addressing the synaptic effects of tau phosphorylation. Preventing pTau at serine residue 396 (relative to full length human tau) effectively blocked neuronal induction of LTD but not LTP, while preventing phosphorylation at other residues had no effect [4] (Fig. 10.3).

These results indicate that an individual phosphorylation site of tau plays an important role in affecting synapse function, though the reasoning for such a specific requirement in LTD is not clear. pTau has been shown to affect its interactions with synaptic proteins [34] and can be necessary for the synaptic recruitment of tau [30], though these effects are not known to be directly linked to tau serine 396 phosphorylation.

The pathophysiological relevance of the above findings is demonstrable through evidence of serine 396 phosphorylation of tau in pathologies [59–61], including the early stages of AD. Given that synapse loss is widely considered to underly early cognitive deficits in AD [62] it is possible that elevated serine 396 phosphorylation of tau is representative of hyperactive LTD, or synapse weak-

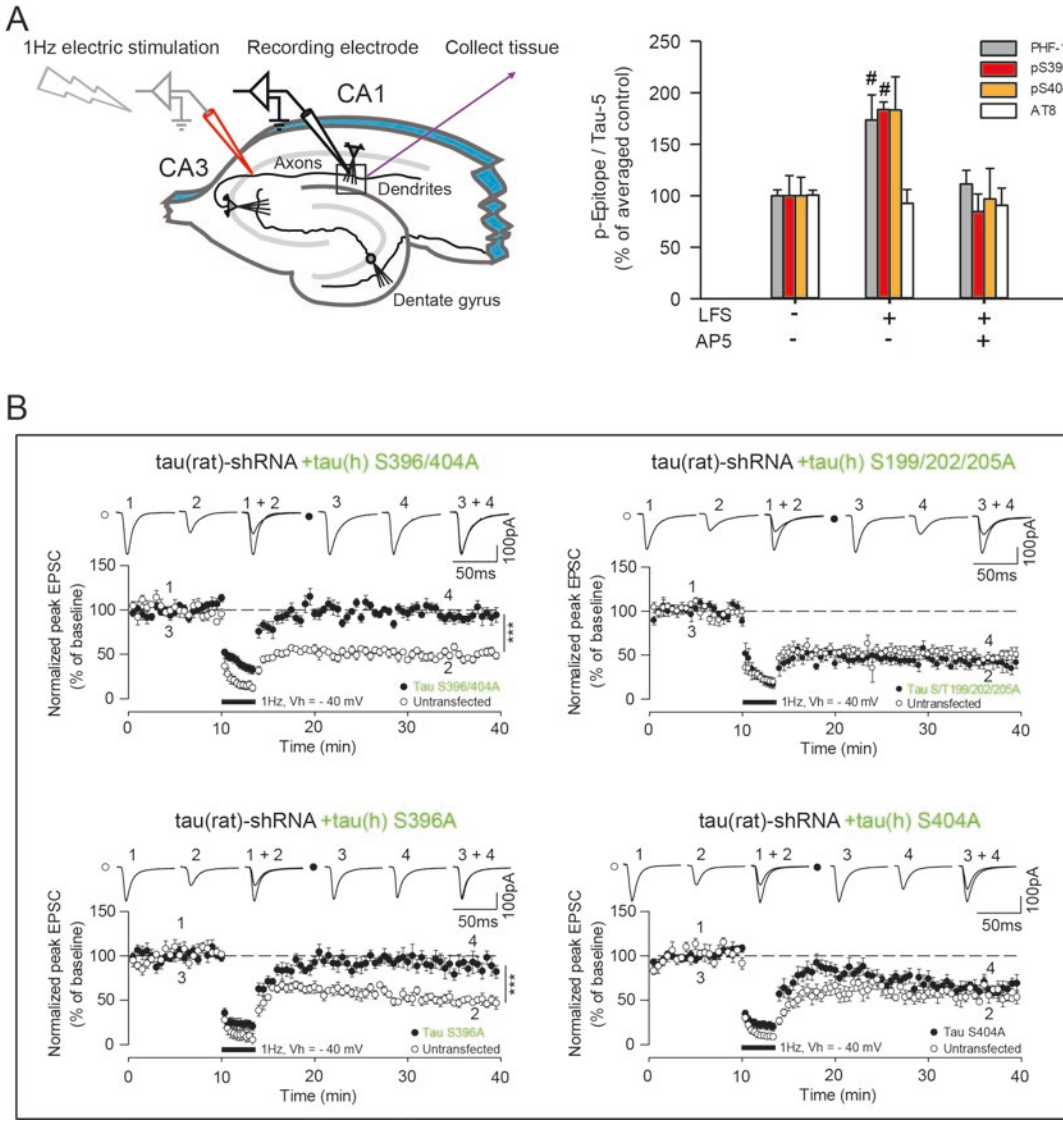

Fig. 10.3 (**a**) Schematic diagram of the microdissection procedure to separate the rat P24 – 28 CA1 somatic and dendritic regions after 1 Hz electric stimulation at CA3 synapse. 1 Hz stimulation induces pTau at the S396 and S404 residues, an effect blocked by the NMDA antagonist AP5. B, LFS (black bar; 200 pulses at 1 Hz, holding voltage −40 mV) induces LTD in untransfected neurons (open circles) but not in neighbouring neurons transfected with rat tau-shRNA plus S396/404A or S396A phosphomutant human tau (closed circles). In contrast, LTD is inducible in S/T199/202/205A or S404A phosphomutant human tau transfected neurons. All data represent the mean ± SEM (***$p < 0.001$). (Figures are taken and adapted from Regan et al. (2015), *J Neurosci 35, 4804–4812* [4])

ening. Credence for this theory comes from AD models, where a caspase-3–Akt–GSK-3 signalling cascade, which drives NMDAR-dependent LTD, is necessary for the Aβ-mediated inhibition of LTP [55, 56, 63]. Thus, pTau, as a GSK-3 substrate, is a putative downstream effector of this pathophysiological synapse weakening signaling. Indeed, tau deletion is protective against the deleterious effects of Aβ on LTP [56]. pTau is also observed in other stages of synapse weakening or degeneration, such as exposure to chronic stress, diabetic retinopathy or hibernation [64–67]. Similar to Aβ, chronic

stress and elevated glucocorticoids inhibit hippocampal LTP in a tau and serine-396 phosphorylation-dependent manner [66, 68]. Clearly, pTau may prove to be a key pathophysiological synapse effector for synapse weakening.

Tau-Mediated Synapse Weakening and Readout of AD Pathology

Central to synapse weakening, several studies demonstrate a molecular mechanism whereby the endocytosis of synaptic AMPARs appears to be pathologically enhanced in AD. Persistence of this aberrant synapse weakening disrupts physiological synaptic plasticity [55, 56] and may be a key pathophysiological entity resulting in AD mediated cognitive deficits. In this context, GSK-3 mediated pTau is a favorable hypothesis for synapse weakening. In animal studies, GSK-3 inhibitors prevent the induction of LTD [57] and neurodegeneration [69]. In clinical trials, a GSK-3 inhibitor has shown cognitive improvements when treated for long periods in AD patients with MCI [70]. This indicates that aberrant GSK-3 mediated pTau causes pathological synapse weakening and this inhibits physiological plasticity (e.g., inhibition of LTP). Indeed, LTP is impaired in AD models [55, 71], and deletion of tau rescues this physiological synaptic plasticity [56].

Tau also interacts with the AD-related proteins such as ApoE, BIN1 and Aβ [72–74]. The relationship between tau and such AD-related proteins reveals a possible mechanism for clinicopathological development of sporadic AD. This suggests that tau plays a key role in the progress of pathology and subsequently cognitive impairment through functional modification of synapses. Aβ deposition is an early pathological signature of AD, but in itself is not sufficient to produce the clinical symptom (see review, Jack Jr. et al. [75]). Arguably, the development of tauopathy is much more closely associated with neuronal silencing [76] and progressive cognitive impairment [77]. Therefore, tau-mediated modification of synapse function may have important implications for diagnosing and treating AD effectively.

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Tau Secretion

11

Zhi Ruan and Tsuneya Ikezu

Introduction

Pathogenic aggregation of microtubule-associated protein Tau, which also been known as intracellular neurofibrillary tangles (NFTs), is a hallmark of multiple neurodegenerative disorders including AD and FTDs. In AD, the severity of dementia is most closely linked with the distribution of Tau aggregates in a hierarchical pattern [5]. The earliest stages of the disease show accumulation of abnormal Tau in the layer II of entorhinal cortex (EC II) whereas later stages show accumulation in the hippocampus followed by neocortical areas. Based on this stereotypical sequential appearance, tau pathology is diagnostically classified into six Braak stages [11]. The EC II is monosynaptically connected to other hippocampal subregions, mainly dentate granular cells through perforant path, and it is trans-synaptically connected with affected regions in the temporal and parietal lobes [114, 121]. One of the most interesting questions in the field is whether pathology of the EC initiates anatomical progression of the disease, or whether pathology dysfunction in hippocampal areas develops independently, and is unrelated to events occurring in the EC. So far, *in vitro* and *in vivo* studies showed that the abnormal Tau seeds could transfer from a "donor cell" to a "recipient cell" and recruitment of endogenous Tau proteins in the latter to generate new abnormal Tau seeds [36, 43, 122], which is similar to the mechanism of prion protein propagation [14, 70]. Based on these findings, the way of Tau secretion and then uptake by recipient neurons has become important mechanism for understanding the spreading of Tau pathology. For the prion spread, it has been shown to not only spread along neuroanatomical pathways but also occur irrespective of the anatomical route or physical location of the prion inoculation site [23]. During the past decades, many pathways were proposed for Tau secretion, but it is unclear how efficient these are for the secretion of aggregated Tau seeds. For example, though 90% of unaggregated Tau is secreted in a free form with only a minority being secreted from extracellular vesicles (EVs), the latter are more efficient for the transfer of Tau aggregates. This highlights the importance of understanding which mechanism(s) of trans-cellular Tau spread plays a role in a physiological condition and which may become more prominent in pathological conditions. On one hand, there are a number of interesting observations that support the trans-synaptic spread hypothesis for the spreading of Tau pathology in AD [27, 71]. On the other hand, the presence of Tau in the interstitial fluid and cerebrospinal fluid (CSF) of Tau transgenic mice and human before neurodegeneration indicates that extracellular Tau can be released by an active process of secretion *in vivo*

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[9, 123]. *In vitro*, recombinant Tau was shown to be secreted by several non-neuronal and neuronal cell lines [78]. In those studies, Tau secretion into the extracellular space could also occurs through exo-synaptic spread way.

Trans-Synaptic Spread Way

Considering Tau proteins is present in presynaptic and postsynaptic terminals especially abnormal Tau enriched in synaptic sites [105], it suggested that Tau could spread through the brain via a trans-synaptic pathway. Such spread pattern of Tau means a trans-neuronal propagation of Tau aggregates from one brain region to the next using the synaptic circuits, which has been first observed by Braak and Braak, whereby Tau pathology manifests largely in a consistent spatiotemporal pattern in human AD brains [11]. In vitro studies have also documented that Tau aggregates have the ability of to move along axons and dendrites in neuronal cultures, both antero- and retrograde, as well as to leave one neuron and be taken up by another [16, 106, 122]. Moreover, the ability of Tau aggregates has been demonstrated to behave similar to prions in that they possess the ability to recruit and ultimately template the naïve monomeric version of the protein [16]. Based on these in vitro results, in vivo models have been generated to better study such spread mode of Tau propagation along synaptically connected networks in the brain. Aggregates of Tau injected into mouse brains appear to move from the injection site to synaptically connected regions [16, 19, 53, 60, 79, 98]. Further, studies in vivo and in vitro have demonstrated that pathological Tau transfers between cells and in between anatomically connected brain regions, leading to the spread of Tau in neurons and oligodendrocytes [20, 21]. Studies also showed that inoculation of Tau induces time-dependent spreading of Tau pathology from the inoculation site to synaptically connected brain regions in human Tau transgenic mice or even in wild type mice [71]. In two lines of Tau transgenic mice, the expression of human Tau under the control of the neuropsin promoter was restricted to the EC, yet Tau aggregates composed of transgenic human Tau and endogenous mouse Tau were detected in neurons downstream in the synaptic circuit such as the dentate gyrus, CA fields of the hippocampus, and cingulate cortex [27]. Because no expression of human Tau mRNA was detected in those regions, the accumulated human Tau in those areas should come from the EC, suggesting that Tau pathology is capable of traversing neural networks in the brains. In addition, abnormally phosphorylated, filamentous Tau derived from the brains of human P301S Tau transgenic mice was sufficient to induce the formation of silver-positive Tau inclusions in ALZ17 mice that overexpress wildtype human Tau, but do not develop Tau inclusions [20], and Tau inclusions as early as 2 weeks after unilaterally infused and showed contralateral hippocampal spread after 1 month [4]. More recently, intracerebral inoculation of Tau fibrils purified from AD brains could result in the formation of abundant Tau inclusions in anatomically connected brain regions in non-transgenic mice [44]. Additionally, these findings suggested that Tau spread through the whole brain was dependent on synaptic connectivity. This trans-synaptic spreading of Tau pathology most likely results from the secretion of Tau by presynaptic neurons and its uptake by postsynaptic ones.

Since new Tau positron emission tomography (PET) imaging has developed, additional studies have been performed to look at the accumulation of Tau pathology in association with brain atrophy using in live patients. The formation of Tau pathology correlates with neuronal dysfunction and ultimately loss in the brain [50, 52, 54, 83, 99, 101]. However, one caveat of this propagation pattern is that EC II project to dentate gyrus, where tauopathies are frequently observed in FTDs but not in AD, which show more frequent tauopathy in the CA1/2 region in the early stage. This discrepancy is not fully understood with the current trans-synaptic mechanism.

Exo-Synaptic Spread Pathway

Extracellular Tau Secretory Pathway

The vast majority of conventional secretory proteins contain a secretory signal sequence that

directs their sorting to the endoplasmic reticulum (ER) from where proteins are transported through the lumen of the ER–Golgi-dependent secretory pathway and then to the cell surface, either the extracellular space or the plasma membrane [67]. However, emerging evidences have documented that there is an "unconventional secretion pathways" for the proteins including Tau without such kind of sequence to be secreted into extracellular space [81]. The presence of extracellular Tau in the AD brain was revealed by its accumulation in CSF during the progression of the disease [47]. In three lines of Tau transgenic mice, they all showed significant age-dependent increases of Tau in the CSF [9]. Additionally, the presence of extracellular Tau in the absence of neurodegeneration was then detected by in vivo microdialysis, indicating that Tau was secreted by neurons *in vivo* [122]. All above interesting findings suggested that the Tau secreted into extracellular space could play a role in the spread of Tau pathology that cause the neurodegenerative process in the AD brain.

As the exist of extracellular Tau was confirmed, their role in the process of tauopathies has also been documented by many groups. In vitro, adding Tau to the culture medium could cause a robust increase of intracellular calcium and cell death of human neuronal SH-SY5Y cells [31]. More recently, Tau oligomers isolated from the AD brain decreased long-term potentiation (LTP) in hippocampal slices [66]. These studies indicated that extracellular Tau can induce neuronal dysfunction. In addition, several recent studies has reported that there was an propagation of Tau pathology after injection of exogenous tau into the mouse brain [20].

During the past decade, accumulating evidence has also indicated that these extracellular secreted pathological Tau could be taken up by neighboring neurons and glia [25, 80, 92], largely depending on the generation of EVs such as exosomes [24, 99]. Meanwhile, other groups also demonstrated that the transfer of such Tau between cells could also occur through intercellular tubular connections [2, 3, 95]. We will review these alternative pathways in the following sections.

Vesicular Mediated Secretory Pathway

The protein degradation of most cellular proteins in eukaryotic cell takes place by two major pathways: through the proteasomal system or autophagolysosomal system. In case of Tau protein, evidences have showed that Tau protein degrades through both systems [119, 124]. However, the protein secretion through EVs recently has been proposed to be an alternative pathway to compensate protein clearance in order to regulate a diverse range of biological processes including facilitating protein discharge, cell differentiation or intercellular communication but also in pathological process [46, 101]. At least two types of EVs, based on their intracellular origin, have been showed to be involved in the Tau secretion: microvesicles (MVs), which are originated by outward budding from the plasma membrane [22] and more frequently exosomes, originated by inward budding of the multivesicular bodies (late endocytic compartments). MVs and exosomes share many protein markers, making it difficult to distinguish between them in the extracellular space or after vesicles have been purified [63]. In addition, both types of EVs use the Endosomal Sorting Complex Required for Transport (ESCRT) machinery in their formation [1]. One of ESCRT-0 proteins hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and signal transducing adapter molecule 1 (STAM) are exclusively involved in the initial phase of exosome biogenesis [24] (reviewed in Colombo et al. [24]). Exosomes may also be formed in an ESCRT-independent mechanism, mainly ceramide-initiated biogenesis [38, 55, 103, 110] and tetraspanin-mediated endosomal sorting [37, 114, 115]. Due to their different biological origins, exosomes and MVs may play divergent roles in physiological and pathological conditions; it is important to differentiate the role in Tau secretion between these two classes of EVs until this point is fully understood.

Both MVs and exosomes have been reported for most cell types if not all types of cells in the body, including the central nervous system (CNS) [85], such as neurons [65], astrocytes [107], and

microglia [91], and have been proposed to play a role in the development of neurodegenerative diseases, such as prion diseases [117] and AD [93]. As larger EVs that directly shed from cells by plasma membrane budding, Tau could be secreted in EVs [33]. In addition, EVs now have served as important mediators of intercellular communication due to their capacity to exchange contents between cell and act as biological signaling vehicles of pathological developments [116].

Microvesicles

As one of major types of EVs involved in intercellular communication, MVs are generated by outward, budding of the plasma membrane [116]. These types of EVs come in many sizes, although not always, but are generally 150–1000 nm in size [24]. First, studies have documented that MVs could play multiple roles in altering the extracellular environment, intercellular signaling, and facilitating cell invasion through cell-independent matrix proteolysis [17, 116]. Furthermore, MVs are also able to modify the extracellular milieu, proximal, and distal recipient cells through their ability to transfer bioactive molecules, including proteins, DNAs [108] and RNAs [112].

The association of Tau with the plasma membrane has been known for many years [12, 61, 68, 109], indicating that Tau could direct the vesicle shedding. As is a soluble cytoplasmic protein, Tau is not directed to the classical secretory endoplasmic reticulum-Golgi secretory pathway in physiological conditions. In addition, considering that MVs are released through cell membrane activation by mediators such as intracellular levels of calcium, inflammatory molecules or oxidative stress, which are involved in the pathology of Tauopathies [8, 32, 86], MVs could be good candidates as the mechanism of secreting Tau protein under pathological conditions. Recently, studies by using in vitro and in vivo in a rat model of sporadic Tauopathies, have demonstrated that Tau protein is present in MVs in the extracellular fluid. Under basal conditions (rat neuronal primary cultures), major free forms of Tau are actively secreted through secretory pathways, predominately secreted in MVs, which are plasma membrane-originating vesicles [33]. It suggested that such specific vesicles, directly emerging from the plasma membrane, enabled cytosolic Tau to be shuttled to the extracellular medium.

Exosomes

Another major type of EVs are exosomes, which are a specific subset of EVs approximately 50–150 nm in size and are released upon depolarization of plasma membrane [30]. Beyond size, exosomes are distinguished from other EVs by their enrichment of the tetraspanin proteins, such as CD63, CD81 and CD9 [64]. Exosomes are often characterized by their high concentration of lipid raft components, such as ceramide and sphingomyelin [28, 94]. Microtubule-unbound Tau was predisposed to be associated with intracellular vesicles. The vesicular association of Tau was especially prevalent in cellular compartments with disorganized microtubules and phosphorylated Tau [68]. Furthermore, vesicular tau has been reported to be colocalized with the lipid raft-associated tyrosine kinase fyn which has been identified in exosomes [68], allowing for the potential of secretion of exosome containing Tau. In addition, Tau was found in exosomes isolated from the CSF [96] and blood of AD patients [35]. More recently, Tau protein released by cultured primary neurons or by mouse neuronal N2a cells overexpressing Tau through the way of exosome secretion [118].

Injection of exosome-enriched fraction isolated from human P301L Tau transgenic rTg4510 mouse brains caused increased Tau phosphorylation and the oligomerization of endogenous Tau [7]. The initiation of endogenous Tau misfolding and aggregation by Tau-containing exosomes was in a threshold-dependent manner [89]. Our recent work has also shown that microglia-derived exosomes can efficiently transmit Tau to neurons in mouse brain. Furthermore, injection of microglia-derived exosomes in the outer molecular layer of dentate gyrus results in the spread of tau accumulation to the dentate granular cells. Inhibition of cremaide-dependent exosome biogenesis by blocking of neutral sphingomyelinase-2 activity

significantly reduced Tau propagation in this model, suggesting that exosomes are indeed a pathogenic agent in the spread of pathogenic Tau [6]. Taken together, these studies indicate that Tau-containing exosomes may play an important role in the propagation of Tau pathology through the brain, which accumulates not only in cell soma but also synapses. Microglia phagocytose Tau-containing inactive synapses from neurons and may eventually secrete them in exosomes when their intracellular clearance is insufficient in an attempt to remain healthy. These pathological protein-laden exosomes could in turn be uptaken by neurons, which are especially vulnerable, resulting in their final death. Neurons can also transmit Tau to each other via exosomes, although this has only been shown in vitro [42, 88, 118]. Exosomes created by these processes also spread pathological forms of Tau throughout the brain, and may eventually secreted in CSF or blood, where their detection can serve as a potential biomarker. Moreover, exosome-associated Tau is also present in human CSF samples and is phosphorylated at Thr-181 (AT270), an established phosphor-Tau biomarker for AD, in CSF samples from patients with mild (Braak stage 3) AD [96].

Overall, above results suggest that exosomes in the case of Tau transmission under pathological condition may be functioning more as a waste-disposal method rather than as a means of cell-cell communication in an attempt by cells to protect themselves.

Tunneling Nanotubes (TNTs)

TNTs are actin-based transient cytoplasmic extensions which are stretched between cells in the form of open ended nanotubular channels (50–200 nm), not always linked to the substrate, and forming bridges that connect remote cells, discovered by Rustom and colleagues [26, 95]. Like EVs, TNTs also represent subtypes and heterogeneous morphological structures [10]. However, biosynthesis of TNTs differs from EVs and is attributed to f-actin polymerization [83]. The regulatory pathways of TNT formation and endosomal trafficking are overlapped, both involving the components of exocyst complex which regulates vesicular transport from Golgi apparatus to the plasma membrane. M-sec, a part of the exocyst complex, interacts with Ras-related protein-A (RalA, small GTPase) and is required for TNT formation. M-Sec in cooperation with RalA and the exocyst complex serves as key factor for the formation of functional TNTs and therefore M-Sec is considered TNT marker [79]. Other studies demonstrate that formation of some TNTs might be actinomyosin-dependent [15, 45]. Perhaps not surprising, motor proteins are required for the generation of some forms of TNTs. For example, myosin10 (Myo10) is required for TNT formation from filopodia, where the overexpression of Myo10 results in increased TNT formation and vesicle transfer between cells [40]. Elevation of Eps8 (an actin regulatory protein) inhibits the extension of filopodia in neurons and increases TNT formation as well as intercellular vesicle transfer [29]. Altogether, these observations indicate that cells may use motor proteins as component of both TNTs and EVs for shipping their cargo.

Emerging data showed that the microtubule-associated protein Tau is a specific constitutive marker of TNTs. This is important because Tau appears beside filamentous actin and myosin 10 as a specific marker of these fine protrusions of membranes and cytosol that are difficult to visualize. Furthermore, extracellular Tau species (monomers, oligomers and fibrils) activate the formation of TNTs that subsequently facilitate fibrillar Tau transfer from neuron to neuron and such extracellular Tau may likely act as a signal for TNT formation as they are found in pathological conditions [106]. In addition, exogenous Tau species could also increase the number of TNTs established between primary neurons, thereby facilitating the intercellular transfer of Tau fibrils [106]. In conclusion, Tau may contribute to the formation and function of the highly dynamic TNTs that may be involved in the prion-like propagation of Tau assemblies.

Species of Tau That Are Secreted

Accumulating data shown that Tau is physiologically present in the extracellular fluid in the form of full-length [59] or truncated forms [13, 57] not only in the culture media of cells over-expressing human Tau [18], primary neurons [18], and iPSC derived neurons [13], but also in the brain interstitial fluid and CSF of mice [122] and human [71, 72]. However, there are many controversies around which specific Tau species are secreted to the extracellular space under pathological conditions. Some studies have shown that Tau propagation could involve species ranging from small soluble monomers to large insoluble fibrils in vivo or *in vitro* [62, 66, 105]. Comparative analysis of CSF from AD and healthy subjects showed a clear increase of amino-terminal (N-terminal) Tau fragments in AD, with no evidence of full-length or carboxyl-terminal (C-terminal) Tau fragments [72]. On the other hand, cell lines such as M1C and Hela cells overexpressing human Tau release a cleaved form of the protein in its C-terminal end [18, 58, 90]. The phosphorylation status of Tau has been also examined. Depending on the cell type, secretion of overexpressed human Tau by non-neuronal cell lines is either phosphorylated or importantly dephosphorylated at several sites [18, 87]. It was reported that intracellular Tau fibrils could be directly released into extracellular space in culture cells and then be taken up by the co-cultured cells in the medium via cell-cell transfer in exosomes or tunneling nanotubes [62]. In vivo studies also shown that intracerebral inoculation of Tau fibrils purified from AD brains could result in the formation of abundant Tau inclusions in non-transgenic mice [44]. Above findings suggest the possibility that Tau fibrils could act as a seed to propagate pathology between neurons in vivo. However, some other groups have identified non-fibrillary Tau forms as necessary for propagation [66, 111]. Tau propagation has occurred after injected human Tau oligomers into the hippocampus of wild type mice [66], as well as Tau aggregation induced by Tau oligomers in human neurons [111]. Additionally, an in vitro study showed that small misfolded Tau aggregates and short fibrils but not the long fibrils were taken up and transported in axons toward the axonal terminals [121].

The Regulation on Tau Secretion

The amount of soluble monomeric Tau released from cells is altered by the various types of external stimuli. For example, starvation or lysosomal dysfunction increases Tau release [73]. In addition, stimulating neuronal activity in either cultured neurons or in vivo also enhances Tau release [34, 90, 123]. This process was calcium-dependent and modulated by phosphorylation. Given that extracellular Tau influences neuronal activity [13], this observation suggests that the activity-dependent release of Tau participates in a positive feedback loop on neuronal activity. More recently, Golgi dynamics were linked to a modulation of Tau secretion by both primary cortical neurons and HeLa cells [74]. Differences in Tau species or isoforms also impact its secretion: phosphorylation, truncation and mutations. These finding of variable Tau secretion suggest the presence of active cellular mechanisms regulating its secretion.

Role of Phosphorylation on Tau Secretion

In AD, there are more than 40 phosphorylated sites of Tau compared to 9 sites in normal patients [49], which means that Tau phosphorylation plays a key role in the Tau pathology. In addition, studies showed that the amounts of phosphorylated Tau of T181 and T231 in the CSF of AD patients were significantly higher than that of normal patients [55], although phosphorylation at other sites such as S199, S202, T205 S396 and S404 remains controversial [71]. It is reasonable to propose that the phosphorylation level might take an effect on the Tau secretion. Recently, an in vitro study has demonstrated that the mimicking of phosphorylation at 12 sites known to be phosphorylated in AD enhanced Tau secretion by Hela cells [83]. Moreover, the Tau secreted by exosomes was shown to be phosphorylated at

several sites found in AD [47] while Tau in the extracellular space would be dephosphorylated in AD brain by tissue nonspecific alkaline phosphatases [31]. Another study also confirmed that M1C cells secrete selectively phosphorylated Tau which is also present in human CSF samples and is phosphorylated at Thr-181 [92]. More recently, expressing the different tau variants CHO cell lines preferentially secreted more phosphorylation tau, which provokes microtubule detachment and increases the availability of free protein inside cells. However, Tau secretion in primary cortical neurons was controversial for that some studies showed the release of unphosphorylated Tau in control conditions [86], other groups reported that both phosphorylated and unphosphorylated could be secreted [83]. Further study is necessary to elucidate the role of Tau phosphorylation on Tau secretion.

Role of Truncation on Tau Secretion

Full-length and various fragments of Tau cleavage have been shown to be secreted in a variety of different cells and animal models [76]. Tau fragments lacking the proline-region are either not secreted or are secreted in a distinct manner to the full-length molecule by COS-7 kidney fibroblast cells [84]. On the other hand, studies have observed the fragments of tau cleaved at the C-terminal presenting in the CSF collected from human AD subjects and from tau transgenic mice [62, 92]. In fact, C-terminal cleavage of Tau at D421 increased Tau secretion [83]. Another study using three distinct neuronal cultures including conditioned medium of N2a cells, induced pluripotent stem cell-derived human cortical neurons, and primary rat cortical neurons observed that the majority of tau released from healthy neurons was C-terminally truncated whereas the microtubule-binding region-containing tau presenting outside of cells was solely due to cell death [56]. In addition, an in vitro study showed that Tau protein fragments secretion to the extracellular space required the presence of the Tau N-terminal domain in a tauopathy model via two distinct mechanisms [62]. The synaptosomes from the AD brains had higher levels of dimerised Tau and a 20-22 kDa fragment of Tau compared to normal samples, and the majority of the tau lacked the C-terminal. Depolarization induced by potassium chloride could significantly enhanced the Tau secretion from synaptosomes in a concentration-dependent manner [98]. In summary, it seems that both full-length and proteolytic fragments of Tau can be secreted by different mechanisms.

Role of Mutations on Tau Secretion

Mutations in the tau gene (MAPT) were demonstrated for the first time to be associated with FTDP-17T (frontotemporal dementia with parkinsonism linked to chromosome 17 and specifically characterized by tau pathology), and a mutation in the MAPT alone could cause neurodegenerative disease and strongly suggested that aberrant Tau plays a pathogenic role in other tauopathies, including AD [50]. To date, at least 107 mutations associated with FTDP-17T or related disorders have been identified, and most of the mutations in the MAPT coding regions are found in in the portion encoding the C-terminal region whereas intronic mutations are located near the alternative splicing site of intron 10, leading to increase the 4R/3R ratio. The C-terminal missense mutations reduce the ability of Tau to interact with microtubules. This partial loss of Tau function may be responsible for inducing the abnormal aggregation of the protein. Several FTDP-17 mutations also could promote the assembly of Tau protein into filaments [77]. In vitro studies demonstrated that Tau mutations not only influenced the rate of Tau secretion, but also affect the form of Tau secretion from the cell, which consisted of the N terminal and microtubule binding repeat length. Moreover, such mutations also produce significantly less extracellular total Tau without altering intracellular total Tau levels [57]. In addition, CSF Tau levels in symptomatic FTD patients expressing a MAPT mutation are slightly elevated as compared to controls but are significantly lower than in AD patients [57]. These results indicate Tau mutations could affect the secretion of certain Tau isoforms from the cell.

Concluding Remarks

Tau can be secreted as is or via EV-dependent pathways, including MVs and exosomes. The secreted Tau can impair synaptic function and cause memory deficits in animal models. This highlights the possibility that the accumulation of Tau in extracellular space could exert detrimental effects on neuron function in the tauopathy brain. It will be interesting to investigate in which way of these extracellular Tau secreted actively, and which pathway will be dominant especially under the pathological conditions. Most importantly, Tau can be not secreted and uptaken by adjacent neurons calls for the development of novel strategies to prevent the propagation of Tau pathology via extracellular pathways. Future studies are needed to elucidate the respective roles of different Tau secretion pathways for the development of tauopathies.

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12 Emerging Connections Between Tau and Nucleic Acids

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Introduction

Tau has long been defined as a microtubule-associated protein (MAP) although early studies have reported its capacity to bind to DNA and RNA. The links between tau and nucleic acids have been largely underestimated until recently when several reports highlighted new key roles of tau in relation with DNA and RNA structure, metabolism and integrity. This chapter will focus on recent research on tau and nucleic acids connections, and their implications in tauopathies.

Tau and DNA Connections

Tau-DNA Complex Formation

The interaction of tau with DNA was first reported *in vitro* in 1980 [1]. Later, nuclear localization of tau and tau-DNA complex formation were observed in various cellular models including neurons (for reviews see [2–4]). In neurons, the tau species described as interacting with DNA in the nucleus is dephosphorylated at the epitope S195–S202, and recognized by the tau1 antibody (Fig. 12.1).

The sequences of both tau and DNA, involved in the Tau-DNA complex formation, have been recently examined in more detail.

Tau Sequences Involved in DNA Binding

The identification, by electromobility shift assays (EMSA) and nuclear magnetic resonance (NMR), of tau sequences interacting with DNA demonstrated the importance of amino acids localized in the proline-rich domain (PRD) and the second repeat (R2) of the microtubule-binding domain (MTBD) [5, 6]. A consensus of research studies supports the hypothesis broad phosphorylation of tau reduces the capacity of tau protein to bind DNA [6–8]. However, the biochemical studies done to date have investigated whether tau interacts with DNA only *in vitro*, which leaves open the question of whether tau is able to directly bind DNA *in vivo*.

DNA Sequences Targeted by Tau

Using gel retardation techniques, recombinant human tau protein was shown to bind and form protein-DNA complexes with double-stranded DNA (either supercoiled, relaxed closed circular or linear) [9], and tau was reported to bind to single-strand DNA by capillary electrophoresis

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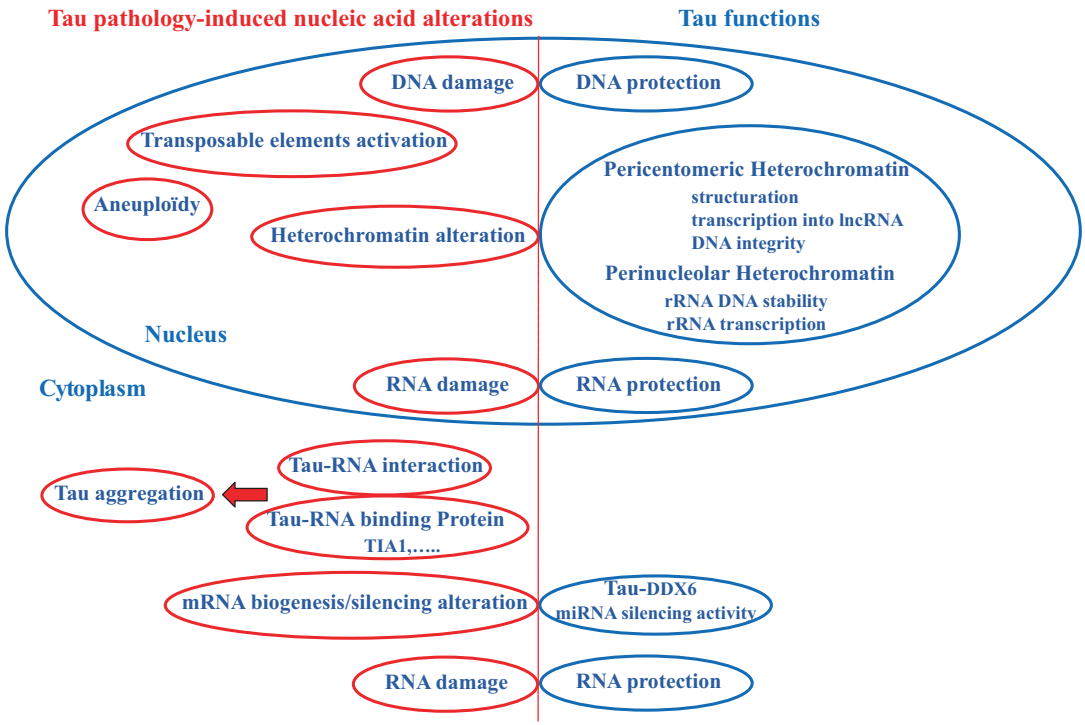

Fig. 12.1 Schematic representation of tau and tau pathology connections with nucleic acids

methods [10]. Further *in vitro* gel retardations assays revealed a preference of tau binding to A/T rich sequences compared with G/C-rich [5]. However, this preference was not confirmed by NMR [6]. In neuronal cultures, results obtained with netropsin, which is a polyamide with antibiotic and antiviral activity, suggested that *in situ*, tau may also interact with DNA through the minor groove of A/T-rich DNA sequences [11]. This work is supported by the analysis of the interaction of tau protein with plasmid DNA by electron microscopy, which showed the formation of regularly spaced bead like structures in a necklace manner [9].

Recently, the first large scale genome-wide analysis of tau interaction with neuronal DNA was published [12]. Specific intragenic and intergenic DNA sequences interacting with tau were identified in differentiated neurons using genome-wide chromatin immunoprecipitation with the tau1 antibody followed by DNA tiling array hybridization (ChIP-on-chip) assays. Tau protein showed a preference for DNA regions overlapping with DNA sequences coding for long non-coding (lnc)RNAs and for DNA regions comprising an AG-rich GAGA-like motifs. The intragenic tau-interacting DNA regions also exhibit functional enrichment for diseases associated with neurological disorders [12]. Interestingly, under heat stress conditions in adult mouse cortex and hippocampus neurons, tau behaved as a repressor of the expression of a subset of tau-interacting genes, although under basal conditions tau protein displayed DNA-binding characteristics different from those expected for a conventional transcription factor. This stress-linked transcriptional repressor role of tau protein was exacerbated by the nuclear accumulation of pathological oligomerized forms of tau protein in hippocampal neurons [12].

Tau and the Maintenance of Genome Integrity

DNA Protection

The capacity of tau protein to protect DNA from free radical induced damage and heat induced denaturation was first reported *in vitro* [5, 9]. *In vitro* experiments also showed that hyperphosphorylation reduced or altered tau DNA binding and folding capacity, impairing the DNA protective effect of tau [13]. These studies led to the hypothesis that the protective activity against free radical attack was linked to DNA folding promoted by tau binding to double stranded (ds) DNA.

Later, the capacity of tau to bind DNA and to protect DNA integrity was highlighted in murine neurons, in primary neuronal cultures and *in vivo* in the hippocampus and cortex, under physiological and stress conditions [11, 14]. These results suggested that the DNA protective function of tau in neurons could rely partly on its capacity to interact with DNA. However, these studies did not exclude an alternative mechanism in which tau contributes to this process by regulating other proteins in the DNA repair pathways [14].

DNA damage is an early feature of neurons developing tau pathology in AD brain. *In vivo* in hippocampal neurons, nucleic acid vulnerability has been linked to the nuclear accumulation of prefibrillar tau oligomers that form in the process of tau pathophysiology [15]. However, the mechanism responsible of the loss of the protective function of monomeric tau in the presence of tau oligomerization has not been deciphered. Small tau oligomers retain the capacity to interact with DNA *in vitro* [8]. Although not demonstrated *in vivo*, a potential role of tau oligomers in competing with tau monomers for DNA-binding has been hypothesized to explain this loss of protective function.

The role of tau in maintaining genome integrity is not limited to neurons. Cells from Bloom's syndrome (BS) patients characterized by mutations in the *BLM* gene, a 3′–5′ DNA helicase, display a strong genome instability. Interestingly BS cells use tau to restrict DNA damage contributing to cell survival [16]. As tau is overexpressed in some cancer cells, it opens up possibility for relevant predictive markers of susceptibility to anti-cancer treatments.

Chromatin Organization

Several reports have highlighted the implication of tau in chromatin organization and a genome 3D structure in neurons, altered in the context of tau pathology and AD [17–20].

Pericentromeric Heterochromatin

Pericentromeric heterochromatin (PCH) is composed of highly repeated major satellite DNA sequences. PCH displays a highly ordered nucleosome distribution rich in particular epigenetic marks such as the trimethylated form of lysine 9 of histone H3 (H3K9me3), giving rise to compact chromatin regions that influence genome stability and gene expression regulation.

Using neurons from wild type (WT) and tau deficient (KOTau) mice, Mansuroglu et al. demonstrated that tau protein interacts with PCH major satellite sequences and regulates PCH structure integrity in differentiated neurons [18]. Also, PCH structures were shown to be altered in *Drosophila* and murine models of tauopathies and in AD brain [17, 18] and a widespread transcriptional increase of genes heterochromatically silenced under physiological conditions, was observed in AD brains [17]. Altogether, these observations suggest that tau pathology affects the physiological PCH regulatory function of tau leading to an alteration of PCH structure and function in neurons.

PCH DNA sequences can be transcribed into non-coding RNAs (ncRNAs) that exist in both, sense and antisense, orientations. Tau has been shown to repress the transcription of the antisense PCH ncRNAs [18]. This repression could modulate the ratio of sense:antisense PCH ncRNAs, which itself would lead to the regulation of the ratio of single- versus double-stranded conformations of PCH ncRNAs in neurons [18]. In the nucleus, the binding of the single strand sense PCH ncRNA to its complementary PCH DNA sequence is necessary for the establishment of the trimethylated form of lysine 9 of histone H3 (H3K9me3) and the recruitment of

HP1alpha protein on major satellite DNA sequences required to initiate the heterochromatization of pericentromeric major satellite DNA. Dysregulation of the physiological equilibrium between sense and antisense PCH ncRNAs, induced by tau deletion or tau pathology, could contribute to the change in PCH structure observed in KO tau neurons and AD brain [18].

In *Drosophila* and mammalian cells, repair of PCH DSBs occurs through two steps. In the first step, DNA damage response proteins, such as the phosphorylated form of the histone variant H2AX (γH2AX) are recruited within the core of clusters of PCH structures named chromocenters. In the second step, the damaged DNA sequences are relocated from the inside to the periphery of chromocenters, leading to the formation of long-lasting γH2AX foci positioned at the periphery of chromocenters. Tau deletion prevented the transition from the first to the second step in stressed hippocampal neurons *in vivo* leading to the permanent accumulation of γH2AX foci and DSBs within PCH structures [18]. These results show that alongside with a role in the organization of PCH, tau protein also participates in the control of the double strand break (DSB) DNA repair (DDR) process of neuronal PCH DNA [18].

Perinucleolar Heterochromatin and rDNA Genes

During the 1990's, the laboratory of Lester Binder described the presence of unphosphorylated tau protein recognized by the tau1 antibody in the nucleolus of interphase human neuroblastoma cells as well as other non-neuronal human cell lines. This nucleolar localization of tau was observed also in monkey cell lines, but not in non-primate cells [21]. Further analyses localized tau protein to the fibrillar region of the nucleolus that contains rRNA genes and corresponds to the region where transcription of rRNA genes occurs. In agreement with these results, in mitotic cells, tau localized to the nucleolar organizer region (NOR) that contains the tandemly repeated DNA sequences coding for rRNAs [22, 23]. In human non-neuronal cells, tau was shown to localize at the periphery of the nucleolus partially colocalizing with constitutive heterochromatin [24]. Studies in neuroblastoma cells suggest that tau regulates rRNA transcription [25]. A nucleolar localization was also described for the phosphorylated epitope (Ser212/Thr214) and abnormal conformation of tau protein recognized by the AT100 antibody in human neurons from the CA1 region that varies during cell aging [19]. The recruitment of tau protein to rRNA DNA loci, along with upstream binding factor (UBTF), has been shown in cells from BS patients. In this case, a protective role for tau protein was described, necessary to preserve rRNA DNA stability and modulate rRNA transcription [16]. Interestingly, important alterations in the protein synthesis machinery involving the nucleolus have been observed in the hippocampus of advance stages of AD [26]. Tau-mediated ribosomal dysfunction caused by pathological tau has also been suggested as a pathogenic process potentially leading to cognitive impairment [27]. Overall these results suggested that tau protein could be involved in rRNA transcription, ribosomal biogenesis and ribosomal function.

Genomic Instability in Tauopathies

Affected neurons in AD are characterized by genomic instability including changes in nucleic acid sequences, chromosomal rearrangements or aneuploidy [28]. A growing body of evidence brought to light the involvement of tau-mediated mechanisms leading to neuronal genomic instability.

Transposable Elements

In contrast to the initial idea that the neuronal genome was a static entity, there is now abundant data showing that rearrangement of transposable DNA elements occurs and modulate genome stability and expression in neurons. Transposable elements (TE) are mobile genetic sequences that can jump from one location to another in the genome. Rearrangement of retrotransposons, transposons with RNA intermediates, have been documented in adult neurons, in human brains [29]. TE expression is tightly

regulated and limited by heterochromatin condensation that transcriptionally silences TE loci, and post-transcriptional events such as clearance via piwi-interacting RNAs (piRNA) [30]. Transposition of TE is abnormally activated in neurological disorders such as TDP43-mediated neurodegeneration [31], and may lead to increased genome instability and transcriptional deregulation. Recently, two different groups reported abnormal TE activation in AD brains and in *Drosophila* transgenic models expressing wild type or mutated (R406W) tau [32, 33], suggesting that pathological forms of tau deregulate TE expression, possibly via heterochromatin relaxation and/or piRNA depletion. Consequently, tau pathology-related TE activation may contribute to genomic instability in tauopathies.

Aneuploidy

Aneuploidy, defined as an abnormal number of chromosomes, represents another form of genomic instability and is strongly associated with tau dysfunction. Cells from patients and mice carrying tau mutations exhibit aneuploidy [34–36]. *Drosophila* overexpressing 4R (4 repeats) tau isoform, but not 3R (3 repeats), exhibit a mitotic block characterized by spindle defects that lead to mosaic aneuploidy [37, 38]. Overexpressing wild type or mutant 4R-tau in neuroblastoma cells also induced monopolar spindles [38]. The relative distribution of 3R and 4R isoforms is crucial during brain development. An imbalance in the expression of tau isoforms may lead to the genesis of aneuploid cells during brain development that survive in adult life that could present a vulnerability leading to age-related neuronal degeneration. Altogether these results suggest the intriguing hypothesis that a developmental origin of tauopathies may exist.

DNA damage and DNA repair dysfunction also appear to alter neuronal chromatin stability in AD [39, 40], suggesting that dysregulation of the roles of tau in maintaining genome integrity (cf. parts 13.2.1. and 13.2.2.) might contribute to the development of genomic instability early in the course of AD.

Tau and RNA Connections

A clear relationship between tau and RNA has been demonstrated by numerous investigations over the past decades. Although most of them were discovered in the context of tauopathies, it further suggests that tau could be involved in some of these mechanisms in physiological conditions.

Tau-RNA Interaction

Early studies demonstrated that RNA could bind to tau *in vitro* and promote nucleation of tau oligomers in the same way as other negatively charged cofactors such as heparin [41, 42]. Zhang et al. reported recently that tau associated with tRNA in different cell types (HEK-293 and human-induced pluripotent stem cells (IPSCs) derived neurons). Mixing tau and tRNA *in vitro* led to the formation of droplets, known as complex coacervates, and creating conditions in which tau may become vulnerable to aggregation (see Chaps. 25 and 26) [43]. Although the existence of these droplets has not been demonstrated *in vivo*, transfection of tau in neuronally derived IPSCs increased the presence of tau in the sarkosyl insoluble fraction. It is not yet known if the interaction of tau with tRNA is involved in other pathological mechanisms, such as the translational inhibition observed with the pathological forms of tau [27].

In a more general way, we observed that tau deficiency, either from knock-out or pathological conditions, triggers alterations in RNA integrity in mouse hippocampal neurons under physiological, heat-stress and ROS-producing conditions [14, 15]. RNA export machinery seems also to be involved in tau-induced neurotoxicity. It has been reported that tau reduced the protein level of Lamin protein to cause nucleoplasmic reticulum expansion in *Drosophila* and human patients with Alzheimer's disease [44]. The authors showed recently that messenger RNA (mRNA) accumulated in these expansions.

Interestingly, pharmacological inhibition or genetic knock-down in *Drosophila* suppressed tau-induced neurotoxicity [45].

RNA Binding Proteins

The relationship between abnormal aggregation of tau and RNA metabolism is not limited to RNA molecules alone but could involve also RNA binding proteins (RBPs). For example, the stress granules nucleating protein T cell intracellular antigen 1 (TIA1) promotes tau misfolding [46] (see Chap. 27 for further details). Part of the translational stress response involves the formation of stress granules (SGs) in the soma and dendrites of neurons. SGs are cytoplasmic aggregates composed of proteins and RNA molecules that repress translation of a subset of RNAs. In pathological conditions, it has been shown that the interaction between tau and TIA1 accelerates the formation and the size of SGs, promoting tau aggregation. Interestingly, the authors showed that TIA1 depletion or pharmacological inhibition reduced SGs formation and pathological tau misfolding. Further work from the same group demonstrated that the interaction between pathological tau and RBPs is not limited to TIA1 but could involve several proteins such as hnRNPA0, EWSR1, PABP, RPL7 and DDX6 [47].

These observations raised questions about whether tau/RBPs interaction is limited to pathological conditions and linked to pathological tau or could be extended to physiological conditions. Indeed, proteomic studies revealed an interaction of tau with several complexes involved in RNA metabolism, including the DEAD-box RNA helicase DDX6 [48, 49]. DDX6 is involved in translational repression, RNA decay and miRNA pathway [50]. It is also required for the assembly of processing bodies (P-bodies) a class of RNA granules found in somatic cells [51]. Tau increased miRNA silencing activities through DDX6 interaction [49]. Post-transcriptional silencing by miRNAs is thought to occur by two distinct mechanisms, first translational repression and second mRNA decay, comprising deadenylation, decapping and 5′-3′ decay of mRNA. Although the mechanisms have been not fully elucidated, it has been demonstrated that CCR4-NOT present in the miRISC complex recruits DDX6 and thus provides a link between translational inhibition and decapping [50].

The Tau/DDX6 interaction increased the translation repression by miRNAs. Importantly, tau mutations (P301S, P301L) found in the inherited tauopathies, frontotemporal dementia and parkinsonism linked to chromosome 17, disrupt tau/DDX6 interaction and impair miRNA silencing [49]. Furthermore, DDX6 accumulates in the cytoplasm of neurons in AD and primary tauopathy Corticobasal Degeneration (CBD) human brains, mainly colocalized with hyperphosphorylated tau, indicating that the level of DDX6 is increased in neurons from tauopathy brains [49]. DDX6 also accumulated in the cytoplasm of some neurons devoid of pathological phosphorylated tau, suggesting that an increase in DDX6 abundance is an early event in the development of tau pathology. Several miRNAs have been involved in the progression of AD or tauopathies. We found in particular that tau/DDX6 interaction increased the translational repression miR-21 and miR-124. miR-21 restored the cognitive deficits in APP/PS1 mice and prevented pathologic features [52]. Recent studies demonstrated that miR-124 decreased in AD [53]. Of interest, BACE1, an enzyme involved in Aβ production, is a direct target of this miRNA [53]. In addition, recent report showed an alteration in several miRNA expression (miR-10a-5p, miR-142a-5p, miR-146a-5p, miR-155-5p, miR-211-5p and miR-455-5p) in tau transgenic mice [54]. However, it is still unknown whether tau, through the interaction with RBPs, regulates miRNA biogenesis or whether these changes are a consequence of the pathology. The identification of several RBPs that interact with tau suggest a more general role in RNA metabolism. However, further studies are needed to elucidate the role of tau/RBP complexes in this evolving field.

Conclusion

Nucleic acids are key macromolecules which hold essential information to maintain normal functioning cells. The growing body of evidence implicating tau and tau pathology in mechanisms regulating genome integrity, chromatin organization and RNA metabolism, suggests that tau is a major player of neuronal homeostasis. Interestingly, the links between tau and nucleic acids are not restricted to neurons and may play also important roles in cancer cells. Clearly, what we currently know about connections between the multifunctional tau protein and DNA/RNA is still an emerging but fascinating research field that definitely needs further investigations.

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13 Tau Interacting Proteins: Gaining Insight into the Roles of Tau in Health and Disease

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Tauopathies Position Tau as a Crucial Protein in Health and Disease

Tau is most intensely studied in relation to its executive role in Tauopathies, a family of neurodegenerative disorders characterized by the accumulation of Tau aggregates [15, 21, 38, 75, 89, 111, 121, 135, 175, 176, 192]. Tau aggregation in the different Tauopathies differs in the affected cell type, the structure of aggregates and Tau isoform composition. However, in all Tauopathies, accumulation of pathological Tau in well-characterized and well-defined brain regions, correlates strongly with symptoms associated with the dysfunction of this brain region. Hence, symptoms of neurodegenerative Tauopathies can range from motoric to cognitive and behavioral symptoms, even extending to deterioration of vital functions when the disease progresses, or combinations of different symptoms governed by the affected brain regions. The most common Tauopathies are corticobasal degeneration (CBD), Pick's disease, progressive supranuclear palsy (PSP) and frontotemporal dementias with parkinsonism linked to chromosome 17 (FTDP-17). However a growing number of diseases are characterized by Tau aggregation amounting to a large family of more than 20 disorders [176]. Most Tauopathies are sporadic, and are hence linked to a combination of environmental and genetic risk factors. However, mutations in MAPT have been identified which are autosomal dominantly linked to Tauopathies, including FTDP, PSP and CBD [94, 163, 185] (Alzforum, https://www.alzforum.org/mutations/mapt). More than 80 mutations have been identified in MAPT, both in intronic and exonic regions of the human MAPT. These mutations can be classified as missense mutations or splicing mutations. Most missense mutations cluster in or near the microtubule binding site of Tau, while most splicing mutations affect the splicing of exon 10 (encoding the R2 domain), and hence affect the 3R/4R ratio. While Alzheimer's disease (AD), is the most prevalent Tauopathy, no mutations in MAPT associated with AD have been identified. Brains of AD patients are pathologically characterized by the combined presence of amyloid plaques and neurofibrillary tangles [171]. Familial forms of AD, termed early onset familial AD (EOFAD) with clinical mutations in APP or PS1/2, have an early

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onset, and are invariably characterized by the combined presence of amyloid and Tau pathology [24, 80, 170]. These EOFAD cases, identify a causal link between APP/PS1 misprocessing and the development of Tau pathology and neurodegeneration [80, 170]. Furthermore, combined genetic, pathological, biomarker and in vivo modelling data, indicate that amyloid pathology precedes Tau pathology, and support a role for Aβ as initiator and Tau as executor in the pathogenetic process of AD [80, 96, 97]. Hence, AD is often considered as a secondary Tauopathy (similar as for Down syndrome patients), in contrast to the primary Tauopathies described above. Tau aggregates in Tauopathies vary with respect to the ratio of different Tau isoforms (3R/4R), to the cell types displaying Tau aggregation and the structure of the aggregates. However, in all Tauopathies a strong correlation between progressive development of pathological Tau accumulation and the loss of the respective brain functions is observed.

Although the presence of Tau aggregates in Tauopathies suggests its involvement in the pathogenetic process, an executive role for Tau in Tauopathies, including AD is substantiated by several arguments listed here. These arguments include: (i) the existence of a family of neurodegenerative disorders all characterized by Tau aggregation [38, 176]; (ii) the strong correlation of the presence and progression of Tau-pathology with disease symptoms in Tauopathies [59, 97, 171]; and most importantly, (iii) the identification of MAPT mutations autosomal dominantly linked to Tauopathies, which indicates that Tau dysfunction is sufficient to drive or cause neurodegeneration (https://www.alzforum.org/mutations/mapt). And interestingly, recent findings have indicated that (iv) Tau pathology can propagate in a prion-like way from one cell to another, and can propagate to functionally connected brain regions [52, 59, 66, 102, 143]. This prion-like or templated propagation of Tau pathology has been consistently and reproducibly shown in in vitro and in vivo models, proving the self-propagating nature of Tau-misfolding and Tau-pathology once initiated [6, 26, 52–57, 59, 66, 72–74, 87, 101, 102, 110, 119, 147, 167, 180, 190, 191]. Prion-like propagation of Tau pathology is a highly compelling explanation for the progressive and characteristic development of Tau pathology, remarkably strongly correlated with symptom progression in AD. Importantly, the presence of Tau seeds has been demonstrated not only in brains of Tau transgenic mice, but also in brains of patients using a sensitive Tau seeding detector assay [55, 88]. Hence, Tau misfolding can initiate a self-propagating process, spreading along functionally connected brain regions.

Based on the above arguments for an executive role of Tau, Tau represents a key therapeutic target for primary as well as for secondary Tauopathies (including Alzheimer's disease, Down's syndrome, dementia pugilistica) [75, 111]. A detailed understanding of the role of Tau in health and disease hence is crucial to identify therapeutic targets for Tauopathies, which can inhibit its pathological role leaving its physiological role (relatively) intact [75]. Importantly, the exact mechanism as to how pathological changes in Tau induce neuronal dysfunction and neurodegeneration are still not understood and are intensively studied. In general, three different mechanisms can be considered: Tau can induce neuronal dysfunction and neurodegeneration through: (i) a loss of its physiological function, (ii) a toxic gain of function of the altered soluble forms of Tau (ranging from monomers to oligomers) or (iii) a toxicity of the larger insoluble Tau aggregates [19, 122]. While the above hypotheses are intensively studied, for either hypothesis it is important to understand the role of physiological and pathological Tau forms. One way to pursue this is to identify the interacting proteins of physiological and pathological Tau forms to gain insight in the mechanisms involved.

We have recently performed a genome wide unbiased identification of the Tau interactome. Hereto, we performed isobaric tags for relative and absolute quantitation (iTRAQ)-based Tau interactome mapping to gain unbiased insight into Tau pathophysiology and to identify novel Tau-directed therapeutic targets. This yielded some interesting starting points for further analysis of the physiological and pathological role of Tau. Within this Tau interactome mapping we identified proteins that can be subcategorized in different functional groups, including cytoskeletal

proteins, proteins involved in post-translational modifications of Tau, synaptic proteins, signaling molecules [193]. Similar ubiased screening approaches have been performed by other groups aiming at the identification of Tau interacting proteins (directly and indirectly) [27, 71, 130, 193]. In addition unbiased screening approaches of Tau modifiers in small animal models encompassing C. elegans and Drosophila have been performed and provided an interesting starting point for further analysis [42, 116]. In this chapter we will review the Tau interactome as known from hypothesis based studies and from the identification of the Tau interactome by broad unbiased experimental approaches and unbiased functional screens as performed by us and others, complemented by hypothesis based studies. Combined these approaches have yielded important insights in Tau's physiological and pathological role. This chapter does not attempt to be exhaustive or to cover all important Tau-protein interactions. We merely aim but to give an overall picture of Tau and its interactors and the potential of the combination of hypothesis based and unbiased screening approaches and functional screens to uncover novel as yet unexplored functions of Tau, or novel interacting proteins of Tau. Excellent reviews about Tau in health and disease have been published (e.g. [8, 20, 75, 121, 135, 176, 192]).

The Structure of Protein Tau as a Target for Protein-Protein Interactions

Interactions between proteins are driven and regulated by specific AA sequences or domains within the protein, their post-translational modifications – including phosphorylation -, the protein isoform, the environmental conditions and the cellular and subcellular localisation of the protein. Tau protein is predominantly expressed in neurons, in axons, while it has also been detected in lower concentrations in dendrites, in the nucleus and in synapses (pre- and postsynaptically) [155]. Tau is encoded by the MAPT gene, located on chromosome 17, comprising 16 exons [148]. Alternative splicing of exon 2, 3 and 10, generates 6 isoforms differing by the presence of 0,1 or 2 N-terminal inserts and the presence of 3 or 4 microtubule binding domains (MTBD) [61–64, 75, 135]. Differing roles of different Tau isoforms are suggested by the predominant expression of the shortest isoform (0N3R) during development shifting to nearly equal 3R/4R ratios and by differences in their regional expression in the adult human brain [60]. And differing proteins have been shown to interact with Tau in interactome mapping approaches [71, 130]. While the differing physiological roles of these different isoforms remain less clear, changes in the 3R/4R ratio of aggregated Tau are observed in Tauopathies [8, 75, 121, 176], even to the extent that Tauopathies display a fixed ratio of 3R/4R Tau characteristic for the specific Tauopathy, highlighting the importance of a correct balance of these isoforms in the brain to maintain healthy conditions.

Tau protein is a natively unfolded protein with a remarkably high hydrophylic profile, and hence water solubility [99, 135, 192]. Tau (4R/2N) predominantly consists of hydrophilic amino acids, containing 80 Ser or Thr residues, 56 negative (Asp or Glu) residues, 58 positive (Lys or Arg) residues and 8 aromatic (5 Tyr and 3 Phe, but no Trp) residues. Although Tau is predominantly a basic protein, it can be subdivided in 4 subdomains, based on their charge and respective motifs [75, 135, 192]. These domains are crucial determinants in the interaction with other proteins and encompass, the N-terminal domain (AA 1–150), a Proline-rich domain (AA151–243), the microtubule binding domain consisting of 3 or 4 imperfectly repeated motifs and a C-terminal tail (AA370–441). The N-terminal region is predominantly acidic, while the C-terminus is neutral. This charge asymmetry and different 'domain characteristics' enable specific protein-protein interactions with other proteins but also intramolecular folding of Tau and Tau aggregation (Fig. 13.1). Furthermore, the specific sequences in the microtubule binding domains (MTBD) are responsible for interaction with specific sets of proteins. The microtubule binding domain of Tau (4R/2N) contains 4 imperfectly repeated regions (R1 to R4, encoded by exons 9–12), separated by 13 to 14 amino acid long spacer regions [67, 120]. The repeats consist of highly conserved 18 amino acids

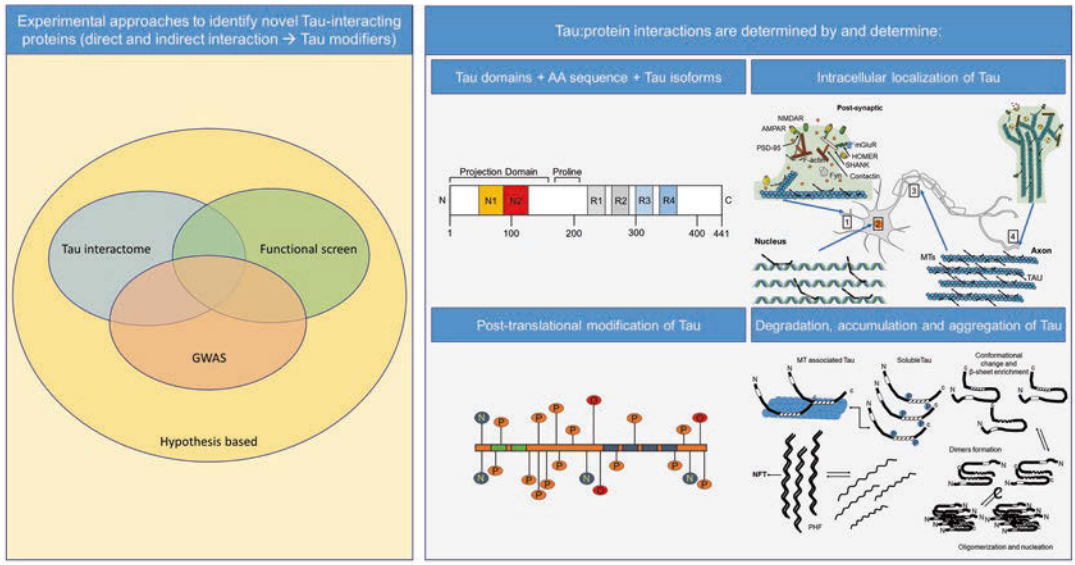

Fig. 13.1 Tau interacting proteins

stretches, which include KXGS motifs, a motif also conserved in other microtubule associated proteins. The interaction between Tau and microtubules heavily depends on positively charged Lys residues on Tau within the microtubule binding domains and the negative charge of microtubules [23, 48, 103–105, 145]. Furthermore, the microtubule binding region is important in Tau aggregation. While Tau is a natively unfolded protein, the second and third MTBDs exhibit a high propensity to form an ordered β-sheet structure, and are important in Tau aggregation [10, 31, 33, 48, 49, 144, 145]. The Proline-rich domain (PRD) contains seven Pro-X-X-Pro (PXXP) motifs, rendering this part involved in signaling pathways, and a target for Proline-directed kinases, as further outlined below. Although Tau is an intrinsically disordered protein, interaction between the different domains may result in dynamic folding of Tau. In this respect, a paperclip structure has been proposed for Tau [98, 99]. In this paperclip structure, the C terminus folds over the microtubule binding domain and the N terminus folds back over the C terminus, bringing both termini in close proximity [98, 99]. This folding of Tau is modulated by binding to interacting proteins, such as microtubules and many others, and by post-translational modifications. Tau interactions with other proteins are mostly described with reference to these domains.

Tau:Tau Binding and Tau Aggregation

As discussed above, Tau aggregation is a core pathological feature of Tauopathies [15] and considered important for their pathogenetic process. Tau can aggregate into paired helical filaments or straight filaments [15, 61]. Interestingly, the domains in Tau involved in Tau-Tau and Tau-microtubule interaction seem to overlap [15, 23, 48, 103, 105, 145]. Binding of Tau to microtubules thereby prevents Tau from self-aggregation, or conversely detachment of Tau from microtubules renders Tau and its MTB domain available for Tau aggregation. The repeat domains of Tau form the core of PHFs, while the C-terminal and the N-terminal regions form the fuzzy coat. The loss of interaction of the MTBD with microtubules, thereby allows Tau to acquire highly ordered β-sheet structures, facilitating Tau aggregation (Fig. 13.1). The hexapeptide motifs of Tau, VQIINK (275–280) and VQIVYK (306–311) in R2 and R3 of the MTBD, are likely to be important for Tau aggregation due to their strong β-sheet forming properties, which has been confirmed in in vitro and in vivo studies. Indeed, disruption of these motifs (e.g., by Pro mutations) abrogates Tau's tendency to aggregate,

not only in vitro, but also in cell and animal models [112, 140]. Conversely, strengthening the β-sheet-propensity by mutations (e.g., ΔK280 or P301L) accelerates aggregation in in vitro and in animal models. However, recently, cryo-EM has identified the core of Tau filaments of AD brains. It was found that the core does not consist of the complete MBD but rather, only amino acid residues 306–378 (i.e., spanning R3, R4, and a part of C-terminal region containing a number of Lys residues), and the remaining portion functions as the fuzzy coat [49]. How β-sheet propensity of R2 domain, and how other domains of Tau affect its aggregation to form this core, as exemplified in the studies listed above, remains to be further analyzed. The structure of 3R Tau only aggregates, as observed in Pick's disease was found to be different from Tau aggregates isolated from AD brains. The part of Tau not belonging to the core of Tau filaments forms the fuzzy coat, which is highly mobile and can even in its aggregated state interact with other proteins.

Tau Interactions with Cytoskeletal Proteins

Tau and Tubulins: The Microtubule Associated Protein Tau

Even before its identification as major component of NFTs, Tau was identified as a microtubule associated protein [194]. Dynamic regulation of microtubules is essential for cellular structure and shape, for intracellular organization, organelle trafficking and chromosome segregation [17]. In adult brain, Tau is predominantly localized axonally in neurons, where it binds to microtubules, which exert crucial functions in neuronal and axonal structure as well as axonal transport. Microtubules are tubular polymers composed of α- and β-tubulin subunits assembled into linear protofilaments. Microtubules consist of 10 to 15 protofilaments, generated by end-to-end polymerization of α/β tubulin dimers, associated laterally to form one hollow cylinder or microtubule, which can further be extended by addition of additional α/β tubulin dimers. This specific organization also accounts for the polarity of the microtubules with a respective plus and minus end [17].

As described above, Tau contains either 3 or 4 MTBDs, depending on the isoform (3R/4R). The MTBD, containing the KXGS motif, which is conserved among microtubule associated proteins, and its flanking basic Pro-rich region, have been shown important for microtubule binding [23, 48, 103, 105, 145]. The exact identification of the interacting sequences in the binding between Tau and tubulin has been hampered by the intrinsically disordered nature of Tau and the dynamic nature of the interaction between Tau and tubulin. A variety of models have been proposed, including (i) binding of Tau to the outer surface of microtubules connecting tubulin subunits either across or along protofilaments [1] as well as (ii) binding of Tau to the interior of the microtubule wall [107]. Using a combination of NMR spectroscopy and mass spectrometry short sequences within the microtubule binding domain were demonstrated to be important for the interaction between Tau and tubulin. These sequences included the imperfect repeat domains 224–237, 245–253, 275–284 and 300–317, while the flanking sequences remain flexible [103, 105, 144, 145]. These regions of Tau were found to bind to the interface between α– and β-tubulin heterodimers. Combined NMR methods optimized for ligand-receptor interactions, demonstrated the competitive binding of the flanking domain downstream of the four microtubule binding repeats of Tau to a site on the α-tubulin surface [103, 105, 144, 145].

Binding of Tau to microtubules, promotes self-assembly of microtubules and their stabilization [14]. Also more subtle effects on microtubule dynamics have been reported, with overstabilization of microtubules by Tau decreasing dynamics and being detrimental for cell viability in vitro [90]. Interestingly, the domains in Tau involved in microtubule stabilization are also involved in Tau aggregation (Fig. 13.1), which may suggest a loss of physiological function following aggregation of Tau [103, 105, 144, 145]. In this respect it is interesting to note, that many modifications associated with Tauopathies (phosphorylation, clinical mutations, altered 3R/4R ratio) alter the ability of Tau to bind to microtubules [76, 77, 136]. While the N-terminal projection domain does not bind to microtubules, its long acidic

domain provides repulsive forces allowing it to exert a spacer function. Similar spacer functions have been noted for domains of other microtubule associated proteins of the MAP family (MAPs: MAP2, MAP2c, ….) [76, 77, 136]. These roles for Tau have been demonstrated unequivocally in cell free systems and in vitro [76, 77, 136]. Subsequent antisense experiments and experiments with primary cultures from Tau knockout mice, have indicated a role for Tau in axonal outgrowth, consistent with a role of Tau in microtubule stabilization and dynamics [35, 47, 135, 154, 192]. However, while these roles for Tau have been identified in in vitro experiments (albeit with some controversy), it must be noted Tau knockout mice display only subtle phenotypic alterations and are surprisingly healthy [93, 152, 183]. This indicates that Tau may regulate these functions but is not essential, although more detailed analysis is still ongoing and will yield more detailed insights. Importantly and most parsimoniously, redundancy between different MAPs may account for the absence of gross developmental and structural abnormalities in Tau deficient mice. These findings should be taken into account when considering loss of function hypothesis in the context of the pathogenetic process for Tauopathies. Recently, it has been reported that acute knockdown of Tau in the hippocampus of adult mice, and thus bypassing developmental compensation, causes learning and memory deficits, which are alleviated when knockdown is lifted [188]. Binding of Tau to microtubules is not only important for regulation of microtubule stability and dynamics, but also indirectly affects microtubule dependent functions, including axonal transport and cellular localization [43, 75, 135, 178, 192]. By binding to microtubules Tau interferes with the roles of kinesin and dynein motors involved in antero- and retrograde axonal transport. When encountering patches of Tau, dynein tended to reverse direction, whereas kinesin to detach at patches of bound Tau [40]. Interestingly, Tau also binds to the p150 subunit of dynactin, a multisubunit protein complex, which binds directly to dynein, and supports dynein dependent axonal transport [133]. Furthermore, Tau as a cargo itself competes with other proteins for axonal transport. Hence different mechanisms have been shown to implicate Tau in dysregulation of axonal transport in vitro. However, also here in vivo compensating mechanisms may exist, as Tau deletion did not affect axonal transport in vivo [196, 197].

Interestingly, using an unbiased approach to identify Tau interacting proteins (iTRAQ) also identified these well characterized Tau interacting proteins within the Tau interactome [193]. Within the Tau interactome mapping, α-tubulin and β-tubulin subunits were identified, as well as proteins from the dynactin complex [130, 193]. This validates the power of these unbiased screening approaches to identify previously characterized and novel Tau interacting proteins. Indirect interactors, i.e. proteins bound in a protein complex, hence in an indirect way, are also identified using these approaches. Dynein and kinesin have been picked up as Tau interacting proteins, probably due to their interaction with tubulins. Hence, this further validates unbiased interactome mapping as an interesting experimental approach. Similarly, several of these proteins have also been identified in a functional unbiased screening for Tau-modifiers. Several unbiased screening approaches have been performed in Drosophila and C. elegans [3, 22, 79]. Within these screens proteins involved in axonal transport and cytoskeletal dynamics have been identified as modifiers of Tau-induced degenerative phenotypes. This highlights the interest of these complementary approaches: proteomics based identification of Tau interacting proteins, unbiased genetic screening for phenotypic modulators of Tau-induced phenotypes, and hypothesis based identification of Tau interacting proteins.

Tau and Actin Cytoskeleton

Besides the microtubules, actin is an important cytoskeletal protein, involved in modulation of cell shape in response to signals, including axonal outgrowth [91, 153, 157]. In fact, the coordinated action of microtubules and actin, is crucial to respond in an adequate way to the cellular environment [41]. Microtubule-associated proteins regulate microtubule dynamics, but can also

bundle and cross-link actin filaments hence representing crucially important proteins for cellular responses [23, 69]. Actin filaments are the smallest type of filaments, consisting of actin polymers organized as a long spiral chain of about 6 nm diameter. Like microtubules, actin filaments have plus and minus ends, with ATP-dependent growth occurring at the positive end predominantly [91, 153, 157]. Actin filaments are often closely situated to the membrane, and involved in modulating or maintaining particular cell shapes [91, 153, 157]. More importantly, actin dynamics are important in synaptic contacts and their maturation, and consequently important for neuronal function [29].

While the interaction of Tau and microtubules has been intensively studied leading to clearcut insights, the interaction between Tau and actin has received less attention. Tau has been found to directly interact with actin. Both sequences within the microtubule binding domain and the Pro-rich domain were identified to interact with actin directly, thereby regulating dynamic actin polymerization and stability [83, 195]. The important role of actin dynamics at the synapse and the role of Tau in actin dynamics, suggests a role of Tau in synaptic function and remodeling. The interaction Tau-actin, could thereby play a role in dendritic spine morphology and postsynaptic reorganizations [92]. A recent study demonstrated that Tau uses several short helical segments to bind dynamically to the hydrophobic pocket between subdomains 1 and 3 of actin [23]. While a single Tau segment is sufficient for binding, minimally two helical segments are required for actin bundling [23]. Phosphorylation of Tau at Ser262 attenuates binding of Tau to filamentous actin, in line with a structural model of Tau repeat sequences in complex with actin filaments. The interaction of Tau with actin filaments, may play a role in Tau–induced dysregulation of neuronal function and synaptic dysfunction, in view of the crucial role of actin filament dynamics in synaptic remodeling. Furthermore, Hirano bodies, which are actin-rich inclusions, containing different proteins, including Tau, have been identified within AD brains [54, 56]. Interestingly, starting from an unbiased functional screening to rescue Tau toxicity in Drosophila, several proteins interacting with the actin and tubulin cytoskeleton were identified [11]. In a different study, it was shown in a Drosophila Tauopathy model that abnormal bundling and accumulation of F-actin mediated Tau-induced neuronal degeneration, indicating a pathological role for the interaction between Tau and actin [54, 56]. Together these data support a role of the Tau-actin and even Tau-actin-microtubule interaction in health and disease conditions [23, 41, 45]. Moreover, within the Tau interactome mapping actin and actin cytoskeleton associated proteins have been identified as Tau interacting proteins [71, 130, 193], further underscoring this interaction and reassuring the relevance of unbiased screens to uncover Tau protein interactors.

Tau Interactions Inducing Post-Translational Modifications

Tau Phosphorylation

Post-translational modifications result from interaction of Tau with other proteins, and subsequently affect the interaction of Tau with still other proteins. In NFTs, Tau is invariably hyperphosphorylated [15, 16, 70], rendering post-translational modifications of Tau, and particularly its hyperphosphorylation an intensively studied topic. Interaction of Tau with kinases and phosphatases alters its phosphorylation status, altering its interaction with other proteins and its aggregation. Protein Tau (4R/2N) contains approximately 80 Ser/Thr residues and 5 Tyr residues, providing 85 potential phosphorylation sites. Approximately 45 of these residues haven been experimentally observed to be phosphorylated [78]. Within the Pro-rich domain of Tau many of these Ser and Thr sites are followed by a Pro residue, and can hence be phosphorylated by Pro-directed Ser/Thr protein kinases. Many different kinases have been demonstrated in vitro to phosphorylate Tau, including GSK-3β

and CDK5. There is substantial support for the Pro-directed kinases CDK5 and GSK-3 being relevant kinases in Tauopathies. In vivo analysis in animal models with Tau pathology, demonstrated respectively aggravated and rescued Tau pathology following their increased and decreased activation respectively, following genetic modification or drug based targeting [16, 18, 46, 85, 124, 125, 132, 150, 151, 177, 184]. In addition, in brains of AD patients, dysregulation of these kinases has been demonstrated [124, 135]. Most of the phosphorylation sites cluster in the flanking regions of the MTBD – the Pro-rich domain and the C-terminal domain – but not exclusively (Fig. 13.1). Tau can be phosphorylated by Pro-directed Ser/Thr-protein kinases, non-Pro-directed Ser/Thr-protein kinases and protein kinases specific for Tyr residues.

In addition, some kinases phosphorylate Tau in or near the repeat domain, these kinases include MARK (microtubule affinity-regulating kinase), CamKII (calmodulin dependent kinase II) and PKA (protein kinase A or cAMP dependent protein kinase) [78]. Particularly phosphorylation of Ser262 of the KXGS motif within the MTBD, has been shown to regulate its binding to microtubules, and to be phosphorylated by microtubule affinity-regulating kinases (MARKs, also known as PAR1 kinases). Tau can become phosphorylated at Tyr18, Tyr29, Tyr197, Tyr310, and Tyr394, with Tyr18 and Tyr394 residues of Tau been detected in NFTs in AD patient brains. Src family kinases, such as Src, Lck, Syk, Fyn and c-Abl have been shown to phosphorylate Tau at these Tyr residues. Tyr phosphorylation, has been shown to occur in brains of Tauopathy patients and animal models of Tauopathies, and to exert diverse effects in the neurodegenerative process in animal models [75, 78].

Obviously, the converse process -Tau dephosphorylation- is crucial in the regulation of its physiological and pathological roles. Protein phosphatase 1/2A/2B/2C and 5 (PP1, PP2A, PP2B, PP2C and PP5) have been implicated in Tau dephosphorylation. Accumulating evidence supports an important role for PP2A as relevant Tau phosphatase. PP2A accounts for ~70% of the human brain Tau phosphatase activity [75, 78, 129]. In vitro and in vivo studies in transgenic mice, further have demonstrated increased Tau phosphorylation and somatodendritic translocation of Tau following PP2A inhibition [75, 78, 114, 131]

Tau phosphorylation is very strongly regulated during development, with fetal Tau displaying high levels of phosphorylation [13, 65, 106]. Tau phosphorylation modulates also cellular localization of Tau within the axon (proximal or distal), its somatodendritic localization (although this is not the sole mechanism [115], its binding to microtubules, its binding to the plasma membrane and its interaction with other proteins. Increased somatodentritic localization and aggregation of Tau has also been detected following increased activation of Tau-kinases in in vivo models. Aggregated Tau accumulating in Tauopathies is invariably hyperphosphorylated, and Tau phosphorylation increases Tau aggregation [2, 39, 186], but other post-translational modifications may also play a role both pro and contra aggregation (see below). Tau phosphorylation is hence not only considered important for regulating its physiological role, but also in the pathogenetic process leading to Tau aggregation [75, 78, 135, 192].

An unbiased approach to identify Tau kinases that phosphorylate specific Tau phosphorylation sites has uncovered novel Tau kinases and novel pathways that affect Tau phosphorylation [27]. Interestingly, unbiased functional screens in C. elegans have identified several of these kinases and phosphatases as modulators of Tau toxicity [116]. Similarly kinases and phosphatases have been picked up in interactome mapping approaches of Tau [130, 193]. The process of Tau phosphorylation and its role in physiological and pathological processes is described in depth in Chap. 6. We here point out the interaction of Tau with this important class of Tau interacting and Tau modifying proteins. These interactions and post-translational modifications not only affect the physiological role of Tau but also affect its aggregation and pathological role.

Other Post-Translational Modifications: Acetylation, Glycosylation, Glycation, Ubiquitination, …

While Tau phosphorylation traditionally has been the most intensively studied post-translational Tau modification, Tau is subject to several other post-translational modifications, including glycosylation, glycation, deamidation, isomerization, nitration, methylation, ubiquitylation, sumoylation and truncation [75, 135, 156, 192]. All these modifications are the result of the interaction of Tau with modifying enzymes and they alter the interaction of Tau with other Tau interacting proteins, potentially affecting its role in physiological and pathological conditions. Furthermore, these post-translational modification may influence one another, as Tau phosphorylation may inhibit its subsequent post-translational modification, whereas acetylation of Tau has been shown to affect its ubiquitiation etc.… . A more extensive discussion of the post-translational modifications of Tau extends beyond the scope of the current chapter (has been provided in [75, 135, 156, 192]). Tau acetylation is discussed in depth in Chap. 7 and Tau truncation in Chap. 8. We here would like to focus on the process of Tau ubiquitination and deubiquitination.

The link between Tau and ubiquitination in AD has been intriguing, ever since the identification of ubiquitin (Ub) in the senile plaques of AD patients and the finding that Tau proteins are highly ubiquitinated in the brain of human AD patients [30, 142, 156, 158]. Increased levels of Ub-Tau were also found in cerebrospinal fluid (CSF) of AD patients [36, 123]. Accumulation of Ub-Tau suggested inefficient proteasomal degradation, as the ubiquitin-proteasome system (UPS) controls degradation of abnormally folded cytosolic proteins [34]. This was further corroborated by the finding that PHF-Tau isolated from human AD brains co-immunoprecipitates with various proteasome subunits, suggesting inefficient processing by the proteasome [109, 146]. Furthermore, proteasome degradative activity has been found to be impaired in post-mortem human AD brains [109, 146]. Together these findings indicate the importance to understand Tau homeostasis and its regulation by ubiquitination and the ubiquitin-proteasome system in the brain [34, 109, 146]. Notably experimental evidence has been obtained for the notion that larger Tau aggregates are degraded by the autophagy-lysosomal system, while more soluble, and smaller Tau forms and Tau oligomers are degraded by the UPS [36, 75, 123, 156].

Poly-ubiquitination occurs by different ubiquitin ligases (E3) in combination with a ubiquitin conjugating enzyme (E2), and defines the cellular fate of proteins including their degradation [168]. Ubiquitin can be ubiquitinated on different Lys residues, leading to polyubiquitin chains with complex topologies. For Tau differently linked (Lys6, Lys11, Lys48, and Lys63) polyubiquitinated Tau forms have been identified in AD brains and models [37, 117]. Several E3 ligases have been identified to ubiquitinate Tau, which include C-terminus Hsp70 interacting protein (CHIP), tumor necrosis factor receptor-associated factor 6 (TRAF6), and axotrophin/MARCH7 [7, 50, 159, 166, 172]. Convincing evidence indicates a modulatory role for CHIP as Tau modulator and Tau-pathology modulator in vitro and in vivo. UbcH5/UBE2D acts as E2 enzyme and Hsp70 as a coenzyme, recognizing the MTBD and PRD of Tau [82]. In line with these findings, deletion of the ubiquitin ligase CHIP leads to the accumulation of phosphorylated Tau species [166]. Furthermore, the high-affinity HSP90-CHIP complex recognizes and selectively targets phosphorylated Tau proteins for degradation. In line with this, overexpression of CHIP attenuates the toxicity of hyperphosphorylated Tau and maintains neuronal survival [82, 172]. In mouse and human brains levels of CHIP and Hsp70 inversely correlate with the levels of insoluble Tau aggregates, strongly suggesting that CHIP-mediated ubiquitination of Tau is an important negative regulator of Tauopathies [166]. CHIP, along with its stress-activated E2s and Hsp70, has diverse target proteins, contributing as an important regulator of the cellular protein folding-refolding machinery

and its degradation machinery. Taken together, targeting Tau degradation by modulation of the HSP90-CHIP complex represents an interesting approach for Tauopathies.

Ubiquitination of proteins is highly dynamic due to the balanced action of ubiquitinases and deubiquitinating enzymes known as deubiquitinases (DUBs) [149]. While Tau ubiquitination has been studied intensively, its deubiquitination remains less well explored. Starting from the Tau interactome mapping we have recently identified, several potential Tau deubiquitinases, and identified Otub1 as a novel Tau deubiquitinating enzyme, with modifying effects on Tau pathology in vivo [193]. Otub1 directly affected Lys48-linked Tau deubiquitination, impairing Tau degradation, dependent on its catalytically active cysteine, but independent on its noncanonical action modulated by its N-terminal domain in primary neurons. Otub1 strongly increased AT8-positive Tau and oligomeric Tau forms and increased Tau-seeded Tau aggregation in primary neurons. Finally, we demonstrated that expression of Otub1 but not its catalytically inactive form induced pathological Tau forms in Tau transgenic mice in vivo, including AT8-positive and oligomeric Tau forms [193].

Taken together these findings indicate that a detailed understanding of the degradation of Tau and different Tau forms by ubiquitination and deubiquitination, yields important insights in the accumulation and degradation of Tau in health and disease. While the UPS selectively degrades normal and abnormally folded soluble proteins, which are tagged by ubiquitin for elimination, the autophagic–lysosomal system (ALS) mainly degrades large protein aggregates or inclusions and organelles [36, 123]. The UPS is thereby considered interesting for removal of smaller soluble oligomeric forms of Tau, often considered as the toxic Tau forms [123]. The ALS conversely can also be involved in the removal of larger Tau aggregates. This topic has been elegantly reviewed in [169] and is discussed in Chap. 20 of this book. Similar as for phosphorylation and ubiquitination/deubiquitination, the post-translational modifications listed above have profound effects on the availability of physiological Tau but also and importantly on the development of Tau pathology. These have been described in detail in Chap. 20.

We furthermore would like to highlight that within the Tau interactome mapping also many Tau interacting proteins involved in its post-translational modification have been identified [11, 130, 193]. These include proteins with already known effects on Tau, but also novel Tau modifiers may still be uncovered by analyzing the role of novel identified Tau interacting proteins. Similarly, within functional genome wide unbiased phenotypic screens, enzymes which post-translationally modify Tau have been identified [11, 116].

Tau Interactions in the Nucleus

Besides its localization in axons, dendrites and synapses, Tau has been detected in the nucleus of neuronal and non-neuronal cells. In vitro and in vivo data in animal models, and data in humans support a nuclear role of Tau, in preserving DNA integrity and potentially other nuclear roles of Tau [137, 173, 181, 189]. Tau has also been shown to protect cytoplasmic and nuclear RNA. In a Drosophila model Tau expression was shown to affect mitosis, mitotic spindle formation and resulted in aneuploidy in Drosophila and cell culture [134]. In addition, Tau aggregates have been detected in the nucleus of AD patients and Huntington disease patients, whereas oligomers have been shown to affect the protective function of Tau in the nucleus [137, 173, 181, 189]. The nuclear role of Tau and the binding proteins involved have been reviewed in detail [173] and are discussed in detail in a separate chapter of the book. Interactions of Tau in the nucleus can be directly with DNA or RNA, but also with DNA/RNA associated proteins. For instance, an interaction between Tau and TIA1 has been found [187]. TIA1 interacts with proteins linked to RNA metabolism. Tau appeared to be required for normal interaction of TIA1 with these proteins, whereas conversely TIA1was shown to induce Tau misfolding. Most recently Tau has been

found to associate with the RNA binding protein Nup98 [44]. Nup98 is part of the nuclear pore complex. Pathological Tau was shown to impair nuclear import and export. Furthermore, Nup98 was shown to accelerate Tau aggregation in vitro. It is interesting to note that different RNA binding proteins appear to be able to induce Tau misfolding and aggregation.

Tau Interacts with Synaptic Proteins and Affects Synaptic Function

While Tauopathies have been initially characterized by large predominantly intracellular aggregates, Tau aggregates range from small soluble oligomers to large Tau aggregates. Smaller soluble oligomeric Tau forms are (also) considered important in the neurotoxic action of Tau. The mechanisms by which this toxic feature is acquired imply most likely the interactions of Tau with neuronal proteins, directly or indirectly linked to synaptic functions. For this reason, great effort has been dedicated in trying to find Tau interacting proteins, eventually unraveling several binding partners potentially involved in a complex picture underlying neurodegeneration. Interestingly, in pathological conditions Tau is increasingly found in pre- and postsynaptic terminals [182]. Hence, important focus is on understanding the interacting proteins and the pathophysiological consequences of this interaction.

Tau Affects Presynaptic Function, through Binding with Presynaptic Proteins

Interestingly, although Tau has been identified predominantly in neuronal axons, it has been also unequivocally identified in synaptic terminals of human neurons, both pre- and postsynaptically. The role of Tau at the presynaptic site has remained relatively long unexplored. Within the Tau interactome mapping many different proteins were found with a synaptic function [28, 193]. More particularly many proteins with a presynaptic function were identified . These proteins included, several proteins involved in presynaptic vesicle recycling including dynamin, amphiphsyin, clathrin, actin and PP2A, synaptotagmin, SV2A among others [130, 193]. While these interactions can be indirect they suggest a presynaptic role of Tau in synaptic vesicle dynamics [130, 193]. Detailed analysis demonstrated that in pathological conditions, Tau dissociates from axonal microtubules and missorts to pre- and postsynaptic terminals [182]. Interestingly, a novel modulatory presynaptic role for Tau was recently demonstrated. Pathogenic Tau was shown to bind to synaptic vesicles via its N-terminal domain, thereby interfering with presynaptic functions, including synaptic vesicle mobility and release rate, lowering neurotransmission in fly and rat neurons [138, 198]. Pathological Tau mutants lacking the vesicle binding domain still localize to the presynaptic compartment but do not impair synaptic function in fly neurons. Moreover, an exogenously applied membrane-permeable peptide that competes for Tau-vesicle binding suppresses Tau-induced synaptic toxicity in rat neurons [138, 198]. These data demonstrate a role for accumulating Tau at the presynaptic terminal in dysregulated neuronal functions. In a follow-up paper, the N-terminal domain of Tau was shown to bind to synaptic vesicles through its interaction with the transmembrane vesicle protein synaptogyrin-3 [138]. Synaptogyrin-3 and synaptic vesicle associated proteins previously had been identified in Tau interactome mapping indicating direct and indirect interactions of Tau with synaptic vesicle associated proteins [130, 193]. In fly and mouse derived models of Tauopathy, reduction of synaptogyrin-3 prevents the association of presynaptic Tau with vesicles, alleviates Tau-induced defects in vesicle mobility, and restores neurotransmitter release [138]. Hence Tau binds through its N-terminal domain to synaptogyrin-3 associated with synaptic vesicles, altering their mobility. The identification of this interaction may be further exploited for therapeutic strategies aiming to counteract Tau induced synaptic dysfunction.

Tau Binds to Postsynaptically Localized Proteins

Although Tau was originally described as a neuronal, predominantly axonal protein, its presence in glial cells, but also in the neuronal somatodendritic compartment and at pre- and postynaptic sites has been identified in physiological and pathological conditions [182]. A synaptic role for Tau first became apparent from the finding that Tau deficiency could rescue Aβ induced synaptic defects mediated at the postsynaptic site [95, 164]. Oligomeric Aβ was shown to induce synaptic deficits and excitotoxicity [126]. An elegant publication demonstrated that Aβ dependent excitotoxicity was dependent on Tau expression, but was also mediated by Tau-dependent recruitment of the SRC family tyrosine kinase Fyn to postsynaptic NMDAR complexes [95, 164]. Tau was shown to interact with Fyn through the PXXP motifs in its Pro-rich domain. At the postsynapse, the Tau/Fyn complex interacts with the PDZ (postsynaptic density, discs-large, zona occludens protein-1) domain of the protein PSD-95 [95, 140], a key scaffolding protein for postsynaptic receptors [113]. While Tau is essential for recruiting Fyn to the PSD-95 complex, it is the localized activity of Fyn that is critical for NMDAR-mediated excitotoxicity [95, 139]. Finally, Fyn can phosphorylate Tau at tyrosine-18. This phosphorylation of Tau is important for the interaction of Tau with Fyn [9], adding another means for regulating the formation of PSD-95/Tau/Fyn complexes (Fig. 13.1). Taken together these findings indicate a role for Tau in mediating Aβ toxicity at the postsynaptic level.

In addition, Tau may exert a physiological role at the postsynaptic site. In line with such a role, induction of LTP induces increased levels of postsynaptic Tau [51]. Furthermore, activation of synaptic glutamate receptors induced translocation of Tau from dendritic shafts into postsynaptic densities, together with Fyn kinase [165]. In addition, Tau has been shown to be released by neurons, following stimulation of neuronal activity, in line with a regulation of Tau by synaptic activity and a role for Tau in its modulation [161].

Taken together, both pre- and postsynaptic roles (and protein-protein interactions) of Tau, have been demonstrated and are increasingly investigated to understand their roles in health and disease in more detail. Broad spectrum, unbiased approaches as well as hypothesis based approaches have in a combined way contributed to these insights.

Tau and Proteins Identified in GWAS

Identification of causal mutations for Tauopathies and related neurodegenerative disorders has provided important insights and cornerstones for their analysis. However, the majority of Tauopathies, including AD, are sporadic forms, driven by interaction between risk genes and environmental factors. Hence there is an important interest in understanding the contribution of risk factors and risk genes in the pathogenetic process, which led to the identification of risk genes for AD. The ApoE4 allele has been identified as important risk gene for AD, and subsequent GWAS studies have identified additional AD risk genes [81, 86, 108, 118]. Genome wide associated studies (GWAS) have identified polymorphisms in or near several genes that are associated with AD risk: ABCA7, CLU,CR1, CD33, CD2AP, EPHA1, BIN1, PICALM, MS4A. Additional loci were identified in a meta-analysis of these large LOAD consortium datasets: CASS4, CELF1, DSG2, FERMT2, HLA DRB5 DBR1, INPP5D, MEF2C, NME8, PTK2B, SLC24H4 RIN3, SORL1, ZCWPW1 [81, 86, 108, 118]. Two genes with moderate to large effects on LOAD risk: PLD3 and TREM2 [32, 100] have been identified in smaller datasets. The identification of common variants with diverging effect sizes on AD risk has begun to create a broader picture of the processes and pathways involved in AD risk. Variants in genes involved in lipid metabolism, the inflammatory response, and endocytosis have been identified through these GWAS [81, 86, 108, 118]. Several of the gene products of genes picked up in GWAS may belong to the Tau interactome.

In this respect an interaction between Bin1 and Tau has been identified [28]. GWAS have

identified a region upstream of the BIN1 gene as the most important genetic susceptibility locus in Alzheimer's disease (AD) after APOE. BIN1 belongs to the family of proteins capable of influencing endocytotic processes by influencing membrane curvature and recruiting dynamin, modulating membrane trafficking and actin polymerization: these proteins are BIN1, amphiphysin and RUS167, also called "the BARs" [25, 28]. BIN1 transcript levels were shown to be increased in AD brains and a novel 3 bp insertion allele ~28 kb upstream of BIN1 was identified, which increased: (i) transcriptional activity in vitro, (ii) BIN1 expression levels in human brain and (iii) AD risk in three independent case-control cohorts [28]. Furthermore, decreased expression of Amph (BIN1 ortholog in Drosophila) suppressed Tau-mediated neurotoxicity. Accordingly, Tau and BIN1 colocalized and interacted in human neuroblastoma cells and in mouse brain [28]. Interestingly, BIN1 variants correlated with Tau pathology in AD brains, but not with amyloid pathology [28]. Subsequent studies demonstrated that (i) BIN1 and Tau bind through an SH3-PRD interaction and (ii) the interaction is downregulated by phosphorylation of Tau in primary neurons [174].

Similarly, clathrin adaptor CALM/PICALM, which has been identified as an AD risk locus, has been shown to be associated with Tau inclusions in AD, PSP and Pick disease [4, 5]. PICALM is a key component of clathrin-mediated endocytosis, and levels of PICALM were shown to correlate with levels of phospho-Tau and autophagy-related proteins [4, 5], suggesting a potential role as Tau modulator.

Along the same line, PTKB2 encodes Pyk2, a tyrosine kinase, which has been shown to act as a direct Tau kinase [128] and as a suppressor of Tau toxicity in the Drosophila eye assay [42] and improves a murine AD model [58]. However, Pyk2 may also affect APP processing [160]. The relative role of PTKB2 in AD related processes involving APP or Tau has been discussed in Polis and Henn [160].

Interestingly, an unbiased modifier screen in Drosophila has been used to analyse the Tau modifying potential of several GWAS identified risk genes for AD. This interesting approach identified several BIN1 and PTKB2 as Tau modifiers [42].

Tau and Pathologically Aggregating Proteins in Neurodegenerative Disorders

Brains of AD patients are invariably characterized by the presence of amyloid plaques and neurofibrillary tangles, composed of aggregated amyloid peptides and aggregated hyperphosphorylated Tau, respectively. Furthermore, biomarker analysis indicated that changes in amyloid pathology precede Tau pathology, providing a therapeutic window [96, 97]. Amyloid positive PET scans provide a high risk for conversion to AD in the following decade [96, 97]. Most importantly, early onset familial Alzheimer's disease patients, carrying EOFAD mutations in APP, which cause Alzheimer's disease, invariably develop Tau pathology [80]. This proofs a link between altered APP processing giving rise to increased amyloidogenic processing and the development of Tau pathology. This finding is further strengthened by similar findings in patients with presenilin mutations, APP duplication, and Down syndrome [80].

Importantly, studies in animal models have invariably proven a link between amyloid and Tau pathology [179]. This includes the first in vivo studies highlighting aggravated Tau pathology in the presence of amyloid pathology, in (multiple) transgenic mice [179]. This included the study by Jada Lewis and colleagues, by generating crosses of mutant Tau and APP transgenic mice, demonstrating for the first time aggravation of Tau pathology in this model compared to the parental strain [127]. This was further supported by data of Jurgen Gotz and colleagues, demonstrating that injection of aggregated Aβ induced Tau aggregation in Tau transgenic mice [68]. Follow-up studies demonstrated that injection of brain extracts of mice with amyloid pathology in mice expressing wild type Tau, could induce Tau pathology in mice which normally do not develop NFTs [12]. The group of Hyman very elegantly demonstrated that amyloid pathology could facilitate spreading of Tau pathology in a model with

entorhinal expression of Tau when crossed to a model with amyloid pathology [162]. These data were further strengthened by studies performed by different groups demonstrating clearcut aggravation of Tau pathology in the presence of accumulated Aβ (reviewed in [179]). Most recently these studies were elegantly complemented by a new milestone paper demonstrating that amyloid pathology facilitated Tau seeded aggregation of endogenous Tau [84]. Taken together, these studies indicate a link between amyloid and Tau pathology, which needs to be understood in detail, to understand the pathogenetic process of AD [179].

The underlying mechanisms linking amyloid and Tau pathology are being studied in detail. Several processes have been demonstrated which could be involved in the link between Aβ and Tau pathology in AD. A detailed discussion extends beyond the scope of this chapter. We have previously reviewed this topic in a detailed way [179], and a detailed discussion of the link between Aβ and Tau pathology is also described in Chap. 20 of this book on Tau biology. While this interaction may or may not involve a direct interaction between Tau and Aβ, it is crucial for the pathogenetic process of AD.

Similarly, pathological interactions between α-synuclein and Tau, and huntingtin and Tau are described in separate chapters in this book.

Conclusion

The original role ascribed to Tau was stabilization of microtubules regulated by phosphorylation and dephosphorylation of Tau. This view has been drastically changed in the past decade through hypothesis based studies on interactions of Tau with itself and other proteins, proteomics analysis in different physiological and pathological relevant conditions and cell types, functional screens for identification of Tau modifiers (which may directly or indirectly interact with Tau). Interestingly, studies of the latter type have been performed in C. elegans and Drosophila and related small animal model organisms. Combination of results from these different types of studies has already yielded important insights in the role of Tau and is anticipated to yield novel and more detailed insights into the role of Tau in health and disease. Importantly and stressed here again, comparing the outcome of different types of studies is more than the sum of parts. It yields novel insights into the physiological role of Tau and the complex mechanisms that are at the basis of Tauopathies, and in turn inspires new hypotheses for further investigation (see Fig. 13.1).

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Part III

Tau and Disease-Related Proteins

14 Relationship Between Tau, β Amyloid and α-Synuclein Pathologies

Lauren Walker and Johannes Attems

Frequencies Increase with Age

It has been shown that up to 55% of older adults that undergo *post-mortem* examination exhibit pathologies associated with more than one neurodegenerative disease [22, 43]. The largest contributing factor for the prevalence of mixed pathologies is age (Fig. 14.1).

The observation that multiple pathologies of differing aetiologies co-exist in the same individual brain has induced a number of cross-sectional studies to establish the prevalence of mixed pathologies in the ageing brain. Most notably, pathologies associated with Alzheimer's disease (intracellular hyperphosphorylated tau (HP-$_T$), and extracellular β amyloid (Aβ)), and Lewy body disease (intracellular inclusions of α-synuclein), which are the most common neurodegenerative diseases known to cause dementia, together accounting for up to 80% of neurodegenerative dementias [21]. Neuropathological studies have also emphasised the presence of pathological protein aggregates in the brains of asymptomatic patients who may represent the prodromal phase of the disease and may account for disease heterogeneity and often complex clinical presentations [22, 23]. Irrespective of clinical diagnosis, in a community-based study (233 cases, age at death 77–87), comprehensive mapping of abnormal protein depositions reported all cases showed some degree of HP-$_T$ burden, with 68.7% of cases harboring Aβ pathology, and evidence of α-synuclein deposits in 24.9% of cases [23].

Mixed Pathology vs Mixed Dementia

The consensus diagnosis of neurodegenerative diseases is based on the concordance of the clinical symptoms observed during life and the signature pathological lesions present at *post-mortem* examination, with the neuropathological element based on the most prevalent pathology, regardless of the presence of other abnormal misfolded proteins. The term mixed or concomitant pathology describes further pathological changes in addition to the primary pathological lesion and should not be mistaken for mixed dementia, where the burden of 2 or more pathological lesions is enough to fulfill the neuropathological criteria for more than one neurodegenerative disease (i.e. NIA:AA criteria, high AD neuropathologic change, and neocortical Lewy body disease (termed mixed AD/LBD)) [3, 19, 33, 34, 51, 55]. Interestingly neuropathologically mixed AD/LBD cases can present with differing clinical phenotypes including AD, DLB, and Parkinson's disease dementia (PDD).

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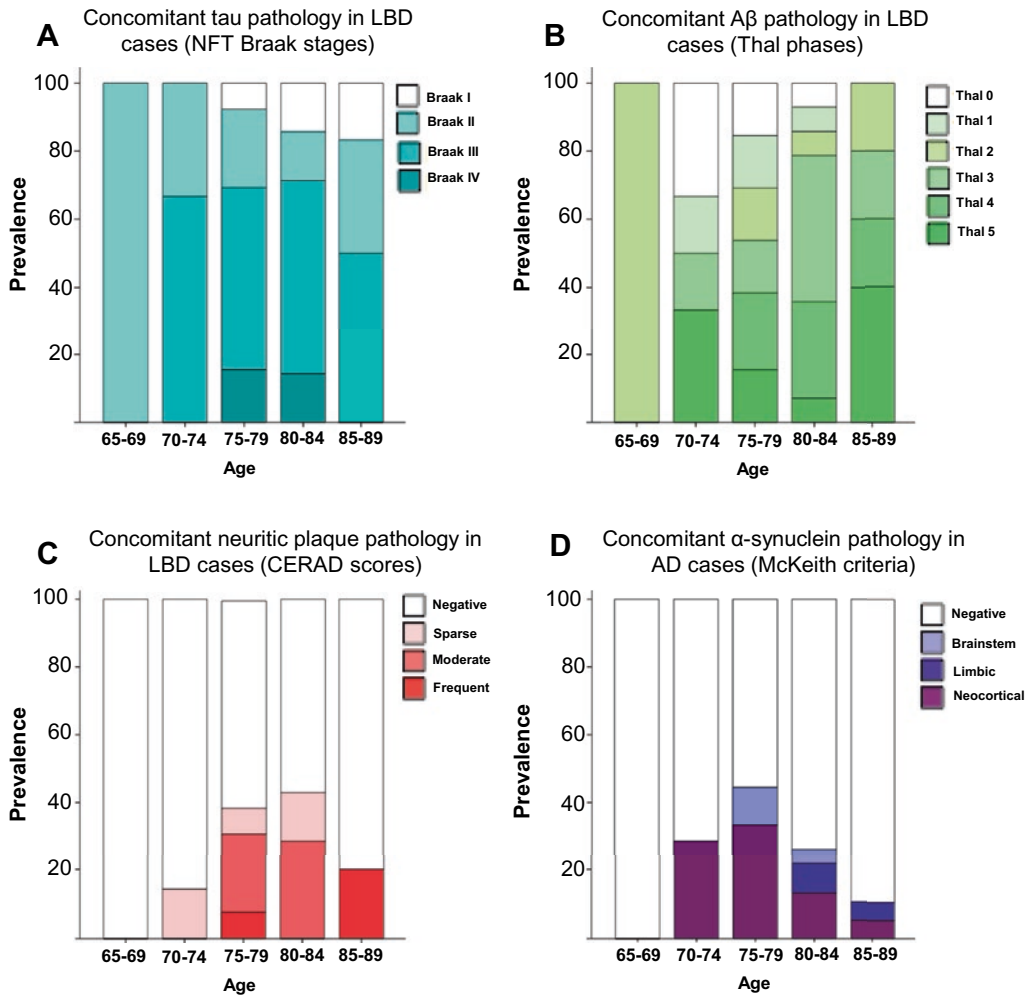

Fig. 14.1 Concomitant pathologies increase in frequency with age. Age related concomitant tau pathology as stratified by NFT Braak stage [3] (**a**), Aβ pathology as stratified by Thal phase [51] (**b**), and neuritic plaque pathology as measured by CERAD score [34] (**c**) was observed in n = 46 LBD cases. Whilst the prevalence of α-synuclein pathology as determined by McKeith criteria [33] (**d**) was observed in n = 61 AD cases. Abbreviations: *LBD* Lewy body disease, *NFT* neurofibrillary tangle, *Aβ* β amyloid, *CERAD* Consortium to Establish a Registry for Alzheimer's disease, *AD* Alzheimer's disease

Clinical Implications of Mixed Dementia

The simultaneous presence of multiple pathologies in the brain is a clinically diagnostic challenge, as the secondary pathology (which on its own is enough to underlie clinical dementia) can often remain undetected. Several studies using neuropathologically confirmed mixed AD/LBD cases have tried to address this; in a cohort of 22 mixed AD/LBD cases, at baseline diagnosis, no subjects were correctly identified as having mixed AD/DLB, and by final diagnosis this increased to just 23%, with the only distinguishing clinical symptom to detect DLB in an AD context being complex visual hallucinations [52]. However, in another study, Savica and colleagues found the overall score for UPDRS II and III and individual components of routine neuropsychological assessment, specifically the Auditory Verbal Learning Test and Boston Naming Test, were able to detect Lewy body pathology in 54

pathologically confirmed AD cases [41]. Of note, it is important to detect the presence of LBs in such cases to identify patients that will experience a more rapid decline in cognition [24, 40, 47, 55], and will have adverse effects to the administration of anti-psychotics [32].

Neuropathological Burden of Mixed Dementia

As neuropathologically mixed AD/LBD cases can present clinically as either AD or LBD, we have previously investigated the burden of pathological protein aggregates (HP-$_T$, Aβ, and α-synuclein) to shed light on the temporal sequence of abnormal protein deposition. Using quantitative techniques mixed AD/DLB cases with a clinical diagnosis of AD exhibited a greater burden of HP-$_T$ pathology, and lower burden of α-synuclein compared to mixed AD/LBD cases with a PDD clinical phenotype [55]. This may suggest that clinical phenotype represents the primary underlying pathology and initial cause of the dementia.

Another key finding from our study is that the topographical distribution of pathological protein aggregates differs between distinct neuropathological phenotypes; when comparing mixed AD/LBD cases to 'pure' DLB cases, the mixed AD/LBD cases exhibited considerable higher α-synuclein burden in the temporal cortex. The temporal cortex also harboured the highest HP-$_T$ loads, and hence HP-$_T$ may have promoted the aggregation and accumulation of α-synuclein [55].

Putative Synergistic Relationships Between Pathological Proteins

Whether concomitant pathologies interact or independently affect clinical disease progression is uncertain, with several studies reporting conflicting results [4, 44]. Although the temporal sequence of the pathological spread of HP-$_T$ and α-synuclein differ in the neurodegenerative diseases they are associated with (HP-$_T$ originating in the entorhinal cortex and α-synuclein initially affecting the medulla oblongata), clinical dementia in both AD and LBD is associated with the progression of both pathologies to the neocortex, with widespread affliction of many anatomically connected brain regions [2, 3, 33]. Of note, the hierarchical deposition of Aβ deposition initially affects the neocortex, however Aβ is only associated with clinical AD when it has progressed to the brain stem and cerebellum, and only in the presence of neocortical HP-$_T$ pathology, which in turn is only seen in the presence of Aβ [35, 51]. Disentangling the underlying aetiology of cognitive impairment in the presence of multiple pathologies is a complex undertaking, however there are a plethora of studies investigating the putative synergistic relationship between pathological protein aggregates, and the combined effect they have on clinical phenotype.

HP-$_T$ and Aβ in AD

Before we address the putative relationships between pathological proteins in cases exhibiting mixed pathology/mixed dementia it is important to understand the neurobiology of individual neurodegenerative diseases which is crucial for the development of effective treatments. Both HP-$_T$ and Aβ are required for a neuropathological diagnosis of AD, and the relationship between these proteins has been scrutinized [7, 46, 53, 56].

However there has been growing interest in post-translational modifications of Aβ, and one species of particular interest is Aβ pyroglutamylated at position 3 (Aβ3(pE)-40 and Aβ3(pE)-42). Recent data suggests Aβ3(pE)-42 plays a crucial role in AD pathogenesis [1], and where similar amounts of non-pyroglutamate modified Aβ are seen in AD and controls (e.g. isoaspartate Aβ), Aβ3(pE)-42 is more abundant, and accounts for up to 25% of total Aβ in AD [17, 36].

A functional connection has been established between Aβ3(pE)-42 and HP-$_T$ as Aβ oligomers containing Aβ3(pE) have been suggested to initiate tau-dependent cytotoxicity [38]. Furthermore,

a study using human *post-mortem* brain tissue demonstrated an association between Aβ3(pE)-42 and HP-$_T$, whilst no associated was observed between HP-$_T$ and non- Aβ3(pE)-42 [28].

HP-$_T$ and α-Synuclein

As HP-$_T$ is frequently seen as a concomitant pathology particularly in LBD, research to elucidate a putative interaction between HP-$_T$ and α-synuclein is of great interest. In a seminal paper in 2003, α-synuclein was shown to initiate the polymerization of HP-$_T$ *in vitro* [13]. Supporting evidence from animal models demonstrated HP-$_T$ deposits accumulate in transgenic mice overexpressing human α-synuclein, which increases in an age-dependant manner [15, 26], whilst phosphorylated α-synuclein aggregates have been detected in the brain of rTg4510 mice that overexpress human P301L mutant tau [50]. A potential seeding mechanism has been suggested as tau oligomers derived from well-characterised progressive supranuclear palsy cases (a 4-repeat tauopathy) and complexes of brain-derived α-synuclein/tau oligomers isolated from cases with Parkinson's disease (PD), enhanced endogenous tau oligomer formation in Htau mice, parallel to increasing cell loss [5]. At the cellular level, the co-occurrence of HP-$_T$ and α-synuclein has been shown using double immunofluorescence [10, 20].

In addition to exacerbating the aggregation properties of each other, the presence of AD related pathology has been demonstrated to accelerate the clinical progression of LBD. In a large clinicopathological correlative study Irwin and colleagues reveal only HP-$_T$ neurofibrillary tangle pathology independently predicted survival time and a shorter interval between motor symptoms and the onset of dementia [19]. This corroborates findings of a PET imaging study, which demonstrated a high frequency of cortical HP-$_T$ pathology in the brains of patients with LBD, which associated with cognitive decline in these patients [14]. Whether pathological proteins of differing aetiologies act individually or synergistically to alter the clinical disease course is a topic of some debate [4, 44]. However, a large study using statistical modeling to extensively analyse whether LB related pathology reacts with AD related pathology revealed the effect of additional pathologies on clinical disease progression was greater in those with intermediate AD related pathology compared to patients with high levels of AD related pathology, which could have implications in the targeted design of therapeutics [4].

Aβ and α-Synuclein

The potential relationship between Aβ and α-synuclein has been studied [11, 29, 30]. Using *post-mortem* brain tissue from 40 PD cases and 20 controls Lashley and colleagues used a combination of semi-quantitative and morphometric assessment to determine Aβ plaque load and LB density, and found a significant correlation between Aβ plaque load and overall LB burden [25]. More recently Swirski and colleagues demonstrated insoluble levels of α-synuclein phosphorylated at serine 129 (α-synuclein pSer129) positively correlated with soluble and insoluble levels of Aβ in a cohort of LBD cases. In parallel experiments, exposure of SH-SY5Y cells transfected with wild-type α-synuclein cDNA to aggregated Aβ42 significantly increased α-synuclein pSer129, suggesting the levels of both proteins are closely related to each other [49].

HP-$_T$, Aβ, and α-Synuclein – A Toxic Triad?

It is essential to examine the relationships between individual pathological protein aggregates, and the cumulative affect they exert on clinical symptoms. However, an important caveat of such studies is that *in vivo* these pathological proteins do not exist in isolation, and this does not accurately reflect the mixed pathology load frequently observed in the brains of the demented elderly, in particular LBDs. This limitation has been addressed by the generation of a mouse model that expresses HP-$_T$, Aβ, and α-synuclein

pathologies by crossing 3xTg-AD mice with A53T mice overexpressing human α-synuclein. The resulting AD-DLB mice exhibited all 3 pathological lesions at a higher load, and an accelerated cognitive decline compared to single transgenic animals [9]. Studies that fully recapitulate these findings in human brains are conflicting; neuropathologically mixed AD/LBD cases experience an accelerated rate of cognitive decline compared to AD and LBD cases without significant concomitant pathology [55], and a combined pathology score of HP-$_T$, Aβ, and α-synuclein being a major determining factor in the development of dementia in a cohort of LBD cases [18]. However, in a separate cohort of LBD cases a correlation between Aβ, and α-synuclein was observed, but not between HP-$_T$ and α-synuclein [10]. Future studies in large, clinically characterised cohorts are warranted to elucidate the mechanistic link between multiple pathologies.

Potential Mechanisms for Interaction

Direct Interactions

In healthy neurons HP-$_T$, Aβ, and α-synuclein do not exist in the same subcellular compartment therefore limiting the potential for a direct interaction. However, in neuropathologic states they have been found in mitochondria, lysosomes, and autophagosomes [6, 8, 16], although whether the proteins are co-localized is yet to be established.

An emerging body of evidence is pointing to the oligomeric forms of HP-$_T$, and α-synuclein as being the major culprits in a putative interaction between the pathologic proteins, with one recent study using novel conformational antibodies demonstrating toxic oligomeric species of HP-$_T$, and α-synuclein can accumulate in LBD [45]. Another study by Castillo-Carranza and colleagues further supports this proposed mechanistic link, as complexes of α-synuclein/tau oligomers enhanced tau oligomer formation with associated neuronal loss [5].

Indirect Interactions

In addition to hallmark pathological lesions observed in AD and LBD, extensive synapse loss in the neocortex is associated with the clinical and pathological profile of both neurodegenerative disorders. Tau neuropathology is associated with a loss of functional synapses in a mouse model of AD [42], and Masliah and colleagues demonstrated a 45% decrease of pre-synaptic boutons in AD cases [31]. Accumulation of pathologic α-synuclein has also been shown to lead to selective decreases in synaptic proteins [54]. These data suggest vulnerable synpases may be more susceptible to further insults in the presence of both HP-$_T$, and α-synuclein.

Phosphorylation of tau and α-synuclein are key events in the pathogenesis of AD and LBD, and numerous kinases have been implicated in the phosphorylation of both proteins including the tyrosine kinase Fyn, and casein kinase 1 [27, 37, 39, 48]. α-synuclein has also been shown to contribute to glycogen synthase-3β mediated phosphorylation of tau [12].

Stratification for Clinical Trials

Identifying the presence of multiple pathological lesions in human subjects will have important implications for the design of effective therapeutic targets for neurodegenerative diseases. Firstly, it will allow for the correct stratification of patients into groups that have similar baseline characteristics, which will strengthen the outcome and confirm endpoints are attributed to any potential drug of interest without any confounding factors that may exacerbate the disease process. Secondly, due to the heterogeneous nature of neurodegenerative diseases, identification of concomitant pathologies (even if clinically silent), and elucidation of basic molecular mechanisms governing the deposition of hallmark pathologies may allow the development of personalised treatment options for patients affected by neurodegenerative diseases associated with clinical dementia.

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Associations Between *APOE* Variants, Tau and α-Synuclein

15

Elena Rodriguez-Vieitez and Henrietta M. Nielsen

Introduction

A common feature of neurodegenerative diseases is the aggregation and deposition of misfolded proteins in the brain. The two most common neurodegenerative diseases leading to dementia are Alzheimer's disease (AD), neuropathologically defined by the presence of amyloid-β (Aβ) and hyperphosphorylated tau aggregates as hallmark pathologies, and dementia with Lewy bodies (DLB) characterized by α-synuclein (α-syn) inclusions. Other synucleinopathies include Parkinson's disease (PD) and Parkinson's disease with dementia (PDD). While each disease is defined by either tau or α-syn as hallmarks, the presence of co-pathologies is very frequent, which leads to a large degree of pathological overlap among diseases [1, 2].

In particular, an overlap of Aβ, tau and α-syn is found in both AD and DLB, which may explain the observed commonalities in their phenotypic clinical presentations, making differential diagnosis challenging [3, 4]. There is also evidence that Aβ, tau and α-syn may act synergistically and that a greater brain load of one type of misfolded protein is associated to a higher frequency of other types of protein aggregates. For example, in a transgenic mouse model of AD, it was shown that Aβ, tau, and α-syn interact *in vivo*, leading to a synergistic accumulation of brain pathology and cognitive dysfunction [5]. These findings add support to the hypothesis that the presence of co-pathologies may be due to the seeding or template effect of one type of misfolded protein that promotes the aggregation of other types of protein. In addition, the results suggest the possibility of common biological mechanisms partly due to shared genetic factors influencing the development of these diseases.

The ε4 allele of the apolipoprotein (*APOE)* gene is the most common genetic risk factor for sporadic AD, and it was also found to increase the risk of DLB, as well as increasing the likelihood of AD pathology in healthy aging [6]. Across many neurodegenerative diseases, the presence of *APOE* ε4 allele is, together with age, a factor that increases the frequency and burden of co-pathologies [1], suggesting that *APOE* ε4 may be a common genetic factor implicated in the biological mechanisms underlying and linking the different proteinopathies. However, tauopathies and synucleinopathies also have differential patterns of co-pathological burden, suggesting that tau and α-syn strains may have a distinct influence on the presence of co-pathologies.

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The apolipoprotein E (apoE) protein, coded by the *APOE* gene, is involved in lipid transport, as well as in processes related to Aβ aggregation and clearance in the brain, and is abundantly expressed in astrocytes. Several apoE isoforms with major structural differences have been shown to influence brain lipid transport, neuroinflammation, glucose metabolism, neuronal signaling and mitochondrial function in various ways. A large body of evidence supports the existence of links between *APOE* ε4 and brain Aβ burden, in particular *APOE* ε4 was reported to promote Aβ fibrillization directly, but it may also contribute to AD pathogenesis via Aβ-independent mechanisms including tau phosphorylation or alterations in α-syn processing. There is also evidence that neuroinflammation, increasingly recognized as an early and key mechanism involved in the pathogenesis of many neurodegenerative diseases, may underlie the links between *APOE* ε4 and proteinopathies [7].

This chapter describes current experimental evidence on the relationships between *APOE* variants, tau and α-syn, from clinical studies on fluid biomarkers and positron emission tomography (PET) imaging, and from basic experimental studies using cellular/molecular biology and animal models. The experimental data summarized here motivates further research using multimodal imaging, fluid biomarkers and genetic factors towards understanding the biological mechanisms underlying these proteinopathies, and to contribute to differential diagnosis and patient stratification for clinical trials.

APOE and the Gene Product Apolipoprotein E

In humans the *APOE* gene is polymorphic and gives rise to three major isoforms of the 299 amino acid apolipoprotein E (apoE); apoE2, apoE3 and apoE4 which vary by only one or two amino acid residues at positions 112 and 158 (apoE2: Cys112, Cys158, apoE3: Cys112, Arg158 and apoE4: Arg112, Arg158). The three alleles encoding these variants, *APOE* ε2, *APOE* ε3 and *APOE* ε4 enable six possible genotypes: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4. The frequencies of the *APOE* alleles in the general population vary slightly between different populations in the world, however the *APOE* ε3 is the most common with a frequency of between 54 and 91% across populations and the *APOE* ε2 is the least common variant with frequencies of nearly zero in native Americans to 15% in Papuans [8]. The *APOE* ε4 allele with a frequency of 9–41% across populations [8], is considered the ancestral *APOE* variant which was proposed to initially have 'acted' like the *APOE* ε3 variant and that due to changes in diet and physical activity evolved to *APOE* ε2 and *APOE* ε3 by some 200,000 years ago [9]. The *APOE* ε4 variant with the amino acid sequence positions 112 and 158 occupied by Arg is the only variant to be found in other mammals like mice, rats, orangutans, chimps and gorillas [10] and the evolutionary development leading to *APOE* ε2 and *APOE* ε3 in humans was suggested to be linked to the increased lifespan in humans compared to other mammals [9].

Apolipoprotein E does not cross the blood brain barrier and it is produced mainly by the liver in the periphery and by glial cells in the central nervous system [11–13]. The apoE in the plasma differs slightly from the one found in cerebrospinal fluid (CSF) due to less sialylation [14]. In both compartments apoE serves as an important lipid carrier which, enabled by its lipid-binding domain, carries cholesterol and through interactions with receptors of the low-density lipoprotein receptor (LDLR) family contributes to the cholesterol homeostasis [15]. Isoform-specific differences in the capacity to carry lipids have been reported with apoE4 proposed to be hypolipidated [15].

In humans, the fluid levels of apoE in plasma and CSF are relatively high (μg/mL range) with ten times higher concentrations found in the plasma. The turnover rate of apoE4 is higher than those of apoE3 and apoE2 in plasma [16–18] and carriers of the *APOE* ε4 variant are known to exhibit low plasma apoE levels [19] which may contribute to the increased risk of neurodegenerative disease in these individuals [20].

APOE Variants and the Risk of Tauopathy and Synucleinopathy

The *APOE* ε4 variant is to date the strongest described genetic risk factor for sporadic onset of AD and DLB, and it increases the likelihood of disease up to 15 versus sixfold respectively. In contrast, the *APOE* ε2 allele appears protective against both AD and DLB [21]. It was further proposed that *APOE* ε4 increases the risk and lowers the age of onset for PD [22] and that it increases the risk of dementia in patients with pure synucleinopathies [23]. Other studies however found no associations between *APOE* and PD [24], hence more studies are needed to establish *APOE* ε4 as a risk factor for PD. One study to date in which a potential association between the presence of *APOE* ε4 and multiple system atrophy (MSA) was investigated reported no significant association between *APOE* ε4 and the disease, a synucleinopathy affecting mainly oligodendrocytes [25].

Whereas the *APOE* ε2 allele appears to be protective against both AD and DLB, a recent study proposed a novel link between *APOE* ε2 homozygosity and the brain load of tau pathology in subjects with progressive supranuclear palsy (PSP). The same study further demonstrated an increased risk of the primary tauopathies PSP and corticobasal degeneration (CBD) in homozygous *APOE* ε2 carriers [26]. The relevance of the different *APOE* variants to the risk of frontotemporal lobe dementia however remains unclear. Taken together, so far the *APOE* ε2 and ε4 variants have individually been associated with the risk of diseases involving Aβ, tau and α-syn pathology.

Evidence from Clinical Studies Linking Apolipoprotein E to Tau and α-Synuclein Fluid Levels

Fluid levels of apoE have long been controversial since conventional immuno-based assays fail to distinguish between the apoE isoforms. Total apoE levels have frequently been reported assuming that the antibody-pairs in for example enzyme-linked immunosorbent assays (ELISAs) have similar affinity for the different isoforms and only one of the isoforms is normally used to produce a standard curve. The technical difficulties to quantify apoE, which furthermore differs slightly in post-translational modifications between the central nervous system compartment and the periphery, are evident from the discrepancy in reported apoE levels [27–31]. Recently, mass-spectrometry based approaches have therefore been employed to in an unbiased manner quantify not only total apoE levels but also the individual apoE isoform levels in both plasma and CSF [32]. Indeed, previous studies reported very poor correlation between mass-spectrometry generated apoE fluid levels and data generated by assays like ELISA (R^2 = 0.22 and 0.59) [27, 32] and although stronger also the correlation between mass-spectrometry data and data from rules based medicine (RBM) was insufficient (R^2 = 0.79) [27]. Data generated from mass-spectrometry assays showed that apoE levels in plasma are directly associated with the *APOE* genotype in which *APOE* ε4-carriers exhibited a strong apoE plasma deficiency attributed specifically to low levels of the apoE4 isoform, as determined in *APOE* heterozygous individuals [19, 33]. Cerebrospinal fluid levels of apoE appeared to not differ amongst the *APOE* genotypes and also not amongst AD and non-AD patients [19].

Studies in which individual apoE isoforms were quantified enabled the investigation of potential correlations between individual apoE isoform fluid levels and concentrations of neurodegenerative disease-associated biomarkers including fluid levels of tau, hyperphosphorylated tau (p-tau), Aβ peptides, and α-syn. Cerebrospinal fluid levels of the apoE3 were found to be positively associated with both total tau (t-tau) and p(Thr181)-tau levels in *APOE* ε3/ε3 and *APOE* ε3/ε4 individuals whereas these associations were absent for the fluid levels of apoE4 in *APOE* ε3/ε4 subjects [19]. The direction of the observed correlations suggests that higher levels of CSF t-tau and p-tau were paralleled by higher apoE in the CSF. Similar results were reported by Toledo and colleagues who further speculated that higher CSF levels of at least

apoE2 and apoE3 may be due to a protective response to neurodegenerative processes associated with AD [34]. Interestingly, a study that utilized transcranial magnetic stimulation over the primary motor cortex to assess long-term-potentiation (LTP)-like cortical plasticity in AD patients with or without the *APOE* ε4 allele demonstrated higher CSF t-tau levels in the *APOE* ε4-carriers versus the *APOE* ε3-carriers [35]. The study further showed that only in *APOE* ε4-carrying AD patients CSF tau levels were correlated with impaired cortical plasticity, cognitive decline and astrocyte survival. Whether the observations were related to the fluid levels of apoE4 was not assessed.

Correlations between fluid levels of apoE isoforms and α-syn remain to be investigated. However it was recently demonstrated that CSF levels of α-syn were dose-dependently associated with the *APOE* ε4 allele in patients with sporadic mild cognitive impairment (MCI) and who within 2 years after initial diagnosis fulfilled the clinical criteria for an AD dementia diagnosis [36], as illustrated in Fig. 15.1a. Furthermore, the same study exhibited a positive correlation between increased CSF α-syn levels and brain Aβ plaque load in Aβ-positive asymptomatic subjects carrying autosomal dominant AD (ADAD) mutations in the *APP*, *PSEN1* and *PSEN2* genes (Fig. 15.1b). Interestingly, when stratifying all mutation carriers into *APOE* ε4-positive and *APOE* ε4-negative, a significant positive association between CSF α-syn levels and brain Aβ plaque load was found in the *APOE* ε4-positive ADAD mutation carriers only (Fig. 15.1c). The described link between CSF α-syn levels and the build-up of brain Aβ plaque load was absent in *APOE* ε4-negative mutation carriers [36]. Together, these results propose a novel link between the *APOE* ε4 allele, higher CSF α-syn levels, and the development of both sporadic and familial AD. The molecular relevance of this relationship remains to be investigated.

Other studies have investigated the association between CSF levels of α-syn, t-tau and p-tau in different neurodegenerative diseases, however the influence of *APOE* ε4 on those relationships is still not well known. In cognitively normal subjective memory complainers, in addition to a positive association between CSF α-syn concentrations and brain Aβ deposition, a positive association was also reported between CSF α-syn and both CSF t-tau and p(Thr181)-tau concentrations [37] suggesting an involvement of CSF α-syn in AD pathophysiological mechanisms in preclinical stages of the disease; the possible influence of *APOE* ε4 was however not investigated. In sporadic MCI and AD groups, a positive association was observed between higher CSF levels of α-syn and both t-tau and p-tau [36], as illustrated in Fig. 15.1d, e; this type of positive association was also reported in familial ADAD [36].

Many studies in different diagnostic groups have found a relationship between *APOE* ε4 and low concentrations of CSF Aβ42 [38], especially in cognitively normal individuals [39] or early stages of AD, but less evident at later stages of the disease. The connection between *APOE* ε4 and tau levels seems weaker with some contradictory findings, and dependent upon the disease stage. In cognitively normal individuals, no association was found between *APOE* ε4 and tau levels in the CSF [39]. However, a positive association between *APOE* ε4 and both t-tau and p-tau levels in the CSF was reported in MCI and AD groups [40].

APOE ε2 is a protective factor leading to lower Aβ pathology as measured either in the brain or CSF, but it did not affect tau levels in the CSF [41], so its protective effect seems to be restricted to the Aβ pathology. The protective action of *APOE* ε2 has been ascribed to its antioxidative and anti-inflammatory effects. There is still very little evidence whether *APOE* ε2 has benefits in terms of reduced tau load, and the mechanism on how these two factors are related is not known [42].

Brain Imaging-Based Evidence Linking Apolipoprotein E to Tau and α-Synuclein

Recently, tau PET imaging has become available and applied to visualize tau deposits in different neurodegenerative diseases [3, 4]. No α-syn PET

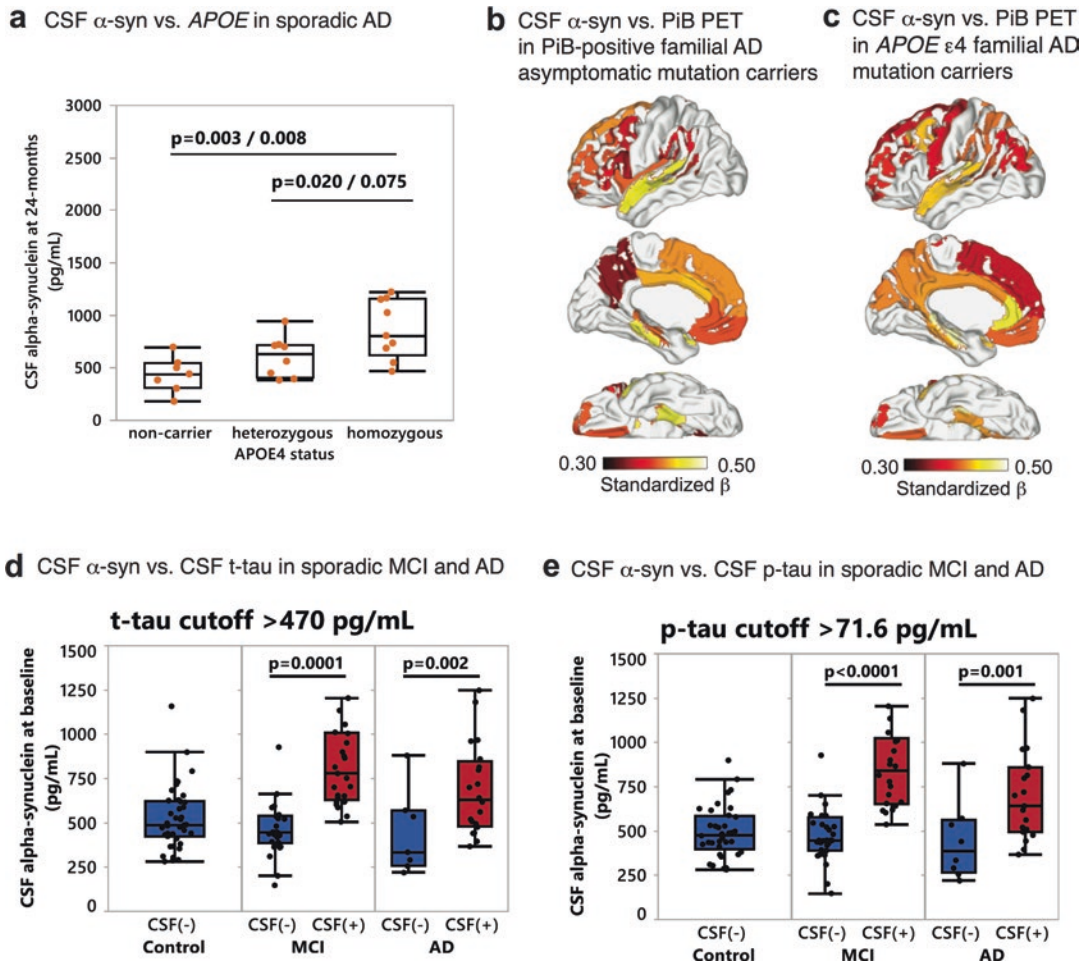

Fig. 15.1 Relationships between *APOE* ε4, α-syn, tau and Aβ in the development of sporadic and familial AD. (**a**) CSF α-syn concentrations as a function of *APOE* ε4 in sporadic AD patients; (**b**) Positive associations between CSF α-syn and regional Aβ uptake as measured by ^{11}C-Pittsburgh compound B (PiB) in PiB-positive familial AD asymptomatic mutation carriers; (**c**) Positive associations between CSF α-syn and regional Aβ uptake as measured by PiB in *APOE* ε4-positive familial AD mutation carriers; (**d**) Higher CSF α-syn concentrations in CSF t-tau positive MCI and AD patients; (**e**) Higher CSF α-syn concentrations in CSF p-tau positive MCI and AD patients. (Figure adapted from Twohig et al. [36])

tracer is yet available, although it is subject of intense research and development efforts [43].

Tau PET imaging studies comparing the brain regional uptake patterns in synucleinopathies versus in other proteinopathies are scarce [44, 45], and much is still unknown regarding the relationships between *APOE* variants and tau PET uptake in different proteinopathies [46].

Previous to tau PET imaging, a vast literature had reported positive associations between *APOE* ε4 and Aβ plaque deposition as measured by Aβ PET tracers especially in healthy aging [38], consistent with *APOE* ε4 being a risk factor for developing AD pathology with aging. In MCI and AD groups, previous studies have reported either a positive association between *APOE* ε4 and Aβ plaque deposition [38], or no correlation [47].

In cognitively normal individuals, the relationship between *APOE* ε4 and tau PET uptake as measured by the ^{18}F-AV1451 PET tracer was not significant, despite a positive association between

APOE ε4 and Aβ PET uptake in the same group [48]. When investigating MCI and AD groups, few studies have looked at the differences in topographical patterns of tau PET uptake depending on the *APOE* ε4 genotype. The limited evidence available points to *APOE* ε4 carriers displaying a more typical AD-like pattern of tau deposits involving the entorhinal cortex, medial temporal lobe and parts of the neocortex, consistent with a more amnestic clinical presentation [46, 49]. In contrast, *APOE* ε4 non-carriers had a more atypical tau topography, showing high tau load in the neocortex including parietal and frontal regions and relatively lower levels of tau in the entorhinal cortex [46, 49]. The total tau load was found to be higher in *APOE* ε4 carriers compared to non-carriers in a small group of mostly atypical AD patients, even after controlling for global Aβ load, suggesting a direct and Aβ-independent effect of *APOE* ε4 on tau deposition [50], consistent with findings that *APOE* ε4 facilitates the phosphorylation of tau [51].

Due to the recent development of tau PET imaging, few studies have yet compared the patterns of tau PET upake across different proteinopathies. The evidence so far has shown higher brain tau load in AD compared to DLB [44, 45], also showing different topographies, with greater tau load within AD-typical regions such as the medial temporal lobe in AD, and a more atypical (non-Braak) pattern of tau PET uptake involving the posterior temporoparietal and occipital regions in DLB compared to AD [44, 45], as illustrated in Fig. 15.2. No PET imaging studies to our knowledge have yet reported on the relationship between *APOE* ε4 and tau PET retention in synucleinopathies, or on the associations between *APOE* ε4, tau PET retention and CSF α-syn in different proteinopathies.

Cell and Molecular Observations Proposing Associations Between *APOE*, Tau and α-Synuclein

The bulk evidence tying *APOE* to neurodegenerative disease supports a strong association of mainly *APOE* ε4 and the apoE4 isoform to AD and Aβ pathology [52]. It is well-described that carriers of the *APOE* ε4 allele silently, in the absence of cognitive symptoms, develop Aβ pathology already in their third and fourth decade of life [53, 54]. Numerous studies have by now shown that apoE may hamper cellular clearance of Aβ [55–57]. The underlying mechanism was proposed to be competition between Aβ and apoE, a heparin-binding protein, for cell surface heparin sulfate proteoglycans (HSPG) [58]. The HSPG pathway was previously suggested to be a common route for cellular uptake of both tau and α-syn [59], hence in theory apoE may be able to

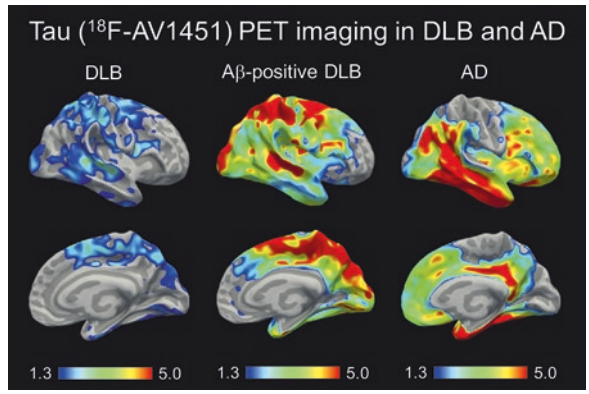

Fig. 15.2 Surface-based comparisons of the regional patterns of tau (^{18}F-AV1451) PET retention in DLB and AD patient groups, compared to healthy controls. Color bars represent $-\log_{10}P$ values for each comparison. (Figure adapted from Fig. 2 in Lee et al. [44] with permission from John Wiley & Sons Inc. © 1999-2019)

also alter the cellular uptake of both these molecules. Indeed, whereas apoE appeared to interfere with cellular Aβ uptake in an apoE isoform-unspecific manner *in vitro* [58], specifically the apoE4 isoform reduced oligodendrocytic uptake of α-syn *in vitro* [25]. Whether the apoE4 effect on cellular uptake of both tau and α-syn is significant enough to cause pathology remains to be investigated. Interestingly, an increase in extracellular levels of α-syn, possibly due to an apoE4-mediated shift in the distribution of the protein between the intra- and extracellular compartments, may in turn promote tau phosphorylation by glycogen synthase kinase-3β (GSK-3β) [60].

A separate line of evidence proposes a direct link between the apoE4 isoform and tau pathology. Apolipoprotein E immunoreactivity was described in association with tau neurofibrillary tangles [61] and specifically an 18 kDa amino-terminal fragment of the apoE4 isoform was shown to colocalize with the neurofibrillary tangle marker PHF-1 in frontal cortex sections from AD patients [62]. It is well-known that the apoE4 isoform is more susceptible to proteolysis than the apoE3 isoform and in addition to the identification of the amino-terminal fragment of apoE4 in AD patients, Rohn and colleagues also identified the same fragment in Pick bodies in the brains of patients suffering from the tauopathy Pick's disease. Based on their results the authors suggested that apoE may have a broader role beyond the association with AD [63]. Other apoE fragment species including C-terminal truncated 29 kDa and 14–20 kDa fragments were shown to accumulate in the brains of AD patients who were *APOE* ε4-carriers, exhibiting higher levels than those of subjects with other *APOE* alleles [51, 64]. Bioactive C-terminal truncated apoE4 fragments were generated in neurons and mainly in brain regions susceptible to AD-associated neurodegeneration. This neuron-specific C-terminal fragmentation of apoE4 was associated with increased tau phosphorylation which the authors proposed may play a key role in AD-associated neuronal deficits [65]. Another study showed that both truncated apoE4 (delta279-299) and full-length apoE4 overexpression caused tau phosphorylation at Ser202 sites by the activation of GSK-3β in the murine neuroblastoma N2a cell line [66].

The notion of a potentially pathological role of neuronal apoE4 expression was supported by results generated by overexpression of the apoE4 isoform specifically in neurons (under the Thy1 promoter) resulting in tau hyperphosphorylation in two independent Thy1-ApoE4 transgenic mouse lines [67]. More recent studies in which P301S tau mice were crossed to human *APOE* knock-out (KO) or knock-in (KI) mice showed higher tau levels and a redistribution of tau from the axons to the neuronal soma in P301S/E4 mice compared to P301S/E2, P301S/E3 and P301S/EKO [68]. The authors attributed their findings to a toxic gain of function of the apoE4 isoform, which led to tau pathogenesis, neuroinflammation and tau-mediated neurodegeneration independent from Aβ. Whether the toxic gain of function observed in this particular animal model resulted from the generation of toxic apoE4 fragments remains to be investigated.

Taken together, experimental evidence supports a direct association between the apoE4 isoform and GSK-3β mediated tau hyperphosphorylation leading to subsequent tau pathology. This cascade may be further amplified if apoE4 affects the distribution between intra- and extracellular pools of α-syn where increased levels of the latter would also promote tau phosphorylation via activation of GSK-3β.

Conclusions

Neurodegenerative diseases are multifactorial, characterized by abnormal protein deposition, and showing high degrees of co-pathologies and overlaps in clinical presentation, which presents a challenge for differential diagnosis. The relationships between genetic risk factors and the different proteinopathies are still not well known. In particular, tau PET imaging is still a young research field [69], and no PET tracer is yet available to visualize α-syn inclusions, although this is a very active field of research. In addition, PET imaging of activated astrocytes and microglia will help to characterize the role of inflamma-

tion and genetic risk factors on the presence and brain distribution of proteinopathies. Future studies should incorporate multiple fluid and imaging biomarkers in longitudinal studies of different neurodegenerative disease cohorts. These studies will help to elucidate the underlying mechanisms by which *APOE* ε4 and other genetic factors may predispose individuals to neurodegenerative dementias.

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Amyloid-β and Tau at the Crossroads of Alzheimer's Disease

16

Gilbert Gallardo and David M. Holtzman

Introduction

Alzheimer's disease (AD) is the most common form of dementia characterized neuropathologically by senile plaques and neurofibrillary tangles (NFTs). Early breakthroughs in AD research led to the discovery of amyloid-β as the major component of senile plaques and tau protein as the major component of NFTs. Shortly following the identification of the amyloid-β (Aβ) peptide was the discovery that a genetic mutation in the amyloid precursor protein (APP), a type1 transmembrane protein, can be a cause of autosomal dominant familial AD (fAD). These discoveries, coupled with other breakthroughs in cell biology and human genetics, have led to a theory known as the "amyloid hypothesis", which postulates that amyloid-β is the predominant driving factor in AD development. Nonetheless, more recent advances in imaging analysis, biomarkers and mouse models are now redefining this original hypothesis, as it is likely amyloid-β, tau and other pathophysiological mechanism such as inflammation, come together at a crossroads that ultimately leads to the development of AD.

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Alzheimer's Disease Introduction (History)

In 1901, Alois Alzheimer first examined Auguste D., a 51-year-old woman at the Frankfurt hospital who was admitted after presenting symptoms of disorientation, paranoia, delusions, hallucinations, psychosocial incompetence and cognitive impairment [1]. Over the ensuing years, Dr. Alzheimer continued to evaluate Auguste D. until her death on April 8, 1906 in Frankfurt. In November of 1906, at the 37th Conference of South-West German Psychiatrists in Tubingen, Dr. Alzheimer presented his clinical studies on Auguste D., where he correlated her neuropsychological symptoms with the neuropathology that consisted of "miliary foci" and "neurofibrils" in the cerebral cortex [1]. These pathological hallmarks would later become known as amyloid plaques and neurofibrillary tangles. The disease itself would become known as Alzheimer's

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disease (AD) used initially to describe presenile dementia.

For the better part of the century, research into AD lay dormant until the mid-1980s. Around this time, it was discovered that the Aβ protein was the predominant pathological component in amyloid plaques isolated from AD and Down syndrome patients [2–4]. Because of this link, it was subsequently hypothesized that the gene encoding the Aβ protein might be located on chromosome 21, given that complete trisomy of chromosome 21 was known to be the cause of Down syndrome. It was also known that Down syndrome patients develop early amyloid plaque pathology, with the vast majority developing dementia by the age of 50 [5, 6]. This hypothesis held to be true as it was discovered that the APP gene encoding a larger type 1 transmembrane protein, from which the Aβ peptide is excised, was indeed located on chromosome 21 [7, 8]. These early breakthroughs in AD research led to the origins of the "amyloid cascade hypothesis" [9]. The amyloid cascade hypothesis posits that amyloid-β aggregation and/or deposition is the starting point of AD development that initiates all associated pathophysiological events. These pathophysiological events include tau neurofibrillary tangles, synaptic dysfunction, inflammation, neurodegeneration, vascular abnormalities and dementia. Following this logic, therapeutic interventions targeting Aβ should ultimately prevent or cure AD-related dementia.

Although the amyloid cascade hypothesis for AD has been highly influential in guiding research toward understanding the biological and pathological roles of Aβ peptide and its precursor protein, its dominance in the field over the past two decades has been criticized. Because of this dominance, the majority of research has emphasized therapeutic strategies aimed at either decreasing Aβ production and aggregation or enhancing Aβ clearance and plaque burden. To date, large clinical trials targeting Aβ have not met their primary endpoints [10]. To some people, these failures in clinical trials have brought to question if Aβ is genuinely the preeminent disease causing agent and have essentially nullified the amyloid cascade hypothesis. On the other hand, the failure of multiple clinical trials to prevent cognitive decline or improve cognitive parameters when targeting Aβ suggest several possibilities. First, the treatments used in trials that have gone through phase III without hitting their primary endpoints have not effectively removed Aβ from the brain. Second, they targeted Aβ ~25 years after it started accumulating when it is likely other mechanisms driving disease [11]. Third, while Aβ may be critical in initiating the process that we ultimately know of as AD, it may serve as a catalyst that initiates a series of pathophysiological events in a time-dependent manner that also includes neurofibrillary tangles. Therefore, consistent with the Aβ hypothesis, Aβ may still be the predominant initiating factor but targeting Aβ production or eliminating amyloid plaques alone may not be sufficient to halt downstream events once significant Aβ accumulation has already taken place. We will review AD pathology and discuss the relevance of Aβ, its influences on tau pathology, and the evidence that either supports or refutes the amyloid cascade hypothesis.

Neuropathological Features of AD

Amyloid Plaques

The defining neuropathological features of AD include senile plaques extracellularly and the intracellular neurofibrillary tangles (NFTs). The primary component of amyloid plaques is the accumulation of aggregated non-fibrillar, fibrillar, or oligomeric Aβ peptide. In AD, there are two types of Aβ plaques; diffuse Aβ plaques and the dense-core plaques. The differences between the two are based on their staining properties with the dyes Thioflavin-S and Congo Red that are specific for the β-pleated sheet conformation [12–14]. Diffuse amyloid-β plaques are Thioflavin-S negative plaques and are commonly present in the brains of cognitively healthy adults.

The dense-core plaques are composed of Aβ fibrils, which are Thioflavin-S positive and are most often found in AD patients [12]. Dense-core plaques are associated with neurotoxicity as they are commonly surrounded by synaptic loss, reactive astrocytes, and microglia. The dense-core plaques also associate with dystrophic neurites, which are distortions in neuronal axons or dendritic processes that contain tau paired helical filament (PHF), also referred to as neuritic plaques. Aβ peptide not only accumulates in senile plaques but also in arteriolar blood vessel walls in the form of cerebral amyloid angiopathy (CAA) [15]. While the contribution of CAA pathology in the development or progression of dementia in AD is not completely clear, it some cases it leads to lobar hemorrhages and likely contributes to cognitive decline in AD when present.

Neurofibrillary Tangles

The neurofibrillary tangles are mainly composed of aggregated hyperphosphorylated tau that ultrastructurally appears as PHF [16, 17]. Tau is a microtubule binding protein that is predominately localized to axons of neurons under normal conditions [18]. Under pathological conditions, tau forms PHF in both AD and a host of neurodegenerative diseases known as tauopathies [19]. There is strong evidence that the hyperphosphorylation of tau plays a pivotal role in the pathogenesis of tau, as phosphorylation liberates tau from microtubules, enabling it to aggregate and translocate to the neuronal processes or cell body [20–22]. In AD, PHF aggregation in the neuronal processes that is associated with neuritic plaques is referred to as neuropil threads [23, 24]. These neuropil threads are linked to profound alteration in the neuronal cytoskeleton [25]. As opposed to the tau pathology in the dendritic and axon processes, the accumulation of PHF in the cell bodies of neurons form the neurofibrillary tangles (NFT) [16]. In AD, the different types of NFTs that are present include pretangles, intracellular tangles (globose or flame shape) or extraneuronal (ghost) [26]. Pretangles are defined by diffuse, punctate tau staining within the cytoplasm of neurons that have well-preserved processes. The pretangles may give rise to the formation of mature intraneuronal fibril tangles consisting of aggregated pathological tau in the neuronal soma that is accompanied with dendritic abnormalities. Ghost tangles are a result of the death of neurons containing NFTs and distinguished by the absence of a nucleus or cytoplasm [26].

The Genetic Basis of AD and the Amyloid Cascade Hypothesis

The Aβ peptide or alternative APP fragments derive from sequential proteolytic cleavage of the amyloid precursor protein (APP), by either α-secretase or β-secretase generating a soluble carboxyterminal fragment (CTF) through either an amyloidogenic processing pathway or a non-amyloidogenic processing pathway respectively. Under normal physiological conditions, 90% of APP is cleaved by the α-secretase, which is non-amyloidogenic as it generates a sAβPPα and an APP CTF83. The CTF83 is cleavable by γ-secretase, which generates a small p3 peptide. The amyloidogenic processing pathway is generated by β-secretase cleavage, resulting in a sAPPβ and a CTF99 fragment that is targeted by γ-secretase at distinctly different sites than the CTF83. This generates different Aβ peptide isoforms 38–43 amino acids in length. Under non-pathological conditions, the predominant isoform is the Aβ40 peptide whereas in AD there is an increase of the Aβ42 peptide, which displays a higher propensity to aggregate.

The discovery that the APP gene localizes to chromosome 21 and the striking resemblance of AD-like neuropathology seen in Down's syndrome played a pivotal role in providing the first genetic evidence for Aβ as the primary agent in AD development. This knowledge lead to the

speculation that perhaps either increased APP expression, mutations in the APP gene, or alteration of APP processing leads to higher Aβ levels and may be the initiator of AD development. Subsequently, missense mutations in the APP gene were indeed identified to be associated with AD and CAA [27–31]. Moreover, mutations in the APP flanking the C-terminus cleavage sites that regulate where Aβ is cleaved from APP lead to an increase of Aβ42 to Aβ40 ratio [32, 33]. This evidence for a higher ratio of the Aβ42 vs. shorter species supported the amyloid cascade hypothesis as the Aβ42 species is more fibrillogenic and is the predominant form in amyloid plaques because of it's propensity to aggregate.

Coinciding with the discovery that an increase in Aβ production and its aggregation are the initiating steps of AD was the discovery of mutations that lead to an excessive production of all Aβ species and are associated with both AD and CAA [34–37]. In addition to mutations, duplication of the APP gene was also discovered in five families with an autosomal dominant early-onset AD as well as a mutation identified near the β-secretase cleavage sites of APP found in a Swedish family [37]. In striking contrast, a mutation only two residues away from the β-secretase cleavage site of APP (Iceland mutation A673T) attenuates the β-secretase interaction [38]. This attenuation significantly lowers Aβ production and thus is protective against sporadic AD (sAD).

In addition to mutations that alter the production or the ratio of the amyloidogenic Aβ42 species, other mutations located within the Aβ-peptide coding region have also been identified in fAD [39]. These point mutations within the Aβ-peptide which are C-terminal to the α-secretase cleavage site increase the propensity of Aβ to form protofibrils or enhances oligomerization. Some evidence suggest these Aβ species are more toxic to neurons [40–42].

Additional support for the involvement of the amylogenic pathway in AD development comes from genetic linkage studies that identified mutations in the presenilin 1 and its homolog, presenilin 2, genes (*PSEN1* and *PSEN2*), both of which are associated with early-onset fAD [43–46]. The *PSEN1* and *PSEN2* genes encode for the polytopic transmembrane protein that forms the catalytic subunits of the γ-secretase complex [47]. This complex is a heterotrimeric complex composed of nicastrin, PEN2, and anterior pharynx defective 1 [48, 49]. The γ-secretase complex belongs to a family of intramembranous aspartyl proteases that cleave transmembrane proteins. Among the γ-secretase complex substrates is the APP transmembrane protein from which γ-secretase activity is necessary for the production of Aβ peptide. Similar to mutations in *APP*, point mutations in the *PSEN1* and *PSEN2* either increase Aβ peptide production or alter the ratio of Aβ species being made and produced in favor of fibrillogenic Aβ42 species.

Aside from mutations related to Aβ processing, the most significant genetic modifier of the late-onset AD is the ε4 allele of apolipoprotein E (*APOE*) [50]. There is strong evidence supporting that the ApoE protein influences amyloid-β plaque deposition [51]. In humans, there are three common alleles of the *APOE* gene (ApoE2, ApoE3, and ApoE4). Carriers of the ApoE4 allele display a dosage response for increased risk of developing AD (~3.7 fold increase for 1 copy of apoE4 and ~12 fold increase risk for 2 copies of apoE4 relative to 2 copies of the apoE3 allele, www.alzgene.org) [52, 53]. ApoE4 carriers display an earlier age of onset for clinical dementia and these patients also show an increased amyloid plaque burden, which is in alignment with the amyloid cascade hypothesis [50, 52]. Studies in genetically manipulated mice expressing human APP mutations and ApoE isoforms provided the first evidence that the ApoE protein influences amyloid-β fibrillogenesis in an isoform-specific mechanism [54, 55]. The expression of ApoE4 displays the highest propensity to induce Aβ plaque deposition whereas endogenous ApoE mouse knockout significantly diminished the fibrillary Aβ plaque deposition [54]. Intriguingly, effects of ApoE do not appear to be restricted to Aβ deposition, as recent studies have demonstrated ApoE4 influences tau pathogenesis

and tau-mediated neurodegeneration independent of amyloid-β by exacerbating a neuroinflammatory response [56]. However, in agreement with the amyloid cascade hypothesis, the genetic evidence appears to support that Aβ deposition is the preeminent initiating factor that leads to AD dementia. The summation of genetic studies suggests either overproduction, altered processing of Aβ42 or increased Aβ/decreased Aβ clearance as the initiators of AD.

The Correlations of Amyloid-β and AD Development

Cross-sectional studies in fAD patients have also provided evidence that abnormal production of Aβ is the initiating factor of AD development. These studies were pioneered particularly by the Dominantly Inherited Alzheimer's Network (DIAN), which took advantage of the fact that the age at which clinical onset occurs for dementia in fAD patients is similar between generations [57]. This parity in the age of onset across generations enabled a pathological timeline to be formed and comparisons of brain atrophy, biochemical measurements of Aβ peptide and tau protein in the cerebrospinal fluid (CSF), positron emission tomography (PET) for fibrillary Aβ-deposition, and cognitive assessment to be made between carriers and non-carriers within families. The first abnormality detected was the levels of Aβ42 in the CSF, which begin to decline from an elevated level ~25 years before the expected onset of dementia and continue to decrease over the ensuing years as the disease progresses. Next in the pathological time-line of AD is Aβ deposition, as visualized by amyloid-PET imaging that occurs within a 10-year period from the decline of Aβ42-CSF levels. The decline in Aβ42 levels in CSF levels is thought to reflect amyloid-β42 sequestration in plaques in the brain [58]. The deposition of Aβ closely coincides with elevated tau and phosphorylated tau levels in the CSF approximately 3–5 years before cognitive decline [59, 60]. The changes in tau CSF levels coincide with cognitive decline, hippocampal atrophy, and cortical hypometabolism [11, 61, 62]. These analyses infer that a genetic mutation in one of the three genes known to cause fAD predisposes individuals to abnormal Aβ processing that is first detectable by a marked decrease in Aβ42 in the CSF followed by the appearance of amyloid plaque deposition, then by change in tau that coincide with neurodegeneration. Thus, abnormal Aβ production is the first of many pathophysiological events in AD development that spans over several years.

In additional longitudinal studies, the amyloid burden in MCI patients seems to predict cognitive decline over the ensuing years [63–65]. This is probably because it reflects those whose cognitive impairment is due to AD vs. other causes which in general, don't progress as fast. In addition to this evidence of a link between amyloid presence and cognitive decline in AD, amyloid-PET imaging studies have revealed a strong correlation between amyloid-β burden and brain atrophy in AD [66–69]. Moreover, individuals that are clinically normal but display amyloid deposition by PET imaging tend to perform lower in cognitive assessments and are associated with grey matter atrophy within the hippocampus and the posterior cingulate [70–73]. This finding suggests Aβ deposition is linked with a pathological state, which primes vulnerable neurons for tau pathogenesis that ultimately drives disease progression.

Amyloid-β Is Unlikely to Drive AD Development Alone

While these studies illustrate that abnormalities in Aβ processing precedes all other AD-related pathologies, several other studies have demonstrated there are disconnects between Aβ plaque deposition and the other pathological events in AD. These studies argue Aβ may be necessary but not sufficient to lead to marked synaptic and neuronal loss. During the course of AD, neuronal cell death begins in the entorhinal cortex and

spreads to the hippocampus and then the neocortex, whereas Aβ plaque deposition originates within the neocortex and spreads inwardly from there [14, 74]. Differing from the pathology of Aβ plaque deposition, postmortem histopathological studies have revealed that tau pathology closely parallels neuronal loss, as tauopathy first appears in the entorhinal cortex and spreads into the hippocampus and then the neocortex [75–77]. There is also a steady increase in tauopathy with normal aging, at least in parts of the medial temporal lobe, thus correlating increased tauopathy with the rate and progression of age-related cognitive decline whereas Aβ plaque deposition plateaus predominantly in the preclinical period of AD [78]. Supporting this, more recent PET imaging tau studies have also revealed tau pathology and cognition are much more closely linked than cognition and amyloid-β plaque deposition [79–81]. These studies suggest tau pathology and its progression is likely responsible for cognitive decline in AD. In addition, the severity of temporal lobe tau pathology is sufficient to predict the levels of cognitive dysfunction in early disease stages of AD, unlike Aβ plaque deposition [80]. Intriguingly, phosphorylated tau is also present within the brainstem nuclei and the coeruleus/subcoeruleus complex in otherwise healthy young adults under the age of 30 with the absence of Aβ plaque pathology [82]. Thus, some tau pathology occurs in everyone with normal aging so that the progression of tau pathology from the brainstem and medial temporal lobe to other brain regions may be driven by Aβ pathology in AD. Although, it is important to note the basis of these studies are neuropathological and are lacking biochemical analysis for oligomeric Aβ or other amyloid-β species known to influence tau phosphorylation [40–42]. Nevertheless, these anatomical and temporal mismatches between Aβ pathology and neuronal cell death indeed demonstrates that there is much more complexity in pathophysiological events of AD, supplementing the argument that Aβ plaque deposition alone is not sufficient to initiate the neurodegenerative process as has been proposed.

Additional evidence that argues Aβ production and or deposition is not the preeminent factor leading to dementia is found in fAD patients. In fAD, individuals are predisposed to abnormal production of Aβ from birth, yet it seemingly takes several decades before the complex symptomatology of AD to manifest. This disparity in age with the onset of disease does not imply Aβ is not neurotoxic but instead implies other pathophysiological factors in the aging process are necessary to decrease neuronal health. However, the age of onset is much earlier in fAD cases as opposed to sporadic AD cases, which suggests that there may be a threshold of the amyloid burden that is necessary before the disease is initiated.

Perhaps most perplexing and most challenging for the amyloid cascade hypothesis is the fact that normal individuals may display substantial Aβ plaque deposition [83, 84]. This may be because it takes time for Aβ accumulation to lead to enough downstream events and damage to lead to cognitive decline. These observations set an intriguing conundrum; on one hand, for a definite diagnosis of a dementia-related to AD, it is necessary to have Aβ plaque deposition. On the other hand, it is possible for otherwise healthy individuals to be amyloid positive without the presence of dementia either at autopsy or by PET imaging. However, AD is an age-dependent neurodegenerative disease and one might be poised to conclude healthy individuals that present amyloid-β plaque pathology are on course to developing AD-related dementia which in fact appears to be the case from longitudinal biomarker studies [85]. Coincidently, cross-sectional biomarker studies spurred from the Alzheimer's Disease Neuroimaging Imitative (ADNI) suggest that the presence of amyloid-β plaque deposition in otherwise healthy individuals are not necessarily asymptomatic, as the presence of Aβ plaques deposits in these individuals increases the risk for developing dementia by ~four-fold in the ensuing years [86–88]. Elderly individuals that are Aβ plaque-positive but otherwise cognitively normal also display accelerated brain atrophy in the cor-

tex and hippocampus [67]. In a more recent study, Alzheimer Disease Neuroimaging (ADNI) enrolled participants that displayed normal cognition but were positive for brain amyloid. These patients showed a faster decline in cognition, brain volume, and brain glucose metabolism than individuals without amyloid pathology within a four-year span [89]. These studies suggest the amount of amyloid plaque in otherwise cognitively normal individuals have not yet reached a certain amyloid threshold or damage downstream of amyloid that is necessary to induce the clinical symptoms. It appears that tau pathology is likely one of the critical downstream factors.

Tau Biology and Tau Pathogenesis

As stated above, tau is a microtubule-associated protein that plays an essential role in microtubule stability. Phosphorylated tau has been identified as the main component of NFT in AD as well as in a host of neurodegenerative diseases such as progressive supranuclear palsy, corticobasal degeneration (PSP), Pick's disease (PiD), cortical basal degeneration (CBD) and certain forms of frontotemporal dementia (FTDP). The tau gene consists of 16 exons and the alternative splicing of exons 2, 3, and 10 generates six major tau protein isoforms. Exons 9–12 encode the four microtubule binding repeats, giving rise to tau protein isoforms with four or three repeats (4R and 3R). Tau isoforms also differ by the presence of zero, one or two inserts of a 29-amino-acid sequence (0 N, 1 N, and 2 N) in the amino-terminal half. In the adult human brain, there is an equal molar ratio in the three-3R and the three-4R isoforms. In AD and FTDP, there is an equal 3R:4R ratio, whereas PSP and CBD predominately display 4R tau inclusions.

Structurally, tau is composed of four domains the C-terminal, N-terminal, a proline-rich domain, and microtubule binding domain (MBD). These domains all play a role in physiological and pathological conditions. In normal conditions, the N-terminal domain known as the phosphatase activation domain is negatively charged and generally hidden in the native protein. However, under pathological conditions, the N-terminal domain of tau becomes exposed which is thought to be an early event in tau pathogenesis [90, 91]. Studies of the C-terminal domain suggest it may play a role in inhibiting tau polymerization, whereas truncation of the C-terminal domain facilitates tau pathology [92]. The MBD domain and the proline-rich domain are involved in protein-protein interactions and signaling through the PxxP motifs that are within the proline-rich domain and that interact with SRC kinases.

During the course of AD, several post-translational modifications of tau occur, the most notable of which is phosphorylation at numerous sites [20–22]. The phosphorylation of serine and threonine residues at sites immediately adjacent to or within the MBD alters its confirmation. This alteration liberates tau from the microtubules, leading to the accumulation in the somatodendritic compartment of pair helical filaments and other abnormal conformations. Neuropathological studies of post-mortem AD patients provided the first evidence that tau tangles occur hierarchically, with the first appearance in the transentorhinal cortex (Braak stages I-II) [14, 74]. Neurons associated with these tangles give rise to the perforant pathway, the major projections to the hippocampus where tau pathology gradually advances into CA1 region (Braak II). Next, tau tangles develop in the limbic structures of the subiculum and inferior temporal neocortex (Braak III) the amygdala and thalamus (Braak IV) finally spreading into the neocortex (Braak V-VI) during the process of AD.

The hierarchical pattern of neurofibrillary tau accumulation is consistent with transmission of tau fibrils demonstrated in mouse models of tauopathy. Injection of brain extracts from diseased tau transgenic mice into the cortex or hippocampus of mice expressing human wild-type tau induced the formation of wild-type tau fibrils that spread to distant neurons [93]. Similarly, the

injection of tau oligomers isolated from AD brain subjects or preformed synthetic tau fibrils into the hippocampus or striatum of tau transgenic mice induce neurofibrillary inclusions that propagated from the injected site to connected brain regions in a time-dependent manner [94]. These studies suggest that pathological tau can propagate and spreads pathology through interconnected brain regions.

Amyloid-β Deposition Influences Tau Pathology

Although tau pathology is more closely associated with cognitive decline and neuronal cell loss then amyloid-β plaque deposition in AD, tau tangles are commonly observed in the brainstem and medial temporal lobe of older individuals who are cognitively normal with no evidence of neurodegeneration. These findings suggest tau tangles alone do not necessarily lead to the development of AD [82]. In AD, where Aβ deposition and tau tangles are both present, neurodegeneration does occur throughout several brain regions, including the hippocampus [95, 96]. This further enforces the suggestion that the combination of Aβ plaques with tauopathy are necessary for the AD degenerative process. Pathologically, there is also evidence to suggest Aβ plaque burden exacerbates downstream tau pathology, as there is a strong correlation between the severity of Aβ pathology and the severity of tau pathology in clinically demented AD patients [80]. Additionally, neuritic plaques that consist of Aβ plaques associated with neurofibrillary tau pathology correlate with neuronal loss and dementia [97]. There is also evidence suggesting the Aβ plaque burden in non-demented cases is associated with the development of dystrophic neurites within neuritic plaques [78]. These cases display an increase in both the number of tangles and rate tangle formation with age. Collectively, the human neuropathological data is supportive of the amyloid cascade hypothesis as it suggests the Aβ plaque deposition exacerbates tau pathology despite an anatomical discord. However, it is important to emphasize that mutations in the tau gene are capable of leading to neurodegenerative diseases independent of Aβ although tau filament ultrastructure and the cell type in which tau pathology occurs as well as anatomical distribution differs from AD [19].

Amyloid-β Deposition Favors Tau Pathology

It is evident from the human neuropathological data that AD is a dual proteinopathy, needing both amyloid plaque deposition together with tauopathy to progress. In early disease, the NFT formation is restricted to the medial temporal lobe. As the disease progresses, tau pathology spreads outside the temporal lobe into the neocortex including many areas filled with Aβ plaques. These findings tend to support that Aβ deposition creates an environment that promotes the formation of tau pathology in line with the amyloid cascade hypothesis for AD.

Further evidence for Aβ deposition influencing tau pathology has come from mouse models of tauopathy. The injection of synthetic Aβ fibrils into the hippocampus of transgenic mice expressing human mutant tau-induced tau pathology in the hippocampus that spread to brain connected regions including the entorhinal cortex and amygdala [98]. Similarly, the injection of Aβ containing extracts from aged transgenic mice overexpressing human APP (hAPP) which harbor mutations that cause fAD, also induced tau pathology in transgenic tau mice [99]. Characteristic to human AD pathology, the injection of Aβ induced tau pathology spatially separate from the Aβ injected region. These findings also coincide with the notion that tau protein displays the phenomenon of "spreading" tau pathology in a non-cell autonomous fashion. This "spreading" progresses from cell to cell through the brain in a well-defined pattern, depending on the location where it starts and the specific disease in which it occurs.

Additional evidence that supports the suggestion that Aβ influences tau pathology has come from double transgenic mice that express a human mutant tau together with a human APP gene harboring a double mutation. The double transgenic mice displayed amyloid plaque formation that was indistinguishable from the single hAPP transgenic line, suggesting tau mutations do not necessarily augment Aβ pathology [100]. This finding is consistent with human primary tauopathies that display tau pathology independent of amyloid plaque pathology. However, the double transgenic mice displayed enhanced NFT in the limbic system and olfactory cortex. Additional studies in double transgenic mice expressing human mutant tau and hAPP mutations revealed the NFT formation displays an earlier onset with a more consistent manner of staging that emanated in the entorhinal cortex and proceeds into hippocampus [101](Lee). The double transgenic line also displayed enhancement of argyrophilic ThioS-positive NFT in the hippocampus and cortical regions which contain an abundance of Aβ plaque deposition. Additional studies have also suggested Aβ potentially promotes an environment that is ideal for the development of a distinct tau species that displays enhanced tau seeding [102]. Collectively, these studies in mouse models of tauopathy and amyloidosis suggest Aβ deposition is upstream of NFT formation. Aβ plaques favor an environment for tau pathology and once tau pathology initiates, it is capable of self-propagating through neuronal projections.

While these studies demonstrate that Aβ plaques influence tau pathology, they rely on double transgenic mice overexpressing human tau mutations found in autosomal dominant frontotemporal degeneration. Therefore, the relevance of tau mutations in AD-dependent dementia remains unclear. In a more recent study, to eliminate potential confounding effects of tau mutations, investigators utilized a paradigm that induces tau seeding and spreading in wild-type mice (endogenous mouse tau) by the intracerebral injections of purified human tau from sAD subjects [103, 104]. The intracerebral injections of purified human tau from AD subjects into plaque-bearing transgenic hAPP mice lead to the formation of tau pathology. Moreover, the tau pathology induced resembled human AD pathology because it exhibited the accumulation of tau aggregates in dystrophic neurites surrounding amyloid plaques, NFTs, and neuropil threads. The distinct tau pathologies also displayed a temporal onset with tau pathology first appearing in the dystrophic neurites, followed by NFTs, and subsequently neuropil threads. Additional neuropathological and behavioral analysis suggested the tau aggregates in dystrophic neurites potentially promote the spreading of tau pathology to distal regions whereas NFTs impair neuronal activity leading behavioral abnormalities like anxiety, for example. However, the mechanism by which amyloid plaques induce distinct tau pathologies and the role of non-neuronal cell types including microglia and astrocytes remains mostly unknown.

At the Crossroads of AD

The influence of Aβ deposition on tau pathology does not go only one way, as there is evidence that amyloid-induced neurotoxicity is dependent on tau protein. Primary hippocampal neuronal cultures from rodents exposed to synthetic Aβ fibrils develop hyperphosphorylation of tau protein that accumulated in the somatodendritic compartment, display axonal retraction, loss of synapses and dendritic atrophy within days [105, 106]. However, primary neurons derived from tau knockout mice or antisense oligonucleotide knockdown of tau expression protects neurons from the Aβ induced abnormalities [107, 108]. In addition to fibrillar amyloid-induced neurotoxicity, several studies have demonstrated soluble oligomers of Aβ display enhanced neurotoxicity in cell culture that are also dependent on

tau protein [108, 109]. The Aβ-induced abnormalities have also been demonstrated *in vivo* as the intracerebroventricular injection of purified Aβ oligomers into adult rodents blocked long-term potentiation (LTP), a model of memory [40]. The Aβ mediated blockage of LTP appears to be dependent on tau protein as acute slice preparation from tau knockout mice prevented the Aβ mediated blockage of LTP [110]. These studies suggest Aβ and tau work together to drive the neuronal abnormalities, and the combination of both induces synaptic dysfunction *in vivo*.

The dependence of tau in Aβ-induced neurotoxicity is also evident in transgenic mice expressing familial AD mutations in hAPP [111, 112]. One line of transgenic hAPP mice display aged dependent deposition of Aβ plaques, spatial learning deficits, hyperactive, and premature death. The genetic crossing of hAPP mice for either homozygous or heterozygous null of the tau allele protects against these behavioral abnormalities. There is a restoration of learning and memory deficits and survival is prolonged without altering the Aβ deposition. These studies imply that over-expression of APP or one of its products such as Aβ initiates a pathophysiological cascade of events leading to behavioral abnormalities that are dependent on tau protein. In addition, tau knockout mice appear to be protected against PTZ induced seizure activity in wild-type APP mice, consistent with the notion that tau protein is capable of modulating synaptic activity. In a more recent study, the hAPP mice expressing human mutant PS1 that display memory impairments, loss of synapses and premature death were also rescued by homozygous tau knockout. However, this particular study differed in which deletion of the tau gene decreased the Aβ deposition, suggesting tau pathology may influence the amyloid burden [113].

Other Pathophysiological Mechanisms That Contribute to AD

In addition to Aβ and tau, emerging evidence suggests other neuronal stressors such as neuroinflammation play a role in AD development. Reactive astrocytes, microglia, and elevated levels of proinflammatory molecules span across the course of the disease that may influence either Aβ plaque clearance or deposition and promote tau pathogenesis. Further evidence has come from whole exome sequencing studies that have implicated the innate immune system as a critical component of AD pathogenesis. Most notably, are the recent findings of rare variants in the microglial-expressed gene TREM2 (Triggering Receptor Expressed on Myeloid Cells2) that increase the risk of developing AD by 2–4 fold [114, 115]. The expression of TREM2 in microglial appears to regulate the microglial-mediated phagocytic clearance of cellular debris and their responsiveness to brain insults [116, 117]. Several studies from mouse models of amyloidosis and postmortem human brain sections have demonstrated TREM2 variants associated with AD, attenuate the microglia responsiveness to Aβ plaques suggesting these variants results in a loss of function of TREM2 [118–121]. Notably, the loss of microglia surrounding the plaques seems to promote diffuse star-shaped amyloid fibrillary plaques that tend to be associated with neuritic dystrophy [120, 121]. Thus, potentially early in disease the microglial responsiveness to Aβ plaques plays a protective role by lessening the impact of Aβ-induced neurotoxicity by trimming plaques or forming a barrier between the plaques and the surrounding neurons. However, additional studies have suggested microglia potentially influence the spread of tau pathology by phagocytosing and exocytosing tau protein [122]. Moreover, microglia deficient in the microglia fractalkine receptor that promotes microglia activation appears to facilitate tau pathology in tau

transgenic mice [123, 124]. While the role of microglial response in AD is complex, microglial appear to be influencing Aβ deposition early in AD development potentially in a protective role, as the disease progresses the amyloid plaque burden exceeds a threshold that induces tau pathology promoting a pathological role for microglia in the spreading of tau pathology.

Reactive astrocytes are also a well-known feature of AD that co-localize with Aβ plaques, NFT, and brain atrophy in postmortem brain tissue [119, 125]. Both Aβ and tau pathology are also commonly found intracellularly in astrocytes and in non-AD dementias, astrocytes may contain tau pathology with distinct characteristics that are pathological hallmarks for many of the primary tauopathies [126, 127]. Although, the mechanisms for either Aβ/tau uptake or the cellular effects for the accumulation of pathological Aβ/tau in astrocytes remains mostly unexplored. However, studies have illustrated the exposure of Aβ fibrils to astrocytes activates an NFkB-mediated response that releases the complement protein C3, which is capable of disrupting dendritic morphology [128]. Both the activation of NFkB and C3 protein are associated with AD development and the inhibition of C3 in mouse models of amyloidosis reduce synapse loss and rescues cognitive impairments [128–130]. Additionally, recent studies have identified a minimum of two different forms of reactive astrocytes based on gene expression profiles [131]. One subtype of reactive astrocytes appears to display a potential reparative role, while a second subtype adopts an aggressive toxic gain of function characterized by an upregulation of several complement cascade genes and neurotoxins detrimental to synaptic integrity, neuronal health, and survival. These disease-associated astrocytes termed "A1", are present in postmortem AD brain tissue and have been recently described in transgenic mice expressing human tau [56, 132]. Although, these studies demonstrate a compelling association between AD and reactive astrocytes, their impact on disease pathogenesis and their therapeutic potential remains largely unknown.

Concluding Remarks

Overwhelming evidence suggests AD is a dual proteinopathy in which Aβ deposition and the accumulation of aggregated tau drive AD pathogenesis. Although there is much clarity needed before fully understanding the relationship between Aβ and tau, it still appears amyloid-β pathophysiological effects occur early in AD development, which seems necessary but not sufficient for priming vulnerable brain regions for the intrusion of tau pathology. It is likely the Aβ deposition exacerbates tau aggregation despite the temporal and spatial separation, which plays an important role in damaging axons, dendrites, synapses, and cellular function and that ultimately leads to neuronal cell death.

In addition, the recent advances in basic research, genetics, and biomarkers are demonstrating AD is a multifaceted degenerative process with potentially several pathophysiological mechanisms. Likely, targeting one protein, one gene or one cell type that is selective for one pathophysiological mechanism may not suffice to prevent the degenerative process of AD. Therefore, it is imperative for understanding the molecular mechanism that regulates Aβ deposition, its influences on tau pathology and the spreading of tauopathy. Moreover, as emerging evidence now suggests neuroinflammation plays a prominent role in the neurodegenerative process of AD and altering the neuroinflammatory response may be beneficial (Fig. 16.1).

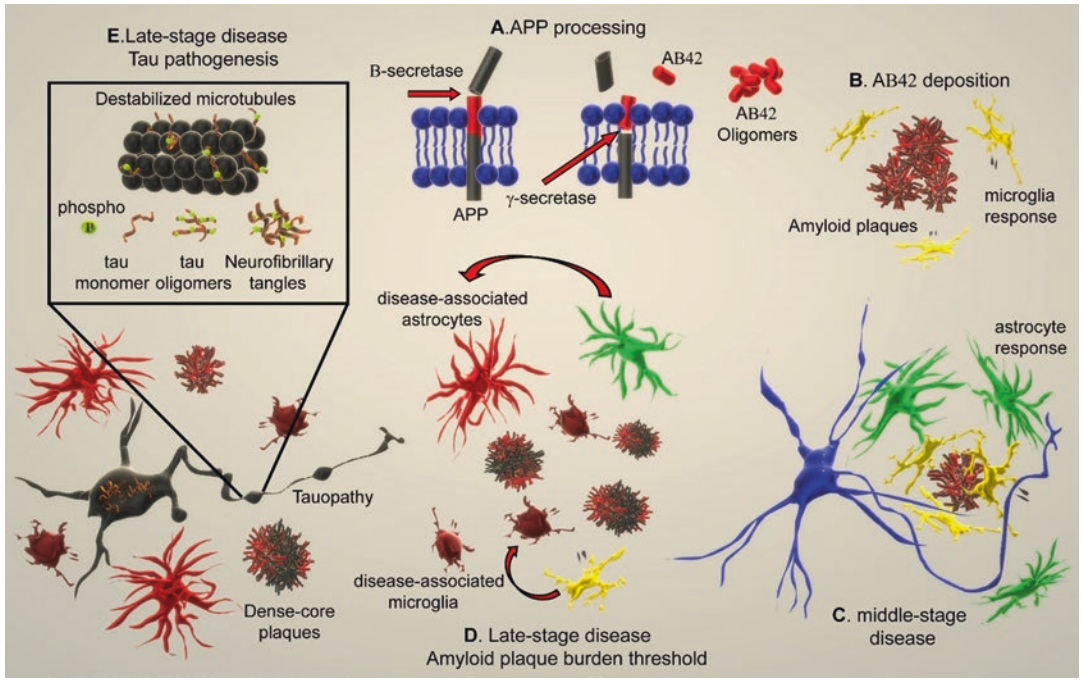

Fig. 16.1 Model of Alzheimer's disease pathology and progression. (**a**) The first pathological event in AD development is the production of the Aβ42 peptide produced from the transmembrane protein APP via cleavage by two enzymes, β-secretase and γ-secretase. (**b**, **c**) Extracellular Aβ42 peptide forms Aβ42 oligomers and initiates Aβ plaque deposition. The Aβ plaque formation leads to a microglial response, which surround the plaque and recruits astrocytes. The glia response appears to be protective by altering Aβ plaque structure and forming a barrier between the plaques and the surrounding neurons lessening the impact Aβ-induced neurotoxicity. (**d**) As the disease progresses the Aβ plaque deposition reaches a threshold forming the dense-core plaques that are associate with dystrophic neurites and influences tau pathogenesis. In late-stage AD, disease-associated microglia may facilitate tau pathogenesis by phagocytosing and exocytosing tau protein and promoting a neurotoxic inflammatory response. In addition, during the late-stage of disease the disease-associated astrocytes exacerbate tau pathology and neurodegeneration by potentially secreting neurotoxic factors. (**e**) Tau pathology spreads to connect brain regions, which drives the neurodegenerative process

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Part IV

Tauopathies; Pathology, Drivers, and Marker

Myotonic Dystrophy: an RNA Toxic Gain of Function Tauopathy?

17

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Dystrophia Myotonica: an RNA Gain of Toxic Function Disease

Myotonic Dystrophy (DM) also referred to as Steinert's disease or DM of type 1 (DM1) is a rare inherited multisystemic genetic disease affecting multiple organs [33]. Worldwide in its most common form, the adult form, DM prevalence is of 1 per 8000. Major symptoms include myotonia, progressive muscle wasting, cardiac conduction defects, endocrine deficiencies as well as cognitive impairments. DM1 is an autosomal inherited genetic disease caused by an unstable expanded CTG repeat sequence located in the 3'UTR of the *DMPK* (dystrophia myotonica protein kinase) gene [8]. The normal allele contains 5–30 repeats whereas over 50 CTGs repeats the unstable mutation is pathogenic. A second gene mutation is responsible for myotonic dystrophy of type 2 (DM2) also known as proximal myotonic myopathy (PROMM). The repeat expansion is a tetranucleotide motif $(CCTG)_n$ ranging from 100 to 11,000 repeats in the first intron of the *CNBP* gene also known as *ZNF9* gene [53]. Both pathogenic repeat expansions are in non-coding region of unrelated genes. However, *DMPK* and *CNBP* are ubiquitously expressed. *DMPK* and *CNBP* expanded alleles are transcribed into RNA but not translated into protein. Mutant RNAs containing expanded $CUG_{(n > 50)}$ or $CCUG_{(n > 75)}$ repeats are defectively exported outside the cell nucleus and consequently accumulate into discrete intranuclear aggregates. Those aggregates composed of mutated RNAs and proteins are referred to as nuclear foci. Expression of expanded CUG repeats in cells [4] or in transgenic animals [56, 76] is necessary and sufficient to reproduce DM1 phenotype suggesting a "RNA toxic gain of function".

Nuclear foci composed of expanded CUGs sequester RNA-binding proteins belonging to the MBNL family of splicing regulatory factors [61]. Nuclear foci are thus labeled by fluorescent in situ hybridization (FISH) using a CAG fluorescent

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RNA probes as well as using antibodies against MBNL [24]. Sequestration of MBNL proteins result in a loss of function and more especially in a splicing misregulation of specific transcripts of tissues affected in DM1. Specific splicing changes in DM1 affected tissues were mainly attributable to the loss-of-function of MBNL1 in the skeletal muscle [42], and both MBNL1 and MBNL2 in the central nervous system and in the heart supporting a functional loss of MBNL family of splicing factors as a central mechanism of RNA gain of toxic function of the CUG repeats in DM1 [13, 31].

This toxic gain-of-function at the RNA level is considered as one of the main pathophysiological hypotheses to explain the multisystemic development of myotonic dystrophy. In addition to the sequestration inside foci of proteins such as members of the MBNL family of splicing factors results in a partial loss-of-function of the MBNL family of splicing factors [42, 45], a gain-of-function of CUG-binding protein and ETR3-like factor 1 (CUGBP1/CELF1) in skeletal or heart muscle, through its stabilization and phosphorylation by PKC, is also instrumental to DM1 pathophysiology [68, 84]. CELF1 and embryonic lethal abnormal vision-like RNA-binding protein-3 (ETR3/CELF2) like factors (CELF) belong to a family of RNA-binding proteins that includes 6 members. While both CELF1 and CELF2 are ubiquitously expressed, the latter is highly expressed in the brain and heart of rodents [47, 48]. The four other members, CELF3, 4, 5 and 6, are enriched in the rodent brain. However, less is known about their expression in the human brain and their potential implication in DM1 pathophysiology.

Also related to the transcription of RNA bearing large tri- or tetranucleotide repeats, an aberrant translational process, named "repeat-associated non-AUG translation" (RAN translation), lead to the translation of DM1 polyglutamine peptides, in human DM1 tissues and mouse models (for review see [16]). However, in DM1 the contribution of the polyamine to the pathology remains ill-defined. Other non-RNA mediated toxic consequences are also reported in DM1. The expanded allele induces an haploinsufficiency of DMPK expression, a defective expression of the surrounding genes such as *DMD* and *SIX5* gene expression, the expression of antisense RNA, microRNA, … that together contribute to the pathophysiology of DM1 (for review see [83]). Moreover, in animal model of DM1 proteomics analyses of the brain also suggest that several molecular deregulations are not directly related to an RNA-gain of toxic function [35]. Thus, the neurological determinism of DM1 and DM2 might be determined by pathological processes shared by these diseases but not yet completely unraveled.

A Multisystemic Neuromuscular Disease with Cognitive Dysfunctions

Adie and collaborators reported in the beginning of the twentieth century the involvement of the central nervous system in myotonic dystrophy [2]. It's only in the 60's that studies including a systematic analysis of brain structures and defective cognitive functions were initiated [28, 55]. Clinical data available describe that DM1 patients experience focused executive and visuoconstructive progressive disabilities [55, 73, 92], frontal cognitive impairment including attentional ability which are worsening with aging [60, 72] together with visual attention, verbal memory, language and executive functions [25]. Impairments of facial emotion recognition is a cognitive deficit trait often reported in the adult form of DM1 [46, 92]. Apart from being principally a muscle disease, DM1 also include a heterogenous but systematic involvement of cognitive brain dysfunctions.

Brain Lesions in Myotonic Dystrophy

Neuroimaging in DM brain provided insights into the effort to link the brain involvement with the cognitive deficits. At the macroscopic level, structural imaging studies using magnetic resonance (MRI) in adult DM1 revealed global

cerebral atrophy with dilated ventricular [6, 12]. Cortical gray matter loss is primarily observed in the frontal, parietal, and occipital lobes and the superior and middle temporal gyrus, whereas the subcortical gray matter loss is detected in thalamic and basal ganglia structures [6, 62, 63, 89]. There was no correlation between brain tissue volumes and the grade of pathology, disease duration or even CTG expansion. However, the areas with grey matter loss are involved in cognitive dysfunctions and personality disorders, such as apathy, depression, anxiety, and deficits in attention, memory, and visuospatial function [6]. MRI examination often reveal hyperintense white matter signals localized principally in the anterior temporal lobe but were originally and etiologically underestimated [30] and latter suggested to be correlated to cognitive impairments [1].

Improvement of brain imaging has provided in depth and extensive description of brain lesions in DM1. Thus, a more widespread cortical and subcortical involvement affecting gray and white matter of all lobes, brainstem and cerebellum is reported (for review see [62]). Moreover, current structural and functional brain imaging network analyses also provide valuable information regarding the link between brain pathology and cognitive impairments. However, up to now the relationship between brain imaging abnormalities and cognitive deficits remains ill-defined and longitudinal studies such as those performed recently for cognitive impairments should be considered [25].

At the neuropathological level, intracellular proteinaceous inclusions in neurons of the thalamus have been reported in DM1 brain [17, 67, 94]. However, the nature of these neuronal inclusions remained unknown. Moreover, their potential incidence upon cognitive functions or other neurological symptoms also remained elusive. Neurofibrillary tangles (NFTs), a neuropathological feature described by Alois Alzheimer [3] are often reported in the limbic system of DM1 patients, including the hippocampus and isocortical areas such as the temporal and frontal cortices [44, 59, 74, 78, 85, 97]. Neurofibrillary tangles were also reported to contain heparan sulfates such as NFT of Alzheimer's disease [78]. Another neuropathological lesion found also in Alzheimer's disease is described in DM1 brain. This neuropathological lesion is the granovacuolar degeneration which are intracytoplasmic vacuoles visualize insides neurons [64, 66, 95]. Lewy bodies made of α-synuclein aggregates were also reported in the substantia nigra of a single case [64]. Interestingly, amyloid deposits or amyloid angiopathy of diffuse amyloid deposition have yet never been reported in DM1 or DM2 [59].

DM1 a Tau Missplicing Tauopahty

NFTs are intraneuronal fibrillar aggregates made of hyper- and abnormally phosphorylated isoforms of microtubule-associated Tau [11, 75]. Tau proteins belong to the family of microtubule-associated proteins. Tau functions are to promote microtubule assembly, their stabilization and their organization, necessary both for axonal transport and neurite outgrowth. Tau proteins are encoded by a single *MAPT* gene located on chromosome 17. It is composed of of 15 exons numbered from 0 to 15 and a canonical and alternative promoter [38]. In the central nervous system, six major isoforms of Tau are expressed and produced by the alternative splicing of exons 2, 3 and 10 [11, 75]. Exon 10 splicing results in the inclusion or exclusion of an additional microtubule-binding sequences leading to Tau isoforms with either three (3R-Tau) or four (4R-Tau) microtubule binding repeats. Thus, Tau proteins with four microtubule-binding domains (4R) bind more avidly and further stabilize microtubules than those with three binding domains (3R).

The alternative splicing of Tau is developmentally regulated, tissue specific and deregulated in several Tauopathies [75]. A single isoform devoid of alternative of exon sequences is express in the foetus whereas alternative splicing occurs post-natally. In DM1, we reported a modified expression pattern of Tau protein isoforms. Instead of the six adult brain isoforms only two isoforms are expressed that are lacking the inclusion of exons 2 and 3 [18, 75]. Tau exon

10 reduced inclusion has also been reported in several brains from DM1 patients [40] as well as in a transgenic animal model of DM1 [32]. However, molecular mechanism leading to the reduce Tau exon-10 inclusion in DM1 and its functional consequences remain unknown. This is of importance since the Tau exon-10 encodes for a fourth microtubule-binding domain. The increased expression of 3R Tau isoforms in DM1 may modify the axonal transport and plasticity, since Tau is suggested to regulate the motor proteins transport along microtubules [22]. Moreover, a growing body of evidences suggests that Tau proteins may also localize to spines of dendrites and regulate glutamatergic transmission [37, 39]. The splicing of N-Methyl D-Aspartate receptor subunit one is altered in DM1 brains [19, 40], which together with Tau mis splicing, may also deregulate the glutamatergic postsynaptic transmission. Thus, reduced Tau protein isoforms expression in DM1 brains and the development of NFT may together contribute the cognitive impairments in DM1.

Tau mRNA alternative splicing is tightly regulated during development. In the human brain, only 3R-Tau is expressed in the fetal stage, while both 3R-Tau and 4R-Tau are expressed in a ratio of approximately 1:1 post-natally and in the adult brain [82]. In DM1, the reversal of the adult Tau splicing pattern to a fetal pattern of Tau splicing is supposed to occur with aging in consequence to the somatic instability of DM1 mutation. Large CTGs expansion are found in the adult brain tissue which may lead to the progressive missplicing of Tau [74].

In frontotemporal dementia, *MAPT* gene mutations is associated with changes in Tau isoforms proportion, leading to the development of NFTs in adulthood (for review see [75]). More than 35 mutations in *MAPT* gene are reported in patients suffering from rare forms of familial frontotemporal dementia with parkinsonism-17 (FTDP-17). Several of these *MAPT* gene mutations located downtstream exon 10 cause aberrant splicing of Tau and leading to a decrease ratio 3R: 4R-Tau [26]. In sporadic FTDP-17 and other Tauopathies, imbalances in Tau isoform ratios are equally observed. For example, Tau aggregates found in Pick's disease consist of 3R-Tau isoforms mainly, whereas in supranuclear palsy, corticobasal degeneration and argirophylic grain dementia, Tau aggregates are mainly composed of 4R-Tau isoforms. It remains unknown whether these specific pattern of pathological Tau proteins are associated with a change of Tau splicing or is it related to selective aggregation of Tau protein isoforms expressed in sub-sets of neuronal populations. A differential splicing of Tau transcript is also reported in Alzheimer's disease [10, 96]. Although speculative, nucleic acid integrity is regulated by Tau and more recently, the nucleotransport have been shown to be disrupted by Tau proteins [23]. Nucleocytoplasmic transport disruption is supposed to be a common mechanism to several neurodegenerative diseases including amyotrophic lateral sclerosis and frontotemporal lobar degeneration [98]. Tau contribution to this defective nucleocytoplasmic transport brings novel insights for understanding the pathophysiology of neurodegenerative diseases, although the specific contribution of Tau isoforms remains to be determined.

Several RNA splicing factors regulate alternative splicing of Tau [5, 54] for review see [70]). They bind regulatory sequences (cis-elements) within or around Tau exon 2 and 10 as well as other specific regulatory proteins (trans-acting factors). Splicing factors regulating Tau belong essentially to two major groups, the hnRNPs and SR/SR-like proteins. The U1 sNRP has been reported to be deregulated in Alzheimer's disease [7]. SRp75/hnRNPG complex regulate Tau exon 10 skipping [87]. The loss of MBNL1 or gain of ETR3 function in DM1 [19, 52], gain of DYRK1A [77], GSK3 [34], CLK2 [29] or hnRNPE3 [87] in Down syndrome or AD is linked to changes in the splicing of Tau exons 2, 3 and 10.

Whereas MBNL1 or MBNL1 both and independently regulate Tau exon inclusion, a synergistic effect of both MBNL1 and MBNL2 is necessary to regulated Tau exon 10 inclusion. This splicing regulatory mechanism may contribute to explain why DM1 is more a muscle disease rather than brain disease since both the loss of MBNL1 and MBNL2 recapitulate the brain dys-

function observed in the disease [31]. Into the complex landscape of Tau splicing regulatory mechanism, DM1 pathophysiology has enable the discovery of master Tau splicing factors which regulate Tau during development, postnatally and in disease conditions. However, the relationship between Tau missplicing, neurofibrillary degeneration and cognitive deficits remains a matter a debate but as in other Tauopathies, Tau splicing can be directly (mutations) or indirectly (splicing factors) deregulated and hence ultimately lead to neurofibrillary degeneration.

Diagnostic and Therapeutic Perspective

Cognitive deficits suggest the involvement of the cerebral cortex as well as subcortical areas, such as the amygdala and the insula. In line with these observations, several protein concentrations were altered in the cerebrospinal fluid of patient with DM1 [36]. Moreover, biomarkers of Alzheimer disease, including amyloid-beta peptide and Tau protein concentrations were significantly reduced and increased in the cerebrospinal spinal fluid, respectively, in DM1 when compared to aged-matched control patients [93]. Structural neuroimaging in DM1 patients reveals the presence of brain atrophy and white matter lesions [20, 21, 43].

Cerebrospinal fluid (CSF) biomarkers reflecting the core pathological features of AD (total-tau reflecting degeneration, phospho-tau reflecting tau phosphorylation and tangle formation, and Aβ42 which inversely correlates with plaque pathology in Alzheimer's disease) have also been studied in DM1 (Concentration of Aβ species in CSF is therefore considered as a valuable peripheral biomarker of the central nervous system. A significant decrease in CSF levels of amyloid Aß42 protein as well as increase in total tau protein was reported in adult and juvenile DM1 [71, 93]. No correlation between the levels of these CSF biomarkers and neuropsychological impairment were found, however. There are so far no reliable blood biomarkers for NFT pathology and neuroaxonal degeneration. For tau, the situation is promising but less clear. Plasma total-tau concentration in AD is increased but less so than in CSF and there is no detectable increase in the Mild Cognitive Impairment (MCI) stage of the disease [57, 58].

The ability to measure Aβ in CSF or by using positron emission tomography (PET) imaging has offered invaluable insights into the early stages of AD [80] and shown great potential as a diagnostic add-on to the clinical workup of patients with cognitive deficits [69]. On the other hand, biomarker studies have shown that the relationships between Aβ pathology and most downstream processes such as glucose hypometabolism, brain atrophy, disease severity, progression, and clinical presentation are modest. Parallel to this, worldwide interest in amyloid imaging has generated great momentum to develop PET tracers that bind to non-Aβ processes such as the microtubule-associated protein tau aggregated as neurofibrillary tangles in AD and other tau-related diseases (e.g. FTLD or chronic traumatic encephalopathy [CTE]). Indeed, tau pathology is known to have devastating effects on synaptic function [81], its temporal and spatial distribution correlates strongly with the clinical evolution of AD [9], and post-mortem tau aggregates are closely associated with cognitive performance during life. Over the past few years, several promising tau compounds have emerged, including 18F-AV-1451 (or 'flortaucipir', previously known as T807, [15]). These tracers consistently demonstrated good in vivo brain penetration, tracer binding to paired helical filaments of tau in AD brain tissue without labelling Aβ, deposits and patterns of tracer retention on PET scans resembling traditional Braak staging (see V. L. Villemagne et al. (2015), for a review [86]). These tracers would be valuable to investigate the natural history of Tau pathology in DM1 or even DM2 with aging and its relationship if any with the cognitives deficits.

No curative treatment is available for DM patients to date. The central hypothesis of DM1 is the RNA gain of toxic function. Consequently most of the therapeutic strategies aim at repress-

ing, delaying or even stopping this toxic effects of CUGs or CCUGs large RNA repeats. Currently under development are the antisense oligonucleotides (ASO) or small compounds targeting the large CUG repeats have which have shown promising beneficial effects in DM1 animal models. Thus, two strategies are proposed. One is based on the degradation and the second by blocking the consequences of CUGs expansion. Degradation of transcripts containing CUG expansions was achieved by using RNase H-active ASO [90, 91] that recognize the RNA target, hydrolyse the target by RNaseH1 and degrade the cleaved-RNA fragments by exonuclease activity. CUGS expansion degradation was also achieved using siRNA oligonucleotides targeting CUG repeats [50, 79].

The blockade of CUGs expansion toxic consequences was achieved either by the use of modified ASO containing CAG-antisense sequences [51, 65, 90] or small compounds [27, 88] that were able to bind specifically to these structures. As a consequence, abnormal protein-CUG repeats interactions were disrupted and toxic consequences were modified. Both strategies were assessed in transgenic mouse model of DM1 (expressing 220 CTG in the 3'UTR of the Human Skeletal Actin gene; [56]) and showed significant to almost complete correction of DM1-associated phenotypes such as splicing defects and myotonia.

Beyond these encouraging animal studies, IONIS has recently completed a phase 1/2a placebo controlled study to assess safety and tolerability of multiple doses of their lead compound in adults with DM1 (clinicaltrial.gov NCT023412011). The compound was delivered subcutaneously and was well tolerated at all doses tested. Despite some modest changes in key biomarkers such as RNA splicing, the clinical benefits of the drug remain yet to be proven. However recent advances in development of ASO generation 3.0, including LICA (Ligand Conjugated Antisense) or peptide-mediated delivery technologies have shown tremendous improvement in delivery and potency in cell and animal models. How they translate in DM1 patient will be reported in the near future.

Overexpression of MBNL1 in the HSA-LR mouse model also reversed the DM-associated phenotypes [14, 41, 42] indicating that CUGexp-RNA toxicity is mediated by the loss of functional MBNL in these DM1 mice. These results suggest that MBNL overexpression could be a valid therapeutic option for DM disorders. However, the expression of MBNL1, 2 and 3 is not ubiquitous but appears to be tissue-specific. Which MBNL protein, which isoform (since there is at least 10 different isoforms for each MBNL) and to what extent they have to be expressed to counteract CUGexp-RNA toxicity, are questions to be answered. Novel tools or small molecules will be necessary to achieve a therapeutic efficacy.

Recently, a well-known anti-diabetic drug Metformin, has been shown to correct some splicing events in vitro and in vivo [49] and to improve the muscle impairment the DMSXL mouse model of DM1. In patient this drug at the maximal tolerated dose provided positive effect on the mobility and gait abilities of DM1 patients.

Promising therapeutic strategies are under clinical evaluation and also open avenues to determine whether those therapeutic strategies would be efficient against the brain dysfunction and brain lesions.

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Tau PET Imaging

18

Makoto Higuchi

Introduction

Neurodegenerative dementias primarily consist of Alzheimer's disease (AD), dementia with Lewy bodies (DLB) and frontotemporal lobar degeneration (FTLD), and are neuropathologically characterized by depositions of aggregated proteins in the brain, as exemplified by fibrillar assemblies of tau, amyloid-β (Aβ), α-synuclein and TDP-43 (Fig. 18.1). The major constituent of protein deposits and their topology in the brain are specific for each illness, as exemplified by accumulations of tau and Aβ in the neocortex and limbic system of AD brains and a large subset of DLB brains. By contrast, neuropathological features of FTLD are formations of tau, inclusions in the frontotemporal regions and subcortical structures including the basal ganglia and brainstem without noticeable Aβ pathologies (Fig. 18.1) [1]. In AD, tau fibrillogenesis is more intimately linked to neuronal death than Aβ aggregation, and is supposed to be initiated decades before the clinical onset of the disease. This raises the possibility that visualization of tau lesions in the living brains provides information serving for diagnosis and staging of AD from an asymptomatic period. Imaging of tau pathologies is also expected to allow classification of FTLDs into neuropathological subtypes at an early stage and assessments of the disease severity in an objective fashion. In consideration of close associations between tau depositions and neurodegeneration, the commencement of an anti-tau treatment in an optimal time frame is supposed to be of critical significance for modifying the disease course, and would be enabled by tau imaging of individual subjects. The necessity of diagnostic and therapeutic approaches to prodromal neurodegenerative dementias has been highlighted in recent days, and it would be largely dependent on biological markers represented by imaging findings whether a disease-modifying treatment such as an anti-tau therapeutic should be started antecedent to the disease onset. Furthermore, it is virtually impossible to evaluate efficacies of a candidate drug in asymptomatic individuals based on clinical manifestations, justifying the use of biomarkers as essential indices for therapeutic effects. In fact, US Food and Drug Administration issued "Early Alzheimer's Disease: Developing Drugs for Treatment; Guidance for Industry" in February 2018, indicating that a drug examined by a clinical trial in presymptomatic subjects would be provisionally approved once its efficacy is proven with changes in a biomarker measure that is capable of rationally predicting progress to the clinical onset. In this context, the effectiveness of this drug to modify the clinical course of the disease would

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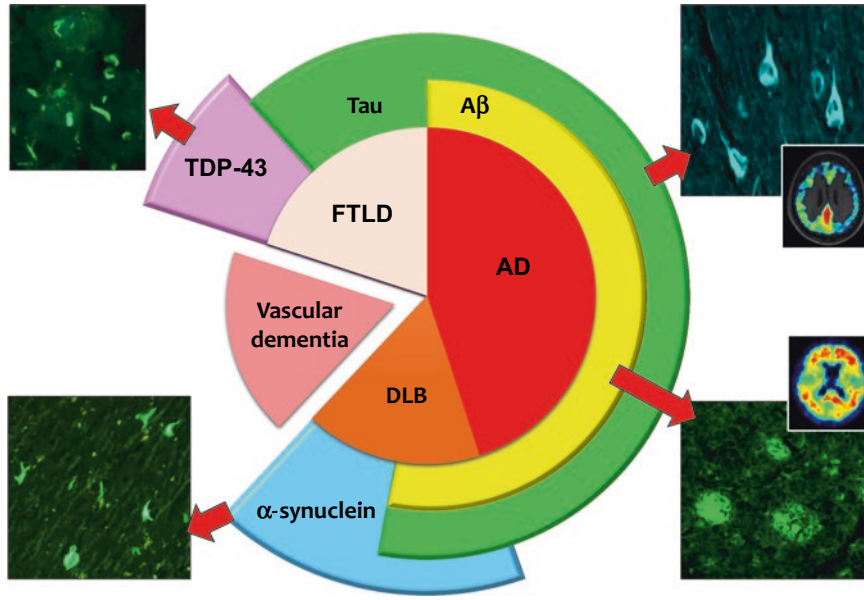


Fig. 18.1 Major classification of dementing disorders and proteins deposited in neurodegenerative dementias. Histochemical staining of brain sections derived from these illnesses with several fluorescent β-sheet dyes, along with PET imaging of tau and Aβ deposits in AD cases are also displayed

be tested through a post-marketing clinical trial. Imaging of tau lesions would offer such a biological index helping prediction of the clinical manifestations, but its utility in the trials as a reliable tool is greatly dependent on the selection of an appropriate tau imaging agent coupled with a robust method for quantification of tau burdens in the brain.

Discovery of a Selective Imaging Probe for Tau Pathologies

Pathogenic proteins such as Aβ, tau, α-synuclein, and TDP43 self-assemble by forming a β-pleated sheet as a secondary structure and are deposited in the brain. It is known that thioflavin-T, thioflavin-S, Congo red, and several other fluorescent dyes can detect amyloid fibrils by binding to β-sheets contained in these molecules. Most of these chemicals are electrically charged, which hampers their entry into the brain through the blood-brain barrier, and are therefore not qualified as in-vivo neuroimaging probes. Meanwhile, a neutralized analog of thioflavin-T was developed in the early 2000s for capturing Aβ plaques in the brain [2]. This compound was dubbed Pittsburgh Compound-B (PiB), and PiB labeled with a radionuclide, ^{11}C, was successfully applied to positron emission tomography (PET) of Aβ deposits in the brains of AD patients [3]. Interestingly, PiB only weakly binds to tau aggregates in contrast to its strong reactivity with Aβ deposits [4], indicating the potential selectivity of a β-sheet ligand for either Aβ or tau assemblies. This notion raises the possibility that structural modifications of such a chemical lead to the generation of an imaging agent selectively capturing tau lesions in a manner opposite to PiB. Amyloid dyes with higher selectivity for tau versus Aβ pathologies are exemplified by thioflavin-S, and a major component of thioflavin-S has a more extended backbone structure than PiB. In our attempt to discover a near-infrared fluorescent tracer for amyloid deposits, we also found that a β-sheet ligand with a long backbone produced a longer emission fluorescence wavelength and tended to show high affinity for tau rather than Aβ aggregates.

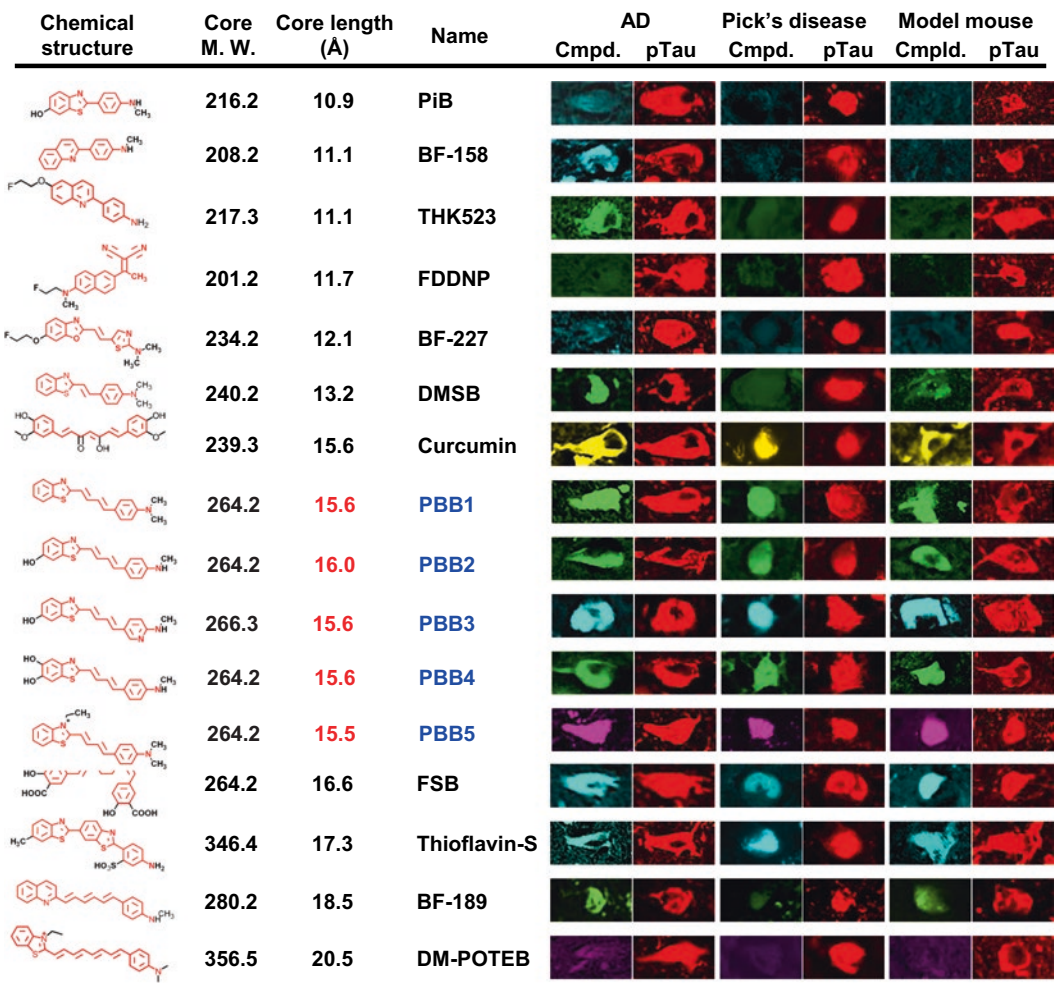


Fig. 18.2 Relationships between the length of a core (backbone) structure of a β-sheet ligand and its binding to tau aggregates in AD, Pick's disease, and tau transgenic mice dubbed PS19. The core structure of a compound is highlighted in red. Ligands are listed in ascending order of the core length. All these chemicals possess self-fluorescence, and their reactivity with tau aggregates can be assessed by fluorescence staining of a brain section. Tau inclusions are doubly stained with the compounds (Cmpd.) and an antibody against phosphorylated tau (pTau), AT8. Compounds with a core length ranging from 15 to 17 Å exhibit versatile reactivity with AD and non-AD tau aggregates. (Modified from Ref. [5])

We then revealed that a near-infrared laser dye, LDS750 (a. k. a. styryl 7), which possesses a core structure seemingly formed by inserting a linker into the middle of a backbone of thioflavin-T or PiB, selectively bound to tau inclusions in diverse disorders such as AD and FTLD including Pick's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) [5]. The backbone lengths of β-sheet ligands were related to their reactivity with various tau aggregates, as shown in Fig. 18.2, and compounds with a core structure approximating 15 A in length detected a variety of tau pathologies with high contrast. Since styryl 7 had a quaternary amine with an electric charge similar to thioflavin-T, its permeability through BBB was modest, impeding the use of this compound for high-sensitivity in-vivo imaging of tau lesions. We accordingly remodeled the structure of styryl 7, resulting in the creation of electrically neutralized chemicals termed PBB (Pyridinyl/Phenyl-Butadienyl-Benzothiazole/Benzothiazolium)

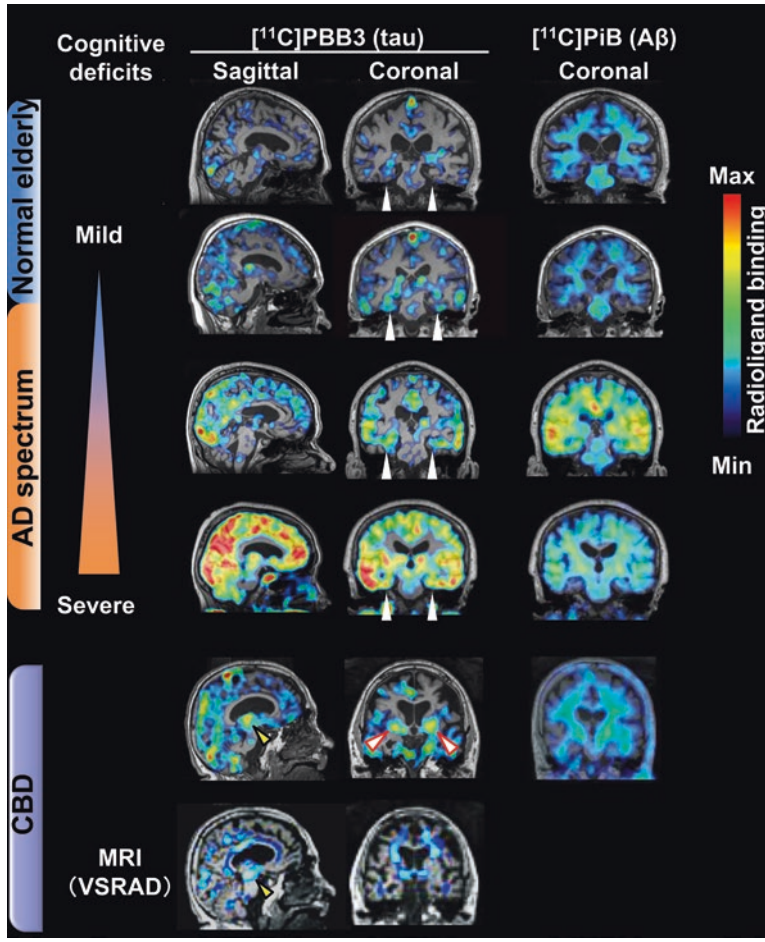


Fig. 18.3 Clinical PET imaging of tau and Aβ accumulations with [11C]PBB3 and [11C]PiB, respectively. PET images reflecting radioligand binding are superimposed on co-registered MRI data. Depositions of tau are noticeable in the hippocampal formation of a cognitively normal individual (second row). In the AD spectrum, Aβ pathology appears to trigger the expansion of tau depositions from the hippocampal formation to the limbic system and subsequently to the neocortex. By contrast, a patient with suspected CBD is PiB-negative and displays tau accumulations in the basal ganglia, subthalamic nucleus, and midbrain with left-right asymmetry. Volumetric analysis of MRI data with software termed VSRAD indicates local atrophy in agreement with tau lesions in this case. (Modified from Ref. [5])

(Fig. 18.2) [5]. Styryl 7 was also renamed PBB5 and was employed for additional assessments.

PBBs were dubbed PBB1–5 in descending order of their lipophilicity, and all of these compounds tightly bound to tau deposits in brain sections derived from almost all tauopathies. We subsequently utilized PBBs as fluorescent probes for intravital two-photon laser microscopic imaging of a tau transgenic mouse expressing mutant human tau to compare performances of these compounds, and found that PBB3 yielded the highest in-vivo contrast for neuronal tau inclusions. Correspondingly, PET imaging of the tau transgenics with ^{11}C-labeled PBB2, PBB3, and methoxy-PBB5 indicated the highest sensitivity of tau detection obtained by [11C]PBB3 [5]. Supported by these nonclinical data, we initiated a clinical PET study with [11C]PBB3, resulting in the revelation that this radioligand was capable of visualizing tau pathologies in AD, PSP, CBD and several other tauopathies (Fig. 18.3) [5].

There have also been PET imaging agents for tau lesions developed by several other industrial and academic research groups. A series of β-sheet

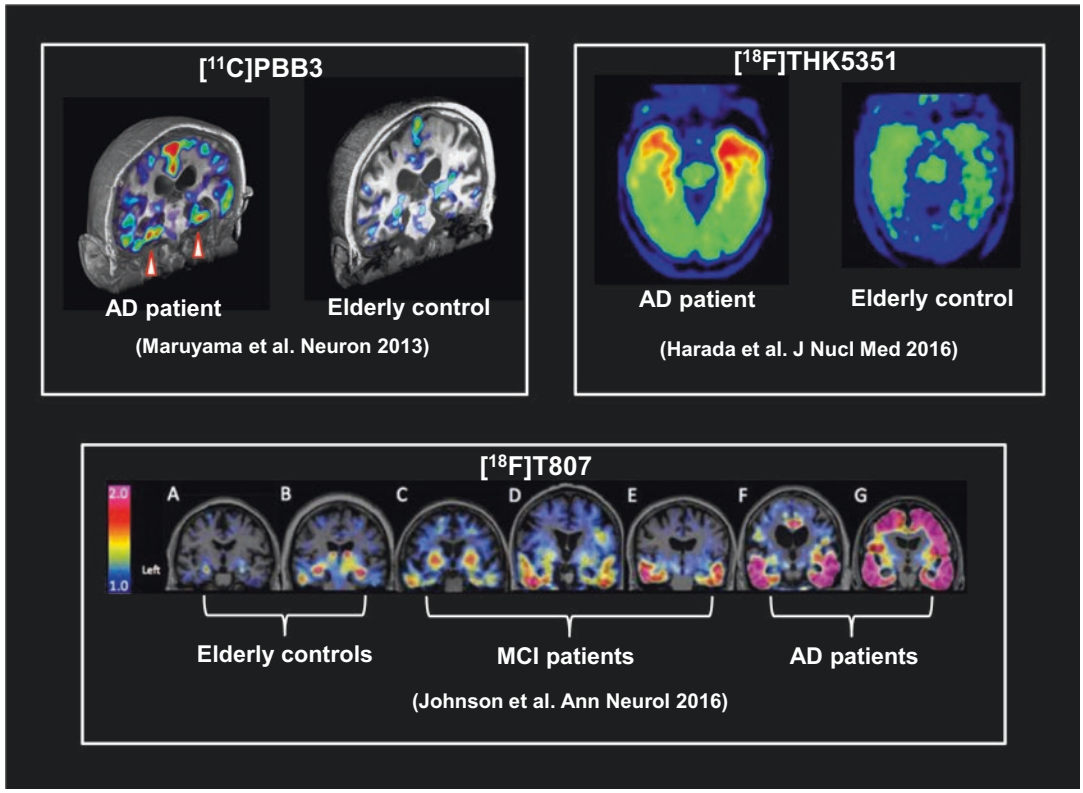


Fig. 18.4 Tau PET images of cognitively normal elderly controls and patients with AD spectrum pathologies with [^{11}C]PBB3, [^{18}F]THK5351, and [^{18}F]T807. (Modified from Refs. [5, 9, 17])

ligands created by BF Institute (occasionally called BF compounds) were evaluated by scientists at Tohoku University, and they demonstrated high-affinity binding of chemicals containing quinoline as a part of its core structure to tau deposits. They accordingly promoted clinical applications of these quinoline derivatives in collaboration with University of Melbourne and some other research sites, and proved that PET radioligands including [^{18}F]THK523 [6], [^{18}F]THK5105 [7], [^{18}F]THK5117 [8] and [^{18}F]THK5351 [9] were able to detect tau accumulations in the brains of living AD patients. Among these BF compounds, [^{18}F]THK5351 was reported to capture tau pathologies in AD [9], PSP [10], and CBD [11] with the highest contrast (Fig. 18.4). Meanwhile, the quinoline derivatives were also documented to have an affinity for monoamine oxidase B (MAO-B), and this may hamper dissociation of tau depositions from neuroinflammatory gliosis in light of the expression of MAO-B in astrocytes [12, 13]. Primarily for this reason, clinical trials for [^{18}F]THK5351 conducted by General Electric Company was discontinued, but there remains a possibility that additional structural modifications of BF compounds will lead to radiotracers with high selectivity for either tau fibrils or astrocytes enriched with MAO-B.

Other efforts to develop tau imaging agents are represented by the development of ligands for tau aggregates including, T807 and T808 by Siemens Medical Solution USA, Inc., on the structural basis of a BF compound, BF-126 [14–16]. A notable feature of their discovery strategy was that candidate chemicals were evaluated by assessing their reactivity with native tau deposits in AD brain tissues but not in-vitro assemblies of recombinant tau proteins. As most β-sheet ligands bind to Aβ, tau, and α-synuclein multimerized in

a test tube, the implementation of an assay system with real brain samples may be more feasible for the screening of test compounds than the use of artificial models of the protein fibrillogenesis. These tau fibril ligands were then acquired by Eli Lilly and Company, and their performances have been further analyzed by Lilly's subsidiary, Avid Radiopharmaceuticals. Since metabolic and pharmacokinetic properties of T807 were superior to those of T808, subsequent developments focused on ^{18}F-labeled T807, [^{18}F]T807, which was also named [^{18}F]AV-1451 in the development by Avid and flortaucipir F-18 in clinical trials by Lilly. [^{18}F]T807 was extensively used in US, Canadian and European research institutes (Fig. 18.4) [17], as exemplified by its application to a multicenter imaging program in the US termed AD Imaging Initiative (US-ADNI) for collection of tau PET data with a large sample size [18].

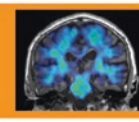
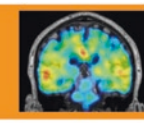
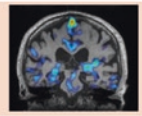
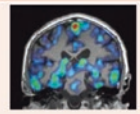
Utility of Tau PET Imaging Demonstrated by Clinical Assessments

PET imaging of plaque lesions revealed Aβ depositions in a significant subset of elderly population, providing a notion that cognitively normal subjects and patients with mild cognitive impairment (MCI) and dementia exhibiting PET-detectable Aβ pathologies are conceived to be afflicted with AD at different stages defined as preclinical AD [19], MCI due to AD [20] and AD dementia [21], respectively, in a continuum called AD spectrum. The tau pathogenesis in aging and AD spectrum has been characterized by the following three features according to clinical PET findings with [^{11}C]PBB3, [^{18}F]THK5351, and [^{18}F]T807 [5, 9, 17, 18, 22]: (1) Accumulations of tau aggregates in the hippocampal formation are detectable in a subpopulation of cognitively intact elderly individuals in a manner independent of Aβ depositions. Aging-related tau pathologies in the hippocampal formation have also been documented by neuropathological assays of autopsied brains, and often precede the formation of Aβ plaques (Fig. 18.3); (2) Aβ aggregations in AD spectrum likely trigger spreading of tau depositions from the hippocampal formation to the limbic system and subsequently to the neocortex (Fig. 18.3). This expansion also occurs with slower advancement in the absence of Aβ plaques, which is referred to as primary age-related tauopathy (PART) [23]. The extent of tau accumulations intimately correlates with the severity of MCI due to AD and AD dementia, bringing an objective index for the disease severity; and (3) Localizations of tau depositions agree with those of atrophies in patients with MCI due to AD and AD dementia, suggesting on-site neurotoxicity provoked by tau aggregates.

The close association of PET-detectable tau deposits with neuronal deteriorations and symptomatic manifestations supports the view that tau PET imaging would enable objective and precise evaluations of candidate therapeutics modifying AD processes from an MCI stage. In addition, the presence of tau PET-positive individuals in the non-demented elderly population indicates the possibility of classifying healthy aged subjects into four categories according to Aβ and tau PET findings (Table 18.1). A disease-modifying therapy at a preclinical stage may be applied to one or more of these categories, and tau PET might be employed for assessing biological outcomes of the treatment in subjects lacking noticeable cognitive declines.

To establish the utility of tau PET for therapeutic evaluations, time-course changes of tau radioligand binding in the brains of the same individuals in transition from an early to an advanced stage of the AD spectrum need to be clarified by a longitudinal clinical PET study. In a cohort study conducted by a research group at Mayo Clinic, spatiotemporal alterations of tau depositions were pursued in 126 subjects by PET with [^{18}F]T807. There was an increase of the tau burden by 0.5% per year in non-demented elderly people with PET-visible Aβ plaques, and this increment was 3% per year in Aβ-PET-positive cases with cognitive deficits [24]. This work also documented that tau PET exhibited smaller variabilities of annual changes among individuals

Table 18.1 Stratification of cognitively normal elderly individuals according to findings in tau and Abeta PET scans. Tau-positive and Abeta-negative subjects may be conceived as having primary age-related tauopathy (PART)

	Aβ- 	Aβ+ 
Tau- 	Normal aging	Preclinical AD
Tau+ 	PART?	Preclinical AD

than did psychometries, leading to a higher statistical power in assessing therapeutic efficacies of a test drug.

For proving the usefulness of a tau PET ligand as a reliable diagnostic agent, the specificity of PET radiosignals for tau aggregates should be demonstrated by analyzing correlations between neuroimaging and neuropathological data in the same subjects. Such assays on imaging-neuropathology relationships with a small sample size were reported with [^{18}F]T807 and [^{18}F]THK5351 in patients with AD spectrum pathologies [25]. We have also carried out neuropathological examinations of the autopsied brains derived from PSP and AD patients who had undergone a PET scan with [^{11}C]PBB3 at multiple imaging sites in Japan, and links between in-vivo radioligand binding and immunohistochemical and biochemical measures of tau deposits are being investigated.

FTLD tauopathies, including PSP and CBD, are characterized by accumulations of tau fibrils in the brain without prominent amyloid plaque formations, and an anti-tau treatment could be evaluated in these disorders without confounding effects of Aβ pathologies. Unlike AD, clinical outcome measures in a therapeutic trial for PSP patients are primarily composed of motor indices rather than cognitive scores. As motor performances could be assessed with smaller variabilities, placebo effects, and individual biases than cognitive functions, clinical trials for PSP relative to AD may yield high precisions for determining efficacies of an anti-tau drug candidate.

These notions also rationalize the application of a PET radioligand for PSP and CBD tau deposits to clinical settings, but it has been pointed out that the reactivity of [^{18}F]T807 with these aggregates is insufficient for in-vivo imaging (Fig. 18.5) [26–28]. It is well known that tau proteins in CNS are comprised of six isoforms, which are classified into four-repeat and three-repeat isoforms according to the number of repeat domains. While AD tau tangles are constituted of all six isoforms, PSP and CBD tau aggregates contain four-repeat isoforms only, in contrast to Pick bodies in Pick's disease formed by three-repeat tau isoforms. The difference in the isoform composition may result in diversity in 3-D structures of tau aggregates, causing selectivity of a PET ligand for tau assemblies in AD versus PSP/CBD or Pick's disease as represented by [^{18}F]T807, which preferentially binds to AD-type tau fibrils.

A neuropathological study of PSP patients following a [^{18}F]T807-PET scan of these cases also indicated a dissociation between topologies of in-vivo PET signals and tau depositions [29]. Although [^{18}F]T807 binding in PET scans of CBD patients was found to be correlated with neuronal and glial tau inclusions assessed post-mortemly in the same individuals [28], in-vivo

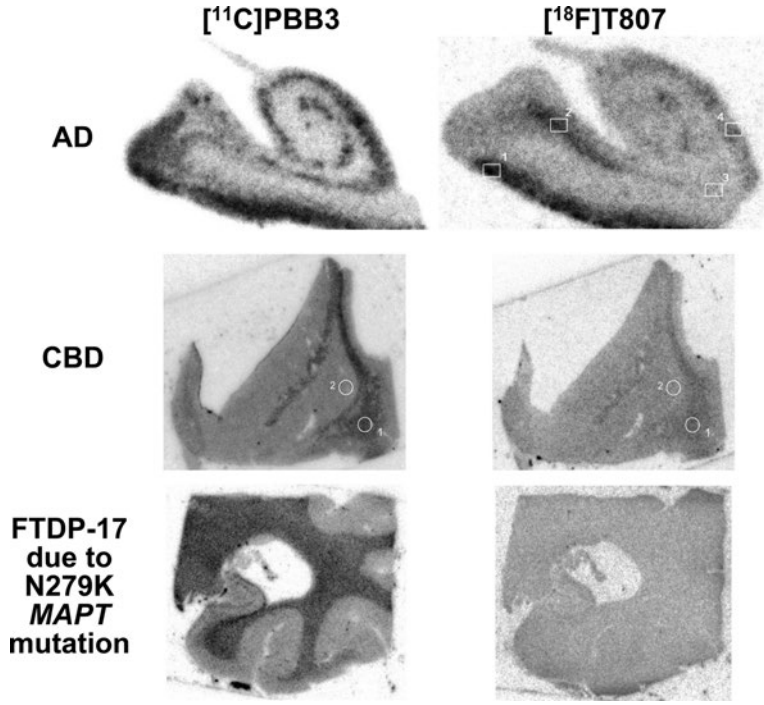


Fig. 18.5 Autoradiograms of brain sections derived from patients with AD, CBD, and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) due to the N279K *MAPT* mutation. Adjacent slices are labeled with [^{11}C]PBB3 and [^{18}F]T807 in a comparative manner. Both radioligands tightly bind to AD pathologies, but subregional distributions of [^{11}C]PBB3 and [^{18}F]T807 radiosignals differ from each other. Binding of [^{11}C]PBB3 in CBD and N279K mutant FTDP-17 brain tissues enriched with four-repeat tau aggregates is greater than that of [^{18}F]T807. (Modified from Ref. [26])

contrast for CBD tau pathologies produced by this radioligand was modest, often impeding differentiation between CBD and control subjects. [^{18}F]THK5351 was demonstrated to accumulate in the brains of PSP and CBD patients with its spatial distributions being characteristic of each disease [10, 11], but it remains likely that these radiosignals were mainly attributed to the binding of this radioligand to MAO-B expressed in activated astrocytes.

PBB compounds were originally designed as ligands for diverse tau aggregates composed of all six isoforms or four- or three-repeat isoforms only, on the basis of structure-activity relationships [5], and high-affinity binding of these chemicals to PSP and CBD tau deposits was shown by our in-vitro experiments (Fig. 18.5) [26]. Moreover, clinical PET examinations have revealed elevated retention of [^{11}C]PBB3 in the brains of PSP and CBD patients [5, 30], along with symptomatic carriers of *MAPT* mutations causative of familial three-repeat and four-repeat tauopathies [31]. However, a signal-to-noise ratio for capturing these non-AD tau lesions with [^{11}C]PBB3 was not sufficient for determining tau positivity or negativity for each individual and for accurate quantification of longitudinal changes in the tau load, necessitating a new tau PET ligand allowing sensitive imaging assays.

Development of Next-Generation Tau PET Probes

PET imaging with [^{11}C]PBB3, [^{18}F]THK5351, and [^{18}F]T807 has provided numerous insights into the tau pathogenesis, while properties of these compounds were yet to be improved.

Since [^{11}C]PBB3 is labeled with ^{11}C, which is a radionuclide with a half-life as short as 20 min, possible improvements of this ligand should incorporate the use of ^{18}F with a half-life of 110 min instead of ^{11}C for wider availabilities and applicability to deliveries from a production site to PET facilities. An additional issue on [^{11}C]PBB3 is a prompt metabolic conversion of this ligand to a major metabolite, hampering the efficient transfer of the unmetabolized compound to the brain [5, 32]. Since a structural domain of [^{11}C]PBB3 with a high propensity to biometabolism was identified [33], we have recently generated a new PBB3 analog, [^{18}F]PM-PBB3, by substitution of this moiety with an ^{18}F-containing side chain. Our exploratory clinical work has shown that [^{18}F]PM-PBB3 is more resistant to metabolic changes than [^{11}C]PBB3, and that peak uptake of [^{18}F]PM-PBB3 in the brain is approximately 1.5-fold higher than that of [^{11}C]PBB3 [34]. Owing to its high reactivity with tau aggregates in the living brain and low background retentions, [^{18}F]PM-PBB3 has been found to yield a more than two-fold higher contrast for tau lesions in tau transgenic mice than [^{11}C]PBB3 [34].

Following these non-clinical assessments, clinical evaluations of [^{18}F]PM-PBB3 were commenced at the National Institute of Radiological Sciences as a principal investigator-initiated PET study. In parallel with this program, a license on a patent covering PBB compounds was granted to a bioventure, APRINOIA Therapeutics, and this company initiated a phase 0 clinical trial for [^{18}F]PM-PBB3 in USA [34]. These human assays have proven that contrasts for AD, PSP, and CBD tau deposits produced by [^{18}F]PM-PBB3 are more than two-fold of contrasts provided by [^{11}C]PBB3. It is of particular significance that four-repeat tau inclusions in PSP and CBD can be sensitively captured by [^{18}F]PM-PBB3, allowing robust quantification of these pathologies at an individual level.

[^{11}C]PBB3, [^{18}F]THK5351, and [^{18}F]T807 are often referred to as "first-generation" tau radioligands, and several pharmaceutical companies have been engaged in the development and evaluation of imaging agents in the next generation with more preferred characteristics. Apart from [^{18}F]PM-PBB3, most of these improved tracers were designed based on the chemical structure of T807. While THK5351 cross-reacts with MAO-B, it has been reported that T807 exhibits off-target binding to MAO-A. Accordingly, analogs of T807 with no or minimal binding to MAO-A and MAO-B have been selected for application to clinical PET imaging. As summarized in Table 18.2, all next-generation tau imaging agents capture AD tau lesions without reacting with MAO enzymes. Among these chemicals, [^{18}F]PM-PBB3 is hitherto the only radioligand capable of visualizing four-repeat tau pathologies in PSP and CBD with high contrast. [^{18}F]PI-2620, a compound developed by Piramal Imaging, was also reported to sensitively detect PSP tau lesions, [34] but more recent investigations have indicated that increased binding of this ligand in the brainstem and basal ganglia is mostly derived from its reaction with non-tau components. It should also be noted that a subset of tau radioligands, including [^{11}C]PBB3, [^{18}F]T807, and [^{18}F]PM-PBB3, accumulate in choroid plexus to a variable extent [5, 34, 40]. Exact mechanisms of this seemingly nonspecific radiosignals remain to be clarified but could be explained by interactions of these chemicals with irons and pigments in choroid plexus [41]. It is also likely that the compounds bind to Biondi bodies, which are amyloid-like protein aggregates deposited in choroid plexus with aging [42].

Despite the superiority of the next-generation tau PET probes to the first-generation compounds due to their low nonspecific binding, it is still uncertain whether these new ligands can indeed offer reliable biological indices in the diagnosis and therapeutic evaluations of AD spectrum patients in comparison with [^{18}F]T807. To address this issue, longitudinal alterations of PET signals and their correlations with postmortem observations would need to be examined with standardized protocols in a collaborative research program involving multiple imaging and neuropathology sites.

Table 18.2 Clinically available tau PET imaging agents. Next-generation radioligands are highlighted in bold

PET tracer	Developer	Contrast for AD tau lesions	Non-AD tauopathy		Tg mouse inclusions	Off-target binding	
			Detectable types	PSP tau contrast		MAOs	CP
[^{11}C]PBB3 [5, 22]	QST	1.2–1.4	4-repeat tauopathy	1.09–1.18	Yes	–	±
			3-repeat tauopathy				
[^{18}F]T807 [15, 17]	Avid/Lilly	1.3–1.6	4-repeat tauopathy	1.03–1.16		MAO-A	+
[^{18}F]THK5351 [13]	Tohoku Univ.	1.4–1.8				MAO-B	–
[^{18}F]PI-2620 [35]	**Piramal**	**2.0–2.5**				–	±
[^{18}F]GTP1 [36]	**Genentech**	**2.0–3.0**				–	–
[^{18}F]RO6958948 [37, 38]	**Roche**	**2.0–2.5**				–	–
[^{18}F] MK-6240 [39]	**Merck**	**2.0–3.0**				–	–
[^{18}F]PM-PBB3 [34]	**QST APRINOIA**	**2.0–2.5**	**4-repeat tauopathy**	**1.25–2.25**	**Yes**	–	+
			3-repeat tauopathy				

Contrasts for AD tau lesions are estimated by calculating ratios of standardized uptake value ratio (SUVR), distribution volume ratio (DVR), or non-displaceable binding potential (BP_{ND}) plus 1.0 in neocortical and limbic areas between patients with MCI due to AD or early AD dementia and aged normal controls. Similarly, contrasts for PSP are determined by calculating ratios of SUVR, DVR and BP_{ND} plus 1.0 in the brainstem, subthalamic nucleus, and globus pallidus between patients with PSP and age-matched controls. PBB3 and PM-PBB3 are the only probes with specific binding to tau aggregates in several tau transgenic (Tg) mouse strains [5]. Some compounds exhibit off-target binding to MAO enzymes and choroid plexus (CP)

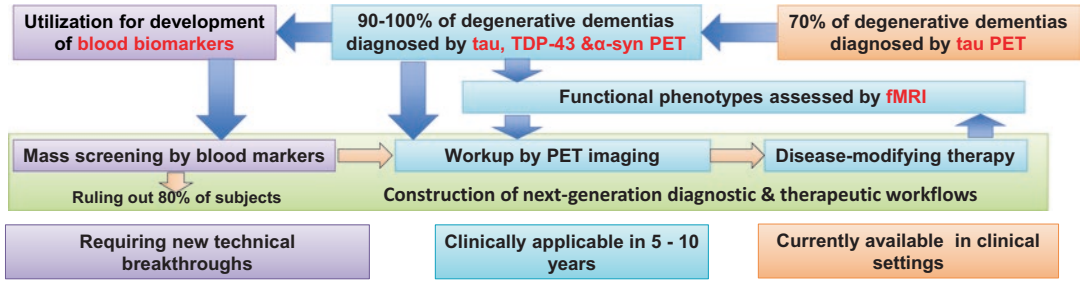


Fig. 18.6 Diagnostic and therapeutic workflows targeting neurodegenerative dementias utilizing proteinopathy PET imaging as a pivotal element

Conclusions

PET imaging of tau pathologies in the AD spectrum and FTLD will cover approximately 70% of neurodegenerative dementias, and this coverage will reach 90–100% with imaging technologies to visualize α-synuclein and TDP-43 deposits. However, multiple PET scans for each elderly subject will not be cost-effective and somewhat time-consuming, and mass screening for dementias would need to be conducted with a handier method such as a blood test. Measurements of tau proteins in plasma could be performed using ultra-sensitive ELISA and related innovations [43], and validations of these liquid biopsies would be facilitated by the use of tau and Aβ PET scans as references. The major purpose of the mass screening with a blood biomarker would be to exclude 80–90% of the subjects from further diagnostic workups, including PET examinations. In the secondary tests for those who are positive in the initial screening, information on pathological protein depositions obtained by PET would be used for the selection of appropriate disease-modifying therapies. The effects of such treatments could also be assessed by PET scans, and functional MRI or similar non-radiological imaging techniques might provide surrogate biological indices reflecting neuronal integrities. In an evolutional diagnostic and therapeutic system for dementing illnesses (Fig. 18.6), proteinopathy imaging represented by tau PET would play pivotal roles as an in-vivo test most reliably probing pathogenetic processes.

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19 Tau Accumulation and Network Breakdown in Alzheimer's Disease

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Introduction

The pathological hallmarks of Alzheimer's disease (AD) are the presence of extracellular senile plaques containing amyloid β (Aβ) peptide, and intracellular neurofibrillary tangles of the microtubule-associated protein tau [1, 2]. Astrogliosis associated with neurodegeneration is also a significant hallmark of AD [3]. However, the roles of Aβ, tau, and astrogliosis/neurodegeneration in developing dementia and their progression pattern have not been fully elucidated.

Recently, several studies have demonstrated that Aβ PET tracers as well several tau PET tracers including flortaucipir, THK5317, and PBB3 will significantly accumulate in the specific areas well correspond to previous pathological data in AD and other tauopathies [4, 5]. Intriguingly, it is also clearly revealed that the process of Aβ accumulation starts 10–20 years before clinical signs of dementia become apparent in AD [6] in accordance with Nun study that pathologically proved the presence of some subjects with good cognition during their lives irrespective of a significant increase in Aβ and tau accumulation corresponding to advanced Braak's stage V and VI [7]. Moreover, the healthy older subject can show increased tau tracer accumulation in the medial temporal lobe with aging [8]. These findings suggest that there are other factors or mechanisms which act as resilience to or compensation against the AD-related pathological changes before the development of dementia.

Advanced magnetic resonance imaging (MRI) technique have provided the insightful mechanisms with visualization of the large-scale functional networks among brain regions [9–11]. A functional network is generally presumed by the correlation between nodal endogenous low-frequency fluctuations of blood oxygenation level–dependent (BOLD) signals using resting state functional MRI (rs-fMRI). The rs-fMRI study is believed to have a potential to delineate the not only decreased but also increased connectivity that may be associated with resilient or compensatory process against dementia and aging process [12, 13]. On the contrary, Jones et al. proposed that such increased connectivity can trigger the local overloads proliferating to

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downstream nodes eventually leading to widespread power or systems failures in AD [14]. Since AD is considered to be a disconnection syndrome characterized by abnormalities in large-scale networks [10, 12, 15], it will be crucial to understanding the relationship between RSNs and accumulation of Aβ and tau related to the maintenance of cognition or development of dementia.

In this review, we summarized the recent advancement of spatial tau PET tracers' retention pattern using data mining approach and disruption of RSNs on MRI in patients with AD.

Spatial Distribution Pattern of Tau Retention in AD

In patients with AD, all available tau tracers showed the retention predominantly in the inferior temporal and parietal cortices corresponding to the distribution of neurofibrillary tangles as well as the clinical phenotype of dementia [4, 6, 16]. The severity of retention also related to cognitive decline in AD [17]. However, first-generation of tau tracers also demonstrated off-target binding in the basal ganglia, midbrain, thalamus, hippocampus, choroid plexus, and venous sinus. As for THK5351, binding to monoamine oxidase B (MAO-B) presumably explains most off-target binding but increased MAO-B is also observed in disease-related brain regions linked to astrogliosis with misfolded protein accumulation [18–20].

Principal component analysis (PCA) is one of the most popular data-driven multivariate statistical approaches to simplify several possible correlated variables into a smaller number of uncorrelated variables. Scaled subprofile model (SSM) is PCA-based approach that can be used to extract disease-related spatial covariance patterns from brain imaging data such as cerebral blood flow and metabolism PET and SPECT [21–23]. It has the advantage to identify the most significant sources of data variation associated with the disease in the first few principal components. The second principal component has the direction which maximizes variance among all directions orthogonal to the first component. Thus, it is expected to obtain not only disease-specific SSM/PCA patterns (patient's patterns) but also normal subject's patterns. Besides, noise elements also will decrease if only the first few eigenvectors that show significant statistical differences between patient and healthy control are selected.

We applied SSM/PCA to the THK 5351 PET images to identify the disease-specific spatial distribution in a patient with AD [24]. To delineate a specific AD-related topography, we limited the analysis to the set of principal components that in aggregate accounted for the top 50% of subject × voxel variability, and for which each principal component contributed at least 10% to the total variance in the scan data [25]. We also generated eigenimages and the associated subject-specific eigenimage scores which represented the similarity of each's preprocessed THK5351 concentration to the SSM/PCA-identified pattern.

Figure 19.1 shows a significant spatial THK5351 pattern corresponding to principal component 1. This pattern accounted for 23.6% of the total subject voxel variance of the data and showed 82.6% sensitivity and 79.1% specificity for differentiating AD from healthy controls. We called this spatial pattern as AD-related THK5351 pattern (ADRTP). The ADRTP was composed of three major clusters. Two clusters were mainly located in the bilateral inferior, middle, and superior frontal gyrus. Cluster 3 was the major component, which included the precuneus, posterior cingulate cortex, inferior parietal lobule, inferior, middle, and superior temporal gyrus, and fusiform gyrus. Among the ADRTP-related regions, the most prominent areas were the precuneus and PCC, followed by the lateral middle and superior frontal gyri corresponding to Braak stage IV and V. The subject scores of ADRTP showed significant correlation with general cognitive performance assessed by ACE-R and CDR score.

Principal component 2 (PC 2) accounted for 11.8% of the total subject voxel variance composed by bilateral basal ganglia, hippocampus, medial prefrontal cortex, and anterior cingulate cortex (Fig. 19.2). The distributions were consistent with non-specific bindings sites of THK5351, which were previously reported [18, 19]. Subject

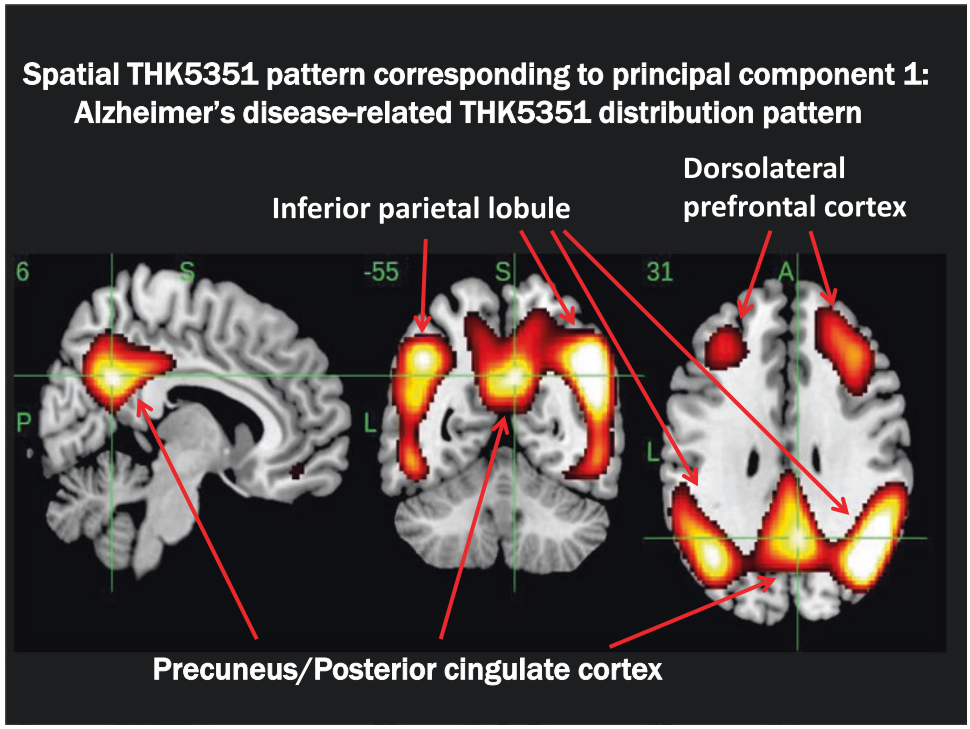

Fig. 19.1 Spatial THK5351 pattern corresponding to principal component 1: Alzheimer's disease-related THK5351 distribution pattern. Scaled subprofile model/principal component analysis showed specific tau retention in the precuneus, posterior cingulate cortex, dorsolateral prefrontal cortex, and infra parietal lobule in Alzheimer's disease compared to control

scores based on PC 2 had 70.8% sensitivity and 52.2% specificity in discriminating healthy controls from AD. There was no significant correlation between PC 2 scores and ACE-R and CDR.

Independent component analysis (ICA) is also a data-driven approach and can separate the imaging signal into several temporally correlated independent networks as spatial maps by multivariate decomposition without any a priori presumptions. Hoenig et al. investigated AV-1451 PET network using ICA in patients with AD [26]. The ICA showed in the detection of 10 independently coherent tau pathology networks with highly functionally connected brain regions such as the precuneus and cingulate cortex. The ten networks spatially resembled established language, frontal control, default mode, visuospatial, and hippocampal networks. The total percent variance explained by all ten components was 95.7%.

Interestingly, SSM/PCA and THK5351 as well ICA and AV-1451 showed very similar spatial distribution pattern in patients with AD. Mainly, precuneus and posterior cingulate cortex were the core regions associated with the progression of dementia. Data mining approach will provide for strong and novel aspects for the pathophysiology of tau PET retention in AD.

More recently, second-generation tau tracers including [18F]RO-948, [18F]GTP1, [18F]PM-PBB3, [18F]AM-PBB3, [18F]MK-6240, and [18F]PI-2620 have been developed [4, 6]. These tracers showed higher affinity for tau pathology, greater signal-to-background ratio, more excellent selectivity, and less off-target binding to such as MAO-A and MAO-B than the first generation. However, the binding specificity to tau protein deposits must be carefully validated in the development of new tau tracers [4]. SSM/PCA and ICA will be beneficial for identifying individual spatial tau tracer's distribution with minimizing the effect of off-target bindings.

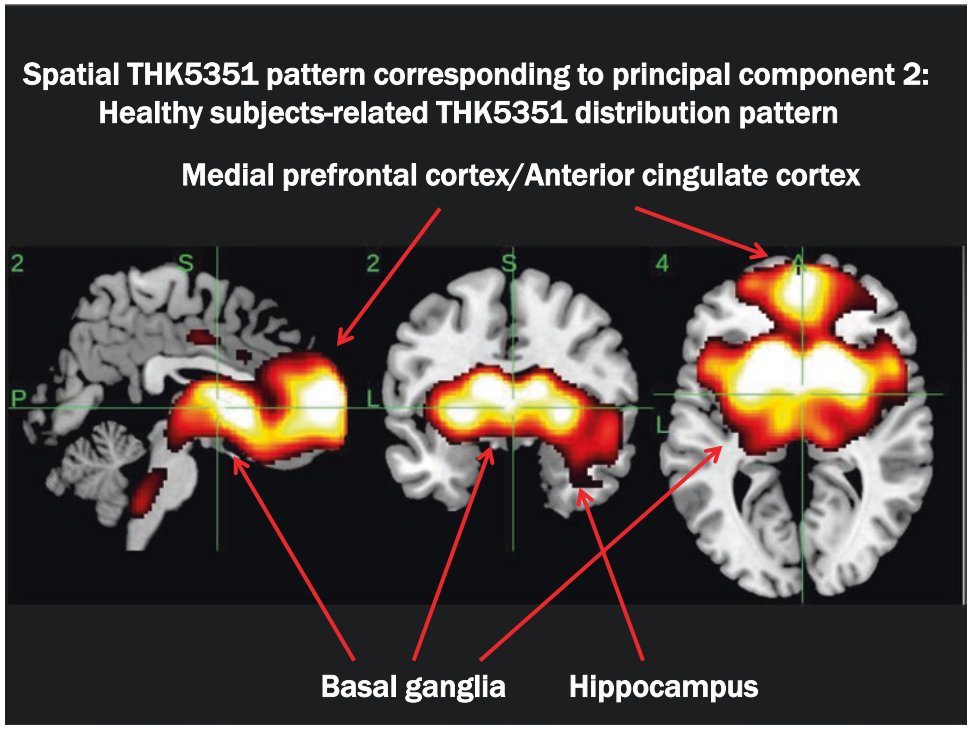

Fig. 19.2 Spatial THK5351 pattern corresponding to principal component 2: Healthy subjects-related THK5351 distribution pattern. Scaled subprofile model/principal component analysis showed specific tau retention in the basal ganglia, hippocampus, medial prefrontal cortex, and anterior cingulate cortex in healthy control

Resting State Networks (RSNs)

Since neurons do not have potential to store internal reserves of glucose and oxygen, hemodynamic response delivering glucose and oxygen in response to a demand for information processing is crucial to their proper function. This cause increasing oxyhemoglobin in the active area, giving rise to a significant change in the regional ratio of oxy- to deoxyhemoglobin (dia- to paramagnetic), providing a difference in magnetic susceptibility between the blood and the surrounding tissue. MRI can visualize the differences of the deoxyhemoglobin content with image intensity that has been termed Blood Oxygenation Level Dependent (BOLD) [27–29].

Resting-state functional MRI measures the intrinsic brain activity as low-frequency fluctuations (<0.1 Hz) in BOLD signals in the absence of any explicit task or an input [9–11]. Functional connectivity (FC) is generally defined as the connection between two spatial regions of interest which have the significant linear temporal correlation of the time-series supporting the idea that they are involved in the same underlying functional process. This low-frequency fluctuation phenomenon relates to some spontaneous neural activity [30] and thought to be related to extremely excessive high resting energy consumption. There are many different measures to analyze rs-fMRI data including seed-based functional connectivity analysis, independent component analysis, and graph analysis.

Seed-based functional connectivity provides the regions correlated with the time-series in a seed region. Seed is generally determined based on a hypothesis or prior results. The computation is a simple and intuitive understanding of the result is easy.

ICA is also frequently used for rs-fMRI analysis. Using ICA, we can evaluate several canonical large-scale networks including default mode

network, salience network, executive control network, dorsal attention network, medial visual network, lateral visual network, sensorimotor network, basal ganglia network, auditory network and so on (Fig. 19.3). Classical default mode network divided into three (precuneus network, dorsal default mode network and ventral default mode network) or four (ventral (precuneus), posterior, anterior ventral, and anterior dorsal default mode networks) parts depending on the ICA results [14].

Graph theory, which has been developed to investigate the properties of complex networks provides a theoretic framework of the local and global brain networks organization. Graph theory has several essential indices. Clustering coefficient describes the level of local neighborhood clustering. High clustering coefficient corresponds to well-connected areas with the same functional specialization. Characteristic path length, which is the average number of connections between all pairs of nodes measures functional integration (efficiency) in brain networks. Degree describes the number of connections of a node. Highly connected nodes within the network are called as "hub (nodes with higher centrality)". Hub will contribute to the large-scale effects of the network.

Relationship Between Tau Retention and RSN Changes in AD

According to the recent network degeneration hypothesis, tau pathology will stereotypically propagate with disease progression spreading along RSNs in AD. We computed the similarity of the distribution between the THK5351 concentration assessed by SSM/PCA, and canonical RSNs generated from the group RSNs obtained

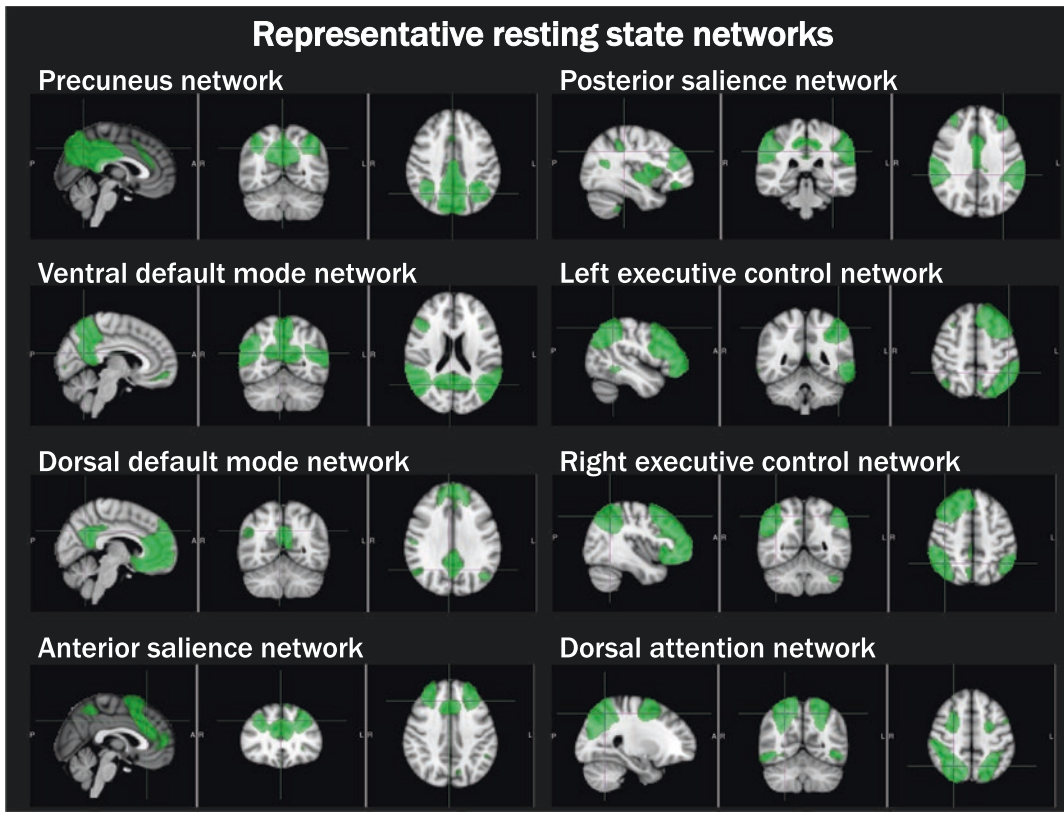

Fig. 19.3 Representative resting state networks. This figure shows the representative resting state networks on magnetic resonance imaging obtained by independent component analysis

using group ICA [24]. THK5351 values and intrinsic connectivity values were extracted from all voxels within the RSNs. Then, the similarity of the distribution between THK5351 intrinsic connectivity was computed using Pearson's correlation. The estimated similarity values were mostly negative in healthy controls indicating that the lower level of THK5351 concentration voxels is associated with higher intrinsic connectivity within RSNs.

On the contrary, in the patient with AD, a shift towards a more positive association. Mainly, precuneus/PCC network, right executive control network, ventral default mode network, visuospatial network, and language network showed significant similarity with THK5351 retention (Fig. 19.4). The results of ICA and dual regression analyses also showed decreased connectivity within the right executive control network, ventral default mode network, visuospatial network, and language network.

Bilateral precuneus/PCC and the left dorsolateral prefrontal cortex (DLPFC) corresponding to hubs showed the most significant difference in THK5351 retention between early AD and healthy controls in ADRTP. According to seed-based connectivity analysis using two ROIs as the seed regions, the intrinsic connectivity of precuneus/PCC significantly decreased in the left middle occipital gyrus, left superior temporal gyrus, left amygdala/hippocampus, and right fusiform gyrus (Fig. 19.5). That of left dorsolateral prefrontal cortex decreased to the left inferior parietal lobule.

As described previously, Hoenig et al. performed seed-based connectivity analysis from regions which showed the maximum z-value of AV-1451 in each of the ten generated tau

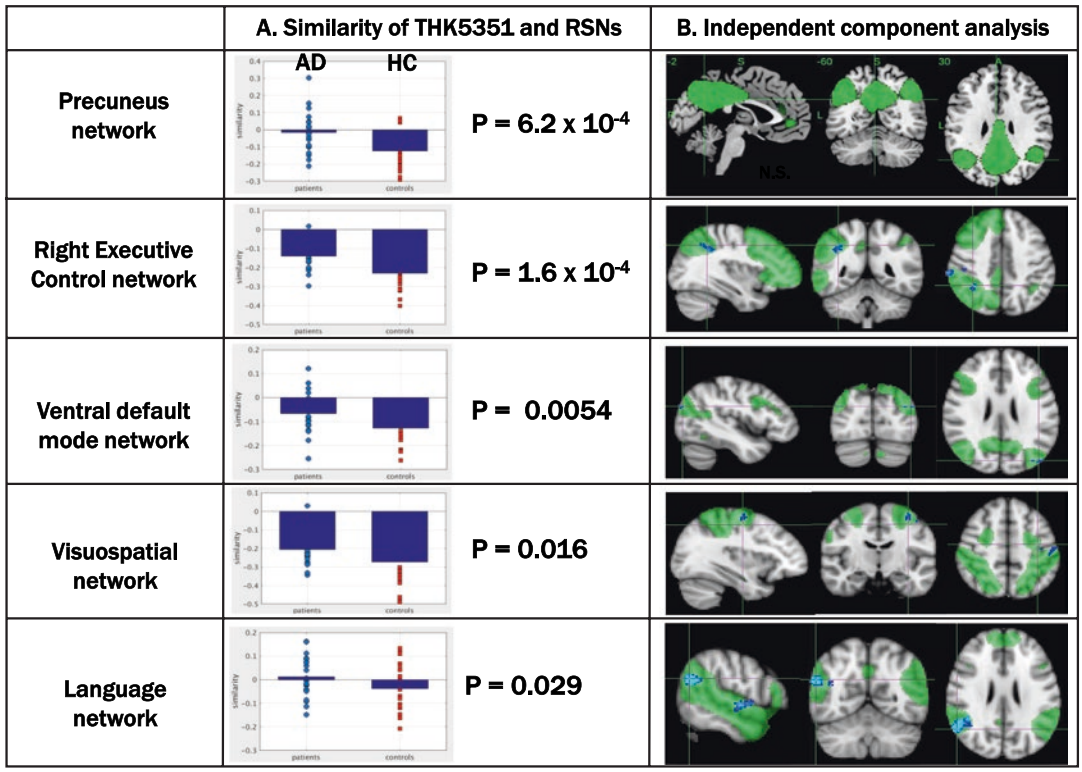

Fig. 19.4 Relationship between THK5351 retention and canonical resting state networks. (**a**) Shows the similarity of the THK5351 retention pattern and each canonical resting state network. (**b**) Is the results of independent component analysis. The green areas indicate each canonical network, and the blue areas indicate decreased connectivity, as identified by independent component analysis of resting-state functional MRI data (Family wised error (FWE) at $p < 0.05$)

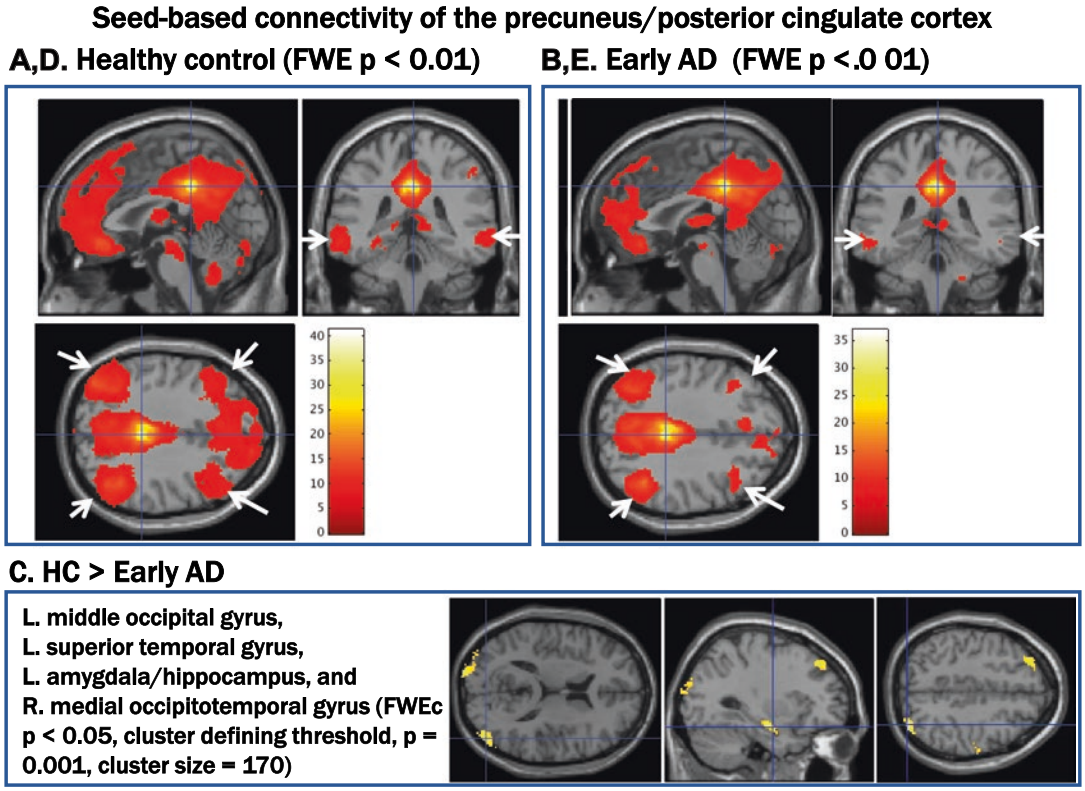

Fig. 19.5 Seed-based connectivity of the precuneus/posterior cingulate cortex. This figure shows seed-based connectivity analysis from ROIs located in the precuneus/PCC (**a**–**c**). (**a**) healthy control; (**b**), early Alzheimer's disease; (c) intrinsic connectivity of precuneus/PCC significantly decreased to the hot areas (Family wised error correction (FWEc) $p < 0.05$, cluster defining threshold, $p = 0.001$, cluster size = 170).

pathology networks extracted from patients with mild-to-moderate AD by ICA in healthy adults (n = 26) [26]. Then, they quantified the spatial overlap between the seed-based networks and the corresponding tau pathology network using the similarity coefficient. Besides, they compare the tau-dependent seed-based networks and canonical RSNs. As a result, the overlap between the tau pathology networks and similar seed-based networks was a fair-to-moderate level. These networks also resembled well-known RSNs, particularly the default mode network.

Hansson et al. investigated the regional distribution profile of AV 1451 pattern and canonical RSNs in two independent samples of prodromal cases (the ADNI study, n = 35) and manifest AD cases (the Swedish BioFINDER study, n = 44) [31]. In manifest AD cases, the typical AV 1451 retention was observed predominantly in the inferior, medial, and lateral temporal cortical areas, precuneus/posterior cingulate, and lateral parts of the parietal and occipital cortex. Based on the standardized maps of RSNs derived from large-scale rs-fMRI data of a healthy adult population, they computed quantitative metrics including the mean Z-score of AV 1451 within each RSN template and assessed the difference between the mean Z-score value of voxels falling within a given ICN template and the mean Z-score value of cortical voxels outside the ICN template as a goodness-of-fit (GOF) score. GOF showed that AV 1451 retention pattern overlapped primarily with the dorsal attention, and to some extent with higher visual, limbic and parts of the default-mode network. Prodromal AD showed highly similar spatial distribution profile but less pronounced.

Hub Vulnerability in AD

These three studies, which investigated the relationship between tau imaging networks assessed by THK5351 and AV 1451 and RSNs showed some differences but generally similar results about the involvements of default mode network particularly precuneus and posterior cingulate cortex. Both the precuneus and posterior cingulate cortex show high levels of metabolism, are vascular boundaries, serve as hub, and key nodes in the classical default mode network [32–35]. Pathologically, the precuneus/PCC is involved in Braak's stage IV, leading to the appearance of initial clinical symptoms. Tau pathology can spread along RSNs and extend to the precuneus, and posterior cingulate cortex could keenly associate with widespread disruption of brain networks resulting in the manifestation of dementia in AD.

Cope et al. investigated the relationship between retention pattern of AV-1451 and rs-fMRI analyzed by graph theory in 17 patients with AD and 12 controls. This study showed that strongly connected nodes displayed more tau pathology in AD, independently of intrinsic connectivity network. Increasing tau burden in AD can be associated with decreasing the connectivity of these same nodes associated with reducing the weighted degree and local efficiency and weaker 'small-world' properties supporting the idea of trans-neuronal spreading. In our study, THK5351 retention was the most prominent in the precuneus/PCC and the dorsolateral prefrontal cortex corresponding to hub.

Stam proposed that hub overload and failure as a final common pathway. Under normal conditions, a brain network constitutes a multilayered hierarchical structure with a hub as vertex. This is a well-designed organization for efficient exchange of information locally and globally. Graph analysis showed that the brain organization corresponds to an optimal balance between the segregation and integration for processing information [36]. Theoretically, nodes failure in the lower level of hierarchy can cause the enlargement of information processing load particularly in the hubs with the highest centrality. This compensatory mechanism will be important to keep its function and associated with resilience to disease or aging process but be also associated with overload of the hubs. If this aberrant rerouting is critical and sustained, the hub nodes themselves may become affected resulting in cascading network failure. It is considered the one of the reasons why hub nodes are vulnerable to damage in various neurological diseases.

Relationship Between Tau Retention and Structural Network Changes in AD

Diffusion-weighted imaging (DWI) can non-invasively provide the findings of white matter fiber architecture in vivo. Mainly, diffusion tensor imaging (DTI) has widely performed for investigating the structural integrity of white matter in patients with AD. Kitamura, et al. showed that PBB3 retention in orbitofrontal cortex associated with cortical thickness in the area and disruption of the orbitofrontal cortex – uncinate fasciculus network, resulting in the development and progression of apathy in AD. This result suggests the significant tau retention is directly associated with neuronal loss and anatomical network changes [37].

However, there are significant limitations in the DTI including crossing-fiber and kissing-fiber if we particularly investigate the global brain network analysis. A recent technique that enables fiber tract-specific statistical analysis using a specific fiber population within a voxel is based on DWI data using constrained spherical deconvolution (fixel-based analysis, FBA). FBA has a potential to characterize the multiple fiber orientations within voxels. Mito et al. demonstrated that FBA showed significant white matter involvement in specific fiber pathways related to default mode network nodes in AD patients. Intriguingly, patients with mild cognitive impairment patients only exhibited within the posterior cingulum [38]. Future study will be needed to elucidate the relationship between tau retention and FBA abnormalities.

Conclusions

In this review, we summarized the results of the spatial distribution pattern of cutting-edge tau PET tracer' retention as well network disruption assessed by resting-state functional MRI and diffusion tensor imaging in AD.

The data drive approach such as SSM/PCA and ICA has great advantage to extract the disease-specific stereotypically spatial pattern from brain tau tracer signals as multivariate decomposition in AD. SSM/PCA and ICA can also be useful for decreasing noise and off-targeted bindings data. It is believed that these methods will be appropriate for not only diagnosis but also better understanding the spatially distribution pattern of tau in AD.

According to recent comparative studies of PET and RSNs, Aβ and tau retention can spread along specific RSNs including default mode network, executive control network, visuospatial network, and language network. Hub lesions of the networks, which can demand higher metabolic and have differential gene expression showed the most prominent tau retention among these networks. Particularly, precuneus and PCC can be one of the most important vital nodes and disruptions of functional connectivity from these hubs to the presence of dementia in AD.

Although the reason why hub regions are vulnerable to AD pathology is not still elucidated, recent studies support the view that AD can be the disconnection syndrome with disruption of a large-scale network. Longitudinal PET and RSNs observation study from healthy aging to dementia will shed light on the mechanism of not only resiliency and compensatory mechanisms against Aβ and tau retention but also the picture of local overloads and failure of specific hubs.

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Stress and the Etiopathogenesis of Alzheimer's Disease and Depression

20

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Introduction

The brain is the most adaptive of all organs. It has a remarkable capacity to respond to a variety of internal and environmental stimuli and to mount pro-survival behavioral responses by orchestrating multiple molecular and biochemical cascades. The latter changes are embraced by the term neural plasticity, the cornerstone of learning and memory [1]. Impairments in neuroplastic mechanisms are commonly found during aging, the primary risk factor for Alzheimer's disease (AD), a disorder characterized by memory deficits. Over their lifetime, individuals experience both good and adverse (stressful) events and notably, stressful events appear to accelerate brain aging [2]. Accumulating clinical and experimental evidence suggests a causal role of lifetime stress in AD. This chapter summarizes current knowledge about how chronic stress and its accompanying high levels of glucocorticoid (GC) secretion, trigger the two main pathomechanisms of AD: (i) misprocessing of amyloid precursor protein (APP) and the generation of amyloid beta (Aβ) and (ii) Tau hyperphosphorylation and aggregation. Given that depression is a well-known stress-related illness, and the evidence that depression may precede AD, this chapter also explores neurobiological mechanisms that may be common to depressive and AD pathologies. This review also discusses emerging insights into the role of Tau and its malfunction in disrupting neuronal cascades and neuroplasticity and, thus triggering brain pathology.

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Stress: A Physiological Tug-of-War – From Adaptive to Maladaptive Responses

Stress is defined as a challenge to homeostasis (physiological and behavioral equilibrium) by physical or psychological events [3]. When challenged by endogenous or exogenous aversive or threatening stimuli (stressors), a series of defensive systems and processes become activated; these include the release of monoamines and GC that initially promote a return to the homeostatic state [4, 5]. The "stress response" normally terminates once homeostasis has been restored, but when the organism is faced with an insurmountable stress (high intensity, contextually inappropriate and/or chronic), it may take

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inappropriate – maladaptive – actions that result in chronically elevated GC secretion. Besides interfering with normal structural and plastic arrangements within the brain, such inadequate responses can have negative consequences for the immune and visceral systems that may ultimately lead to multiple disorders, including neuropsychiatric and neurological diseases [6–9].

The endocrine response to stress is orchestrated within the so-called hypothalamo–pituitary–adrenal (HPA) axis (Fig. 20.1). Stress, perceived by cortical areas of the brain, triggers the release of corticotropin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus (PVN) which, in turn, induces the secretion of adrenocorticotropic hormone (ACTH) release from the pituitary and GC (cortisol in humans and corticosterone in rodents) from the adrenal glands. This sequence of events is normally curtailed by negative feedback of GC at central sites; however, the nature of the stressor and/or impairments in negative feedback mechanisms (e.g. during aging) may block this crucial feedback loop, resulting in supraphysiological exposure to GC. A key area among the brain regions involved in the regulation of the HPA axis is the hippocampus; this area, which also plays a pivotal role in learning and memory, sends inhibitory projections to the PVN (and other hypothalamic nuclei). Similarly, the frontal cortex mediates GC negative feedback effects on the HPA axis, whereas the GC activation of the amygdala results in a positive drive on this axis (see Fig. 20.1).

Mechanisms and Consequences of GC Action in the Brain

Corticosteroid actions in the brain are mediated by glucocorticoid (GR) and mineralocorticoid (MR) receptors. The previously-referred feedback effects of GC during stressful experiences primarily depend on activation of GR, expressed throughout the brain, but at highest density in the hippocampus [10]. Indeed, almost all we know about GC actions in the brain are GR-mediated. Less is known about the role of MR, although, they have a ten-fold higher affinity for GC compared to GR and are believed to play an important role in GC feedback under physiological (non-stressful) conditions [11]. In addition, MR have been implicated in "protecting" the brain against GR-mediated cytotoxicity [12, 13] and behavioral maladaptation [14]. Interestingly, the central expression of GR and MR is subject to regulation by stress and age [15, 16].

While GC have been shown to modulate synaptic activity through non-genomic mechanisms [17–19], GR and MR are better known as potent ligand-dependent transcriptional regulators, i.e. control gene expression/repression [20–23]. The unliganded receptors are located in the cytoplasm in association with chaperone proteins (e.g. the heat shock proteins Hsp90 and 70 and the immunophilins FKBP51 and 52) [24]. Ligand binding results in conformational change of the GR-chaperone complex and subsequently, receptor translocation to the nucleus and binding to specific regions of DNA containing glucocorticoid response elements (GRE) within the promoters of target genes [25]. Gene transcription or repression is then determined by the recruitment of co-activators and repressors [26] as well as by post-translational modifications of the receptor [27–29].

In the brain, MR and GR differentially regulate the expression of genes, in a site-specific manner; these include genes responsible for the regulation of the HPA axis (*CRH* and *CRH receptors* and *pro-opiomelanocortin [POMC],* from whose gene product ACTH is cleaved) as well as pro- and anti-apoptotic genes [13] and, importantly, genes with roles in neural energy metabolism, structure and synaptic transmission, the synthesis of rate-limiting neurotransmitter enzymes and receptors as well of various neuropeptide, growth factors and cell adhesion molecule [30–34]. While all of these GC-initiated transcriptional events contribute to neural plasticity, stress and GC result in the manifestation of visualizable effects, namely, alterations in neuronal morphology. As reviewed by Lucassen [35], stress and GC lead to changes in the rate of neurogenesis, cell death, and neuronal connectivity as well as astroglia-neuronal interactions. In particular, numerous studies have highlighted how

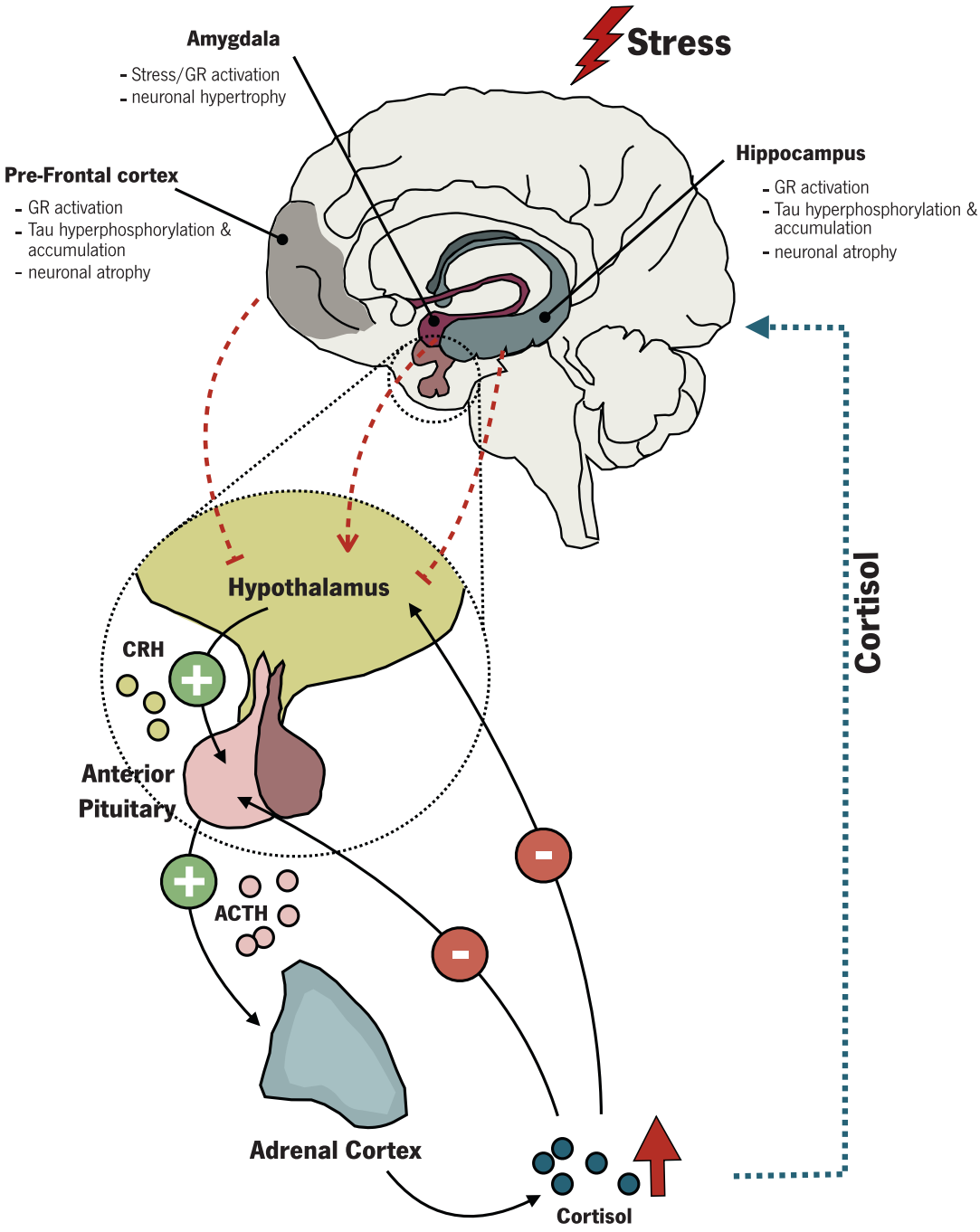

Fig. 20.1 The hypothalamo-pituitary-adrenal (HPA) axis and the response to stress in the healthy state. Stressors perceived in higher brain centers trigger the release of corticotropin-releasing hormone (CRH) from neurons in the paraventricular nucleus (PVN). Carried via a portal vein system, CRH reaches the anterior pituitary where it stimulates the secretion of adrenocorticotropin hormone (ACTH) which, in turn, stimulates the production and release of glucocorticoids (GC) [cortisol in humans and corticosterone in rodents] from the adrenal cortex. The secreted GC access peripheral and central tissues via the general circulation where they serve to mount adaptive responses to the initiating stimulus (stress) after binding to glucocorticoid receptors (GR). Eventually, GC secretion and action is restrained by inhibitory feedback of GC on central (chiefly, the frontal cortex, hippocampus, hypothalamus and pituitary) components of the HPA axis

stress, acting through GC, impacts on dendritic arborization and synaptic number; this aspect of GC actions is considered in the following section. First, in the context of AD as a disease that develops as age progresses, it is important to briefly mention the growing view that stress and GC leave long-lasting "memories" of past experiences via epigenetic mechanisms; these are thought to contribute importantly to the organism's physical and mental health trajectory [36–39]. Notably, epigenetic mechanisms have recently been implicated in the lasting effects of lifetime adversity in humans [40, 41].

Stress, Glucocorticoids and Neural Plasticity

Functional plasticity in the brain is generally preceded by structural plasticity, typically, dendritic and synaptic remodeling. Basal levels of GC are crucial for maintaining synaptic plasticity in the hippocampus in the form of long-term potentiation (LTP) [42], a well-documented mechanism involved in memory formation [43]. On the other hand, high levels of GC such as those experienced during stress impair LTP induction and facilitate long-term depression (LTD) [44]. An important role for *N*-methyl-d-aspartate (NMDA) receptors and shifts in calcium flux has been suggested in both, LTP and LTD modulation by stress/GC [45–48].

One of the best-described forms of stress-evoked structural plasticity is dendritic retraction, with a pioneering study revealing that chronic stress interrupts connectivity between hippocampal CA1 neurons and neurons in the medial prefrontal cortex (PFC) [49, 50]. The latter work followed previous demonstrations that chronic stress causes atrophy of apical (but not basal) dendritic complexity in CA3 pyramidal neurons [51]. Meanwhile, other studies have reported that stress can also increase dendritic length in certain brain regions such as the orbitofrontal cortex, amygdala and bed nucleus of the stria terminalis (BNST, also known as the "extended amygdala") [52, 53]. Interestingly, chronic stress has also been associated with a loss of mossy fiber synapses, increased surface area of the post-synaptic density, and rearrangements of synaptic mitochondria and vesicles at the presynaptic terminals [54]. Further, dendritic spines, which have an important role in information storage, are severely reduced by stress [55] but can mostly be rapidly reversed after a recovery period or subsequent training ([56, 57]; but see [58] for exceptions).

New work from our labs indicates that Tau, a key factor in AD pathology, is essential for chronic stress to disrupt neuroplasticity. Briefly, we showed that mice in which *Tau* has been deleted are spared from the deleterious behavioral (e.g. deficits in learning and memory, depressive-like behavior and anxiety) and neurostructural (namely, dendritic atrophy disconnection of the hippocampal-prefrontocortical pathway) of chronic stress and GC [59, 60].

As already alluded to, stress and GC also influence neuroplasticity by modulating the production of new neurons in adult brain [61]. Several studies indicate that stress/GC related effects on neurogenesis have the potential to affect mental health, including susceptibility to depression [62, 63] and AD [64, 65].

Chronic Stress: Etiopathogenic Role and Mechanisms in AD

A sizeable literature suggests that elevated GC and chronic stress – a state that an increasing proportion of the population finds itself in today – may increase the risk for developing AD pathology and related dementias [66, 67] and may even advance the age of onset of the familial form of AD [68–70]. Indirect support for the link between high GC exposure and AD includes reports that AD patients produce and secrete higher-than-normal levels of cortisol [67, 71–74]. Interestingly, transgenic mouse models of AD also display high levels of GC [75, 76]. Nevertheless, while the direction of the cause-effect relationship between AD-like pathology and hypercorticalism remains unclear, it is worth recalling (see previous section) that the hippocampus is responsible for mediating the negative

feedback effects of GC on the HPA axis; thus, any damage to this brain region is likely to uncouple this control mechanism and unleash unrestrained GC secretion.

To put these findings into context, it is worth noting that there is evidence that GC levels correlate with the rate of cognitive decline [35, 77] and the extent of neuronal remodeling in AD subjects [78]. Such remodeling is especially marked in the hippocampus, the area in which most studies on stress/GC effects on neuroplasticity have been conducted in rodents, and the brain area which clearly displays the first signs of AD neuropathology – deposits of amyloid β (Aβ) and accumulation and aggregation of hyperphosphorylated Tau [79–81]. The hippocampal lesions induced by these deposits correlate with the extent of deficits in declarative, spatial and contextual memory [82].

Consideration Regarding How Chronic Stress and High GC Levels May Contribute to AD Pathology

In this section, we will review some of the evidence for a link between GC/stress and AD and consider some of the possible underlying mechanisms. After briefly considering stress/GC effects on amyloidogenesis, our attention will focus on how chronic exposure to stress or high levels of GC influence Tau biology, culminating in its malfunction and dendro-synaptic toxicity.

As noted earlier, AD neuropathology is characterized by overproduction of Aβ that forms deposits into senile (amyloid) plaques, and by accumulation of hyperphosphorylated forms of Tau protein that becomes insoluble, aggregates and forms neurofibrillary tangles (NFT) [79–81]. Aβ is the proteolytic product of amyloid precursor protein (APP), a large transmembrane protein called, that is sequentially cleaved by β-secretase (BACE-1) and γ-secretase (a complex of enzymes, including presenilin) to yield Aβ; this post-translational pathway is often called APP misprocessing. Studies have shown that extended exposure to immobilization stress increases the load of extracellular Aβ deposits and exacerbates memory deficits in mice expressing an aggressive (human) mutant form of APP V717ICT-100 [76, 83]. Similar observations were made when young 3xTg-AD mice (expressing APP Swedish, P301L-Tau, and PSEN1 M146V mutations) were treated with the synthetic GC, dexamethasone [76]. That the effects of stress are most likely transduced by GC was demonstrated by experiments on dexamethasone-treated neural cell lines (N2A [76] and differentiated PC12 cells [84]). Consistent with these reports, our own studies in wildtype rats demonstrated that chronic stress and/or treated with GC increases APP misprocessing along the amyloidogenic pathway by upregulating BACE-1 and Nicastrin (a component of the γ-secretase complex) to produce neurotoxic and cognition-impairing effects [85]; in this regard, it is worth noting that high exogenous levels of GC upregulate the transcription of *APP* and *βACE-1*, the promoters of which contain a glucocorticoid response element (GRE) [76]. Lastly, experiments that attempted to mimic intermittent stressful events (the effects of which may be cumulative over the lifetime) showed that GC potentiate the APP misprocessing pathway [85].

In recent years, an increasing amount of attention has turned to Tau pathology, especially its hyperphosphorylated forms, in a range of neurodegenerative diseases. Among the first reports to indicate a relationship between stress/GC and Tau was a study by Stein-Behrens et al. [86] who found that GC exacerbate kainic acid-induced hippocampal neuronal loss with a contemporaneous increase in Tau immunoreactivity. A later study showed that chronic treatment of 3xTg AD mice with dexamethasone leads to the somatodendritic accumulation of Tau in the hippocampus, amygdala and cortex [76]. Our own *in vivo* studies demonstrated that chronic stress or GC increase the levels of aberrantly hyperphosphorylated Tau in the rat hippocampus and PFC, both in the presence and absence of exogenous Aβ [87]. Importantly, the hyperphosphorylation occurred at certain Tau epitopes that are strongly implicated in cytoskeletal dysfunction and synaptic loss (e.g., pSer262) [88, 89] and hippocampal atrophy (e.g., pThr231) [90] in AD patients.

Here, it is pertinent to note that the extent of phosphorylation at Thr231- and Ser262-Tau correlates strongly with severity of memory impairment, speed of mental processing, and executive functioning in AD patients [91–93]. Although chronic stress and GC treatment exert similar, but not identical, effects on individual Tau phosphoepitopes *in vivo* and *in vitro* [84], the overall evidence points to GC as the key mediator of the AD-like pathology induced by stress. On the other hand, some studies have suggested a role for at least one other stress-related molecule, namely, corticotrophin-releasing hormone (CRH) as deletion of the *CRH receptor 1* gene in mice prevents the detrimental effects of stress on Tau phosphorylation [94, 95]. Supporting these links, are the results from *in vitro* experiments which indicate that the GC effects on Tau involve activation of glycogen synthase kinase 3 (GSK3) and cyclin-dependent kinase 5 (CDK5), two principal Tau kinases [84].

Transgenic mice expressing human P301L-Tau (the most common Tau mutation), also helped strengthen the evidence that chronic stress can exacerbate Tau pathology. Briefly, we found that stress stimulates the aberrant hyperphosphorylation and aggregation of insoluble Tau [96]. Further, we demonstrated in the latter work that chronic stress enhances caspase 3-mediated truncation of Tau at its C-terminal in the hippocampus, with the protein misfolding and adopting a conformation [96] that facilitates its nucleation and recruitment of other Tau molecules into neurotoxic, pre-tangle aggregates of Tau (see [97–100]. Importantly, experiments also showed that GC contribute to AD pathology by reducing the degradation of Tau, thereby increasing its accumulation [84]. The latter is likely to result from dysregulation of molecular chaperones (e.g. Hsp90 and Hsp70) that are responsible for Tau proteostasis [96]. As noted previously, these same heat shock proteins serve to maintain GR in a high affinity state; thus, they may represent a point at which GC signaling intersects with the cellular machinery that regulates Tau degradation.

While Kobayashi et al. [101] showed Tau may be synthesized *de novo* in the somato-dendritic compartment, earlier work by Ittner and colleagues [102] demonstrated that hyperphosphorylated Tau is missorted to synapses which subsequently become dysfunctional. The missorting of Tau to synapses is now acknowledged as an early event in AD, preceding the manifestation of detectable neurodegenerative processes [102–104]. It is important to note that this series of events depend on Tau hyperphosphorylation [103, 105, 106] and results in the targeting of Fyn (a member of the Src kinase family) to postsynaptic sites [102] where it selectively modulates the function of GluN2B-containing NMDAR (GluN2BR), by phosphorylation of the GluN2B at the Y1472 epitope [102, 107]. The latter stabilizes GluN2B at postsynaptic sites, thus increasing the risk for excitotoxicity [102, 107].

Since NMDAR are known to mediate stress- and GC-driven neurotoxicity [108] and neuronal remodeling [109], we were prompted to examine whether the mechanistic scenario just described also applies to the actions of stress and GC. Indeed, we found that chronic stress and GC also trigger Tau accumulation at synapses with subsequent increases of Fyn at postsynaptic sites [59, 110] (see also Fig. 20.2).

Other mechanisms that may underlie the ability of stress/GC to contribute to AD pathology have been coming to light in the last few years. One of these is autophagy. As the guardian of cellular homeostasis, autophagy is now seen to play a pivotal role in the pathology of a number of neurodegenerative disorders [111, 112]. Briefly, autophagic mechanisms are responsible for the degradation of misfolded proteins and aggregates such as Tau aggregates; interruption of autophagy leads to the accumulation of protein aggregates, a pathological features shared by a range of neurodegenerative disorders [113, 114]. Our investigations demonstrated (summarized in Fig. 20.3), for the first time, that both, chronic stress and high GC levels inhibit autophagic process, thus explaining how these conditions contribute to the accumulation and

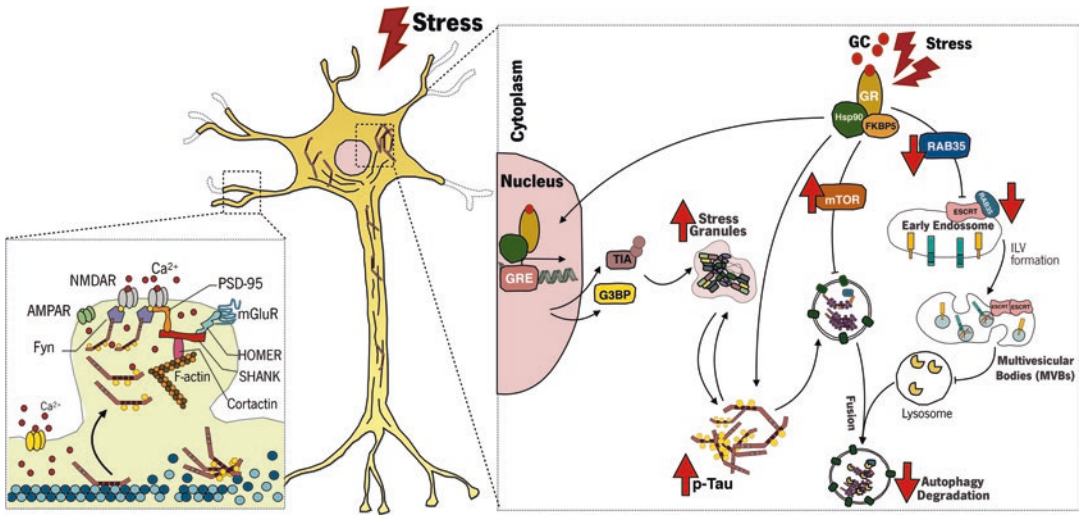

Fig. 20.2 Multiple mechanisms contribute to the induction of Tau pathology and AD by chronic stress. The scheme summarizes the potential mechanisms through which chronic stress and GC activate processes that result Tau accumulation, aggregation and neurotoxicity. Stress leads to increased activation of glucocorticoid receptors (GR) by GC; GR transcriptional activity depends on an interplay of a variety of molecular chaperones (e.g. Hsp90, Hsp70, FKBP51) and HDAC6, a protein that may lead to cytoskeletal instability by reducing the acetylation of tubulin and cortactin. As described in the text, GC induce hyperphosphorylation of Tau and its consequent detachment from microtubules, leading to microtubule destabilization and cytoskeletal disturbances that, together with HDAC6, may contribute to: (i) the formation of stress granules (SG) that promote Tau aggregation and (ii) the inhibition of autophagic process that also contribute to Tau accumulation and aggregation. Interestingly, stress/GC inhibit mTOR, a crucial signaling molecule in the initial phases of autophagy

aggregation of Tau [115]. In fact, defective autophagy is suggested to be major player in AD pathology [116–118]; although Tau itself is a proteosomal substrate [119, 120], it is thought that Tau inclusions and aggregates may be inaccessible to the ubiquitin-proteasome system [121, 122]. Our results showing that chronic stress and GC increase mTOR signaling and reduces the ratio of the autophagic markers LC3II:LC3I and accumulation of p62 [115], indicate that chronic stress inhibits the autophagic process by activating the mTOR pathway; these findings are in line with previous reports that chronic stress stimulates mTOR activity in the hippocampus [123], an event associated with increased total Tau levels in the brains of AD subjects [124, 125]. In addition, support for our interpretation comes from the finding that inhibition of mTOR signaling ameliorates Tau pathology [126, 127] while we demonstrated that inhibition of mTOR blocked the GC-triggered Tau accumulation and aggregation [115].

New research has implicated the endolysosomal pathway in neurodegenerative diseases such as AD and Parkinson's disease in which Tau accumulation is a pathological feature [128–130]. As shown in Fig. 20.2, Tau has been identified as a substrate of the endolysosomal degradation pathway [131]. We demonstrated that *in vitro* or *in vivo* exposure to elevated GC levels block this pathway, accompanied by increases in the build-up of Tau, including that of specific phospho-Tau species. Further, we showed that the involvement of the small GTPase Rab35 and the endosomal sorting complexes required for *transport* (*ESCRT*) machinery that delivers Tau to lysosomes via early endosomes and multivesicular bodies (MVBs). The ESCRT system mediates the degradation of membrane-associated proteins such as epidermal growth factor receptor [132],

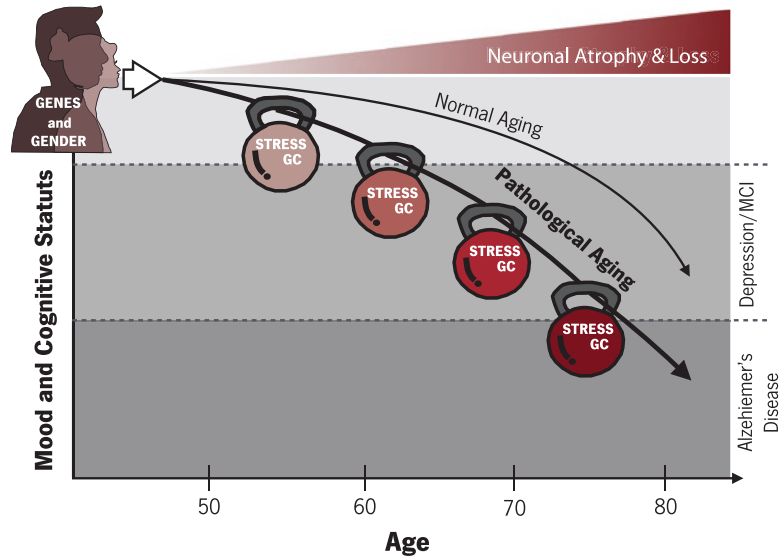

Fig. 20.3 Cumulative effects of stress and glucocorticoids on normal and pathological aging. In this hypothetical representation of brain aging, cognitive and mood status may decline over time. Chronic exposure to stressful conditions, associated with higher exposure to GC, lead to cumulative effects that accelerate brain aging by imposing an increasing allostatic load on brain function by causing neuronal atrophy and synaptic loss, modified by other factors such as genetics and sex – the latter also influence the magnitude of the stress load by modulating the activity of the hypothalamo-pituitary-adrenal (HPA) axis. The model assumes that elements of the HPA axis serve as part of a threshold-regulator mechanism (represented by thick line). Note that brain areas important for regulation of HPA axis (e.g. hippocampus, prefrontal cortex) also appear to be subject to impairments triggered by presymptomatic AD pathology (e.g. mild cognitive impairment, depression), thus feeding into a vicious cycle that further drives GC secretion and neuronal damage. The shaded grey area represents the threshold-transition area where a subject may progress from depression (with or without MCI symptoms) to Alzheimer's disease (AD)

but is also implicated in the degradation of cytosolic proteins GAPDH and aldolase [133]; these findings are of particular relevance for Tau, which has both cytosolic and membrane-associated pools [134, 135], and has been shown to localize to different neuronal sub-compartments, depending on its phosphorylation state [103, 110]. Interestingly, not all phosphorylated Tau species are equally susceptible to degradation in the Rab35/ESCRT pathway. In particular, we found that pSer396/404 and pSer262, but not pSer202, phospho-Tau species undergo Rab35-mediated degradation, indicative of preferential sorting of specific phospho-Tau proteins into the Rab35/ESCRT pathway [131]. Importantly, we demonstrated that high GC levels suppress *Rab35* transcription, and thus, result in an accumulation of Tau due impaired degradation of the protein (Fig. 20.2). Further, overexpression of Rab35 reverses GC-induced Tau accumulation and rescues hippocampal neurons from the dystrophic actions of chronic stress [131].

RNA-Binding Proteins and Stress Granules Facilitate Stress-Induced Tau Pathology

Stress granules (SG) have been recently implicated in the Tau pathology that accompanies AD and fronto-temporal dementia with parkinsonism-17 (FTDP-17) in humans as well as in various transgenic mouse models of Tau-related disorders [136]. The eukaryotic stress response involves translational suppression of non-housekeeping proteins and the sequestration of

unnecessary mRNA transcripts by RNA-binding proteins (RBP) into SG. These macromolecular complexes constitute a protective mechanism against cellular stress (e.g. oxidative stress) that help protect mRNA species and enable the fast production of cytoprotective proteins [136–138]. However, prolonged SG induction can become pathological and neurotoxic; in neurodegenerative diseases such as AD, SG promote the accumulation of Tau aggregates [139–142]. In fact, SG are suggested to accelerate Tau aggregation in a vicious cycle wherein Tau stimulates SG formation, with the RNA binding protein TIA1 playing a lead role in Tau misfolding and aggregation [143]. Notably, while hyperphosphorylation and aggregation-prone mutations of Tau can enhance SG formation, they are not essential for this event [143].

We recently showed that chronic stress and high GC upregulate various RBP and SG markers in soluble and insoluble fractions in the hippocampus of P301L-Tau Tg animals and primary neuron cultures. Specifically, tissues from animals exposed to chronic stress displayed increased cytoplasmic (soluble and insoluble) levels of several RBPs and SG-associated markers (e.g. TIA-1, PABP, G3BP, FUS, DDX5) that contributed to the formation of insoluble Tau inclusions and Tau accumulation. As noted above, TIA-1 plays a prominent role in Tau aggregation: under stressful conditions, TIA-1 is trafficked the nucleus to the cytospasm where it interacts directly with Tau (and other RBP such as PABP and EWSR1) to stimulate its aggregation and accumulation [143–145].

In other recent work, we showed that Tau missorting and accumulation in the dendritic compartment, such as is found in AD pathology [102], is also triggered by chronic stress/GC exposure [59, 110]. This is interesting because Tau missorting is hypothesized to facilitate formation of SG as part of the translational stress response [143]. While the temporal profile and precise mechanisms underlying stress/GC-evoked dysregulation of RBPs and the associated SG cascade remain to be elucidated, Fig. 20.2 illustrates our current working model, designed to explore more about the biology of RNA-protein interactions in stress-related pathologies.

Tau and Its Malfunction in Stress-Related Brain Pathology: Beyond Alzheimer's Disease

Stress pervades all our lives and most of us will respond to daily life stressors in an adaptive manner. However, as noted by Selye as early as 1936, mounting a transient and adaptive response may not be possible in all circumstances and the stressful experience may become chronic and maladaptive. The negative impact of chronic stress (and the associated rise in circulating GC levels) on brain structure and function (e.g. cognition, mood, emotion) is now well recognized. In addition to the role of chronic stress/GC in the development of AD pathology, chronic stress is causally related to major depression which, as in AD, may reflect defects in neuroplastic mechanisms [1, 5, 146]. As briefly mentioned above, major depressive disorder appears to predispose to AD [147]. The body of evidence supporting the latter clinical observation includes findings of potentially common neurobiological mechanisms in the two disorders [84, 85, 148, 149]. Given this, it is interesting that epidemiological studies implicate depression as a risk factor for the development of AD [5], with support for this coming from the observation that previously depressed subjects have increased amyloid plaque and neurofibrillary tangles (NFT) loads [150]. Indeed, since clinicians are sometimes faced with the challenge of distinguishing between patients suffering from depression and AD, several authors have attempted to develop assays based on the detection of various APP cleavage products that might help such distinction [151–154], albeit with little success.

We previously referred to neurogenesis as a phenomenon that contributes neuroplasticity, with impaired neurogenesis being implicated in the pathogenesis of depression [155] as well as

AD [156]. Neurogenesis declines with age (also in humans [156]) and is disrupted by stress and high GC levels [62]. In light of the previously-referred interactions between stress/GC and Tau, it is therefore interesting that our recent research suggests that Tau plays an essential role in stress-driven suppression of birth of neurons (but not astrocytes) in the adult dentate gyrus (DG, a hippocampal subfield) [62]; specifically, chronic stress is unable to impair the propliferation of neuroblasts and newborn neurons in the DG of mice in which the *tau* gene is deleted. Interestingly, *tau* ablation does not interfere with stress-induced suppression of astrocyte proliferation. This finding is likely related to the differential expression of Tau in neuronal *vs.* astrocytic precursor cells – Tau is expressed in neurons at much higher levels than in astrocytes [157]. These observations suggest a novel mechanism through which stress can remodel the adult brain. Interestingly, our investigations also showed that chronic stress increases the 4R-Tau:3R-Tau isoform ratio in the DG. Given that 3R-Tau has a lower affinity for MT than 4R-tau, neuroblasts may be endowed with greater cytoskeletal plasticity than mature neurons since 3R-Tau is more abundant in the former. Here, it is also relevant to note that it was recently shown that higher levels of 4R-Tau are associated with increased Tau phosphorylation and brain pathology [158]. Moreover, an increased 4R/3R-Tau ratio is associated with cytoskeletal disturbances and tauopathies such as AD [64].

Summary/Conclusions

The evidence reviewed in this chapter suggest that deficits in Tau function and Tau proteostasis may be critical and cooperative mechanisms through which stress (whose actions are executed by GC) remodels neural circuits (cell birth and death, dendritic and synaptic atrophy/connectivity), thus inducing impairments mood and cognition. Importantly, we suggest that Tau lies at the core of a set of common neurobiological mechanisms that link stress with AD and other brain pathologies such as depression.

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Tau, Diabetes and Insulin

21

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Overview on Tau and Tauopathies

Tau protein which was discovered in 1975 [310] became of great interest when it was identified as the main component of neurofibrillary tangles (NFT), a pathological feature in the brain of patients with Alzheimer's disease (AD) [39, 110, 232]. Tau protein is expressed mainly in the brain as six isoforms generated by alternative splicing [46, 97]. Tau is a microtubule associated proteins (MAPs) and plays a role in microtubules assembly and stability, as well as diverse cellular processes such as cell morphogenesis, cell division, and intracellular trafficking [49]. Additionally, Tau is involved in much larger neuronal functions particularly at the level of synapses and nuclei [11, 133, 280]. Tau is also physiologically released by neurons [233] even if the natural function of extracellular Tau remains to be uncovered (see other chapters of the present book).

Tau protein sequence contains more than 85 phosphorylated or phosphorylable sites [266]. As a result of hyperphosphorylation, conformational changes in Tau protein notably impair its ability to bind to microtubules. Consequently, accretion of free monomers of misfolded Tau leads to accumulation, oligomerization and aggregation. During the aggregation process, repeated domains of Tau adopt a beta sheet conformation [28] and form filaments [155]. Tau aggregates can deposit in NFT a pathological feature of a group of diseases called Tauopathies [169, 232, 273], divided into primary Tauopathies (Pick's disease, progressive supranuclear palsy, fronto-temporal dementia…) and secondary or mixed Tauopathies (Alzheimer's Disease…) characterized by different clinical features and pathological hallmarks reviewed elsewhere [168] (see other chapters of the present book). Tauopathies particularly differ from the cell types exhibiting NFT and Tau isoforms aggregation [266]. Notably, in AD, NFT are observed early in life and increase during aging [37]. The spatiotemporal progression of NFT from the entorhinal cortex and the hippocampus to the isocortical areas has been shown correlated with cognitive deficits [73], supporting a pivotal role for Tau pathology and spreading in AD-related

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memory impairments [72]. Remarkably, some AD patients also present alterations in hypothalamus, a brain structure that plays a key role in the central control of energy metabolism [193]. Indeed, in a study investigating 28 patients with AD, 22 showed plaques and NFTs in the hypothalamus [193]. Braak in its description of the different stages of AD reported plaques and tangles in the hypothalamus at stages III and IV [37]. Interestingly these stages correspond to early-moderate AD, whereas disturbances in metabolism, such as weight loss, which is regulated by the hypothalamus, are often described to appear prior to cognitive impairments. The latter indicates that factors different from Tau and Aβ accumulation in the hypothalamus could play a role in metabolic deregulation. Indeed, studies of early cases of AD show neuronal loss in several nuclei of the hypothalamus such as the supraoptic nucleus (SON), and the paraventricular nucleus (PVN), and more so in the suprachiasmatic nucleus (SCN), with minimal deposits of Tau or Aβ [17, 281]. SCN degeneration in AD was correlated with circadian rhythm deregulation of body temperature [115]. Notably, deregulation of body temperature is well characterized to promote Tau pathology *in vivo* [75, 104, 295, 303]. Hyperphosphorylated Tau as well as NFT [173] and atrophy [276] in the hypothalamus have also been reported in mouse models of Tauopathies.

Hyperphophorylated Tau is the result of an imbalance between kinases and phosphatases. More than 30 kinases have been described as regulating Tau phosphorylation *in vitro* while only a few have been confirmed *in vivo*. Among them, GSK3-β is an important kinase which phosphorylates Tau on more than 30 sites and seems to play a pivotal role in AD and NFT development [14]. All serine/threonine phosphatases in the brain are capable of dephosphorylating Tau *in vitro* except PP2C [100, 101, 177, 240, 319], with PP2A contributing to 71% of the phosphatase activity on Tau [294]. Interestingly, several kinases and phosphatases of the Tau protein are particularly involved in the signalling of insulin pathway such as GSK3-β, AMPK, ERK, JNK, PP1 and PP2A.

Physiology of Insulin

Peripheral Insulin

Insulin Biosynthesis

Insulin is a peptide hormone composed of 51 amino acids which was discovered in 1921 by Banting, Best, Macleod and Collip from dog pancreatic extracts (for history Rosenfeld [254]). Mature insulin is produced in the beta cells of the islets of Langerhans and is formed of two peptide chains connected by two disulfide bonds: the A and B chains that are composed of 21 and 30 amino acids, respectively. It derives from the proteolytic processing of a precursor molecule called Proinsulin that is cleaved into mature insulin and C-peptide by the prohormone convertases PCSK1 and PCSK2 (for review Steiner et al. [275]). After cleavage, mature insulin is stored in secretory granules before being secreted into the bloodstream by exocytosis (for review Fu et al. [85]).

Insulin Secretion

The main stimulus for insulin secretion is the increase in blood sugar. When glycemia increases, glucose enters in β-cells of pancreatic islets through the glucose 2 receptor (GLUT2). Subsequently, glycolysis and Krebs cycle at the mitochondrial level occur leading to an increase of intracellular ATP. This is followed by a membrane depolarization, caused by the closing of K^+ channels, sensitive to ATP. Voltage dependent Ca^{2+} channels then open and induce a massive influx of calcium ions leading to the secretion of insulin by exocytosis of the secretory granules (for review [70, 122]). Other factors may also stimulate insulin secretion such as Glucagon-like peptide-1 (GLP-1) and other digestive tract hormones (secretin and gastrin) [163, 249]. Insulin secretion and action are also regulated by the autonomic nervous system (ANS), parasympathetic fibers directly stimulating insulin secretion through muscarinic receptors M3 present at the surface of the β-cell. The main effect of sympathetic innervation is an inhibition of insulin secretion through β adrenergic receptors.

Insulin Signaling

The insulin receptor (IR), which is widely expressed in peripheral tissues, is a heterotetrameric membrane glycoprotein belonging to the tyrosine kinase receptor family. It is composed of two α- subunits at the extracellular level that bind insulin, and two β-subunits generating the intracellular signaling cascade of insulin. When insulin binds to α subunits, a conformational change occurs, leading to the receptor autophosphorylation of tyrosines on the β-subunit. Insulin signaling through its receptor involves the tyrosine phosphorylation of cellular substrates, including several insulin receptor substrates (IRSs) [313] as well as other scaffold proteins such as Src-Homology2/a Collagen (SHC) [145] and Grb2-associated docking protein (Grb2-SOS) [125]. The IRSs ensure metabolic effects of insulin through the activation of PhosphatidylInositol 3-Kinase (PI3K) which recruits both the Ser/Thr 3-phosphatidylinositol-dependent protein kinase (PDK) and protein kinase B (PKB or Akt) to the plasma membrane, where Akt is activated by PDK1- and PDK2 [57]. At the end of this pathway, mTOR links insulin signaling to nutrient sensing [284]. Mitogenic effects of insulin involve the recruitment of mitogen-activated protein kinases (MAPKs) through small G-protein Ras leading to the translocation of ERK to the nucleus, where it controls gene expression [92].

Insulin Physiological Functions

The main insulin biological role is to lower the level of blood glucose in case of hyperglycemia, following a meal for example. For this, insulin increases the use of glucose by peripheral tissues such as skeletal muscle or adipose tissue, stimulates hepatic glycogenogenesis and inhibits hepatic neoglucogenesis (for review Barthel and Schmoll [24]). Glucose uptake by muscle and fat cells is made possible by the binding of insulin to its receptor leading to the translocation of GLUT4 glucose transporters to the cell membrane. Insulin then stimulates the use of glucose by peripheral tissues as an energetic substrate for glycolysis or promotes its storage in the form of glycogen or triglycerides. Skeletal muscles capture the majority of glucose in case of hyperglycemia, while adipose tissue captures only 10% of plasma glucose [66]. Glucose that is not used or stored as glycogen can be converted into fatty acids in liver and adipose tissue under the influence of insulin (for review Newsholme and Dimitriadis [205] and Smith [270]) through a process called *de novo* lipogenesis [296]. Additional functions of the hormone have been evidenced in the brain, particularly in the regulation of food intake, reproduction, learning and memory and are detailed in section "Brain Insulin Physiological Functions".

Insulin in the Brain

Origin of Brain Insulin

Insulin was first detected in the rat brain through immunostaining in 1978 [118]. In this study, levels of insulin were higher in the brain compared to plasma and seemed to be independent of the peripheral concentration [117]. Immunoreactive insulin was also found in dogs brain and seemed to be correlated with basal plasma levels [322] and dependent on feeding state, being lower during fasting [279]. Insulin mRNA was then identified by *in situ* hybridization in various brain areas including hypothalamus, hippocampus and olfactory bulb in rodents [67, 324], suggesting a putative role of the CNS in the production of the hormone. While central insulin production is still controversial, insulin transport from the periphery to the brain is well documented. Transport of insulin through the blood brain barrier (BBB) was first reported in rabbits using radiolabeled insulin [71]. Insulin transport necessitates binding of the hormone to the IR and transcytosis of the IR insulin complex through brain endothelial cells allowing insulin to cross the BBB without being degraded. This transport can be regulated by high fat diet, astrocyte stimulation or nitric oxide inhibition [107].

Brain Insulin Receptors

Radiolabeled ^{125}I-insulin allowed the detection of the receptor in the brain of monkeys, pigeons, humans and rats [69, 144, 234]. IRs found in the brain and liver showed different patterns of

migration during electrophoresis suggesting two different isoforms [180]. In addition, conversely to peripheral IRs, brain receptors are not down-regulated by insulin excess [119]. IR in the brain is present at all stages of development, although its distribution and concentration vary between embryonic and adult brain suggesting a role of insulin in neurogenesis [144]. *In situ* hybridization of IR mRNA in the adult rat brain revealed high gene expression in the hypothalamus, more particularly in the anterior nuclei, in the hippocampus, the olfactory bulb and the choroid plexus [189], regions concerned with olfaction, appetite, cognition and autonomic functions [311]. Although insulin receptors in the brain possess kinetic [326], pharmacological and signaling characteristics very similar to those present at the periphery, a smaller size of the β subunits and a more large glycosylation of insulin receptors in periphery is however notable [119, 140, 180].

Brain Insulin Physiological Functions

The biological role of insulin in the CNS is difficult to study and is thus not fully characterized. However, receptor localization and *in vivo* and *in vitro* studies have established several insulin brain functions.

One of the best-known roles of central IR activation is its involvement in the regulation of glucose metabolism and feeding behavior. For instance, study of the neuron-specific knockout mouse model of the insulin receptor (NIRKO mice) showed that inactivation of IR in the CNS led to increased food intake and obesity, together with insulin resistance, hyperinsulinemia and hypertriglyceridemia [43]. Further studies using selective inactivation of IR in specific neuronal populations of arcuate nucleus in the hypothalamus showed that agouti-related peptide (AgRP)-expressing neurons, but not pro-opiomelanocortin (POMC)-expressing neurons were required for central insulin control of hepatic glucose production [160]. In the same manner, inactivation of IR in Neuropeptide Y (NPY) expressing neurons induced an obese phenotype, leading to the conclusion that insulin signaling in NPY neurons controls food intake and energy expenditure [179]. In addition to the hypothalamus, insulin signaling was also shown to play a role in reward dopaminergic circuitry, since inactivation of IR in tyrosine hydroxylase-expressing cells resulted in increased body weight, elevated fat mass, and hyperphagia and seemed to be dependent of dopaminergic neurons in the Ventral Tegmental Area (VTA) and Substantia Nigra (SN) [159]. Experiments using intranasal insulin, which allow the uptake of insulin from olfactory nerves [250], bypassing the BBB, were of significant importance to isolate insulin effects on the brain from the periphery. In Humans, intranasal insulin has been shown to regulate responses to food cues and smelling capacity [82], increase satiety [114] and to decrease food intake [135]. In animal models, brain insulin has been reported to be involved in satiety [18–20], reduction of lipolysis in adipose tissue [261], and regulation of insulin sensitivity in skeletal muscle and liver (brain insulin is required for glucose production) [209]. In the rat brain, insulin regulates enzymes of cerebral glucose metabolism such as hexokinase and phosphofructokinase [128]. Like at the periphery, insulin is involved in glucose uptake in the brain, although to a lesser extent since the most abundant glucose transporters in the brain, GLUT-1 (entire brain astrocytes and endothelial cells), GLUT-2 (hypothalamus), and GLUT-3, (the major glucose transporter in the neurons of the cerebellum, striatum, cortex, and hippocampus and some glial and endothelial cells) are not insulin dependent (reviewed in Blazquez et al. [33]). Indeed insulin dependent GLUT-4 is found in much lower amounts in selective areas of the brain, including the olfactory bulb, dentate gyrus of the hippocampus, hypothalamus, and cortex [76]. The neuron-specific glucose transporter GLUT-8 (also known as GLUTX1), also insulin dependent, is expressed in several areas of the brain, in particular in the hippocampus where it is thought to contribute to glucose homeostasis in neurons [225, 246].

Insulin in the brain also seems to play a role in reproduction. The same mice previously described as being deleted for insulin receptors only in neurons (NIRKO mice) have reduced fertility, due to impaired spermatogenesis and maturation of ovarian follicles following luteinizing

hormone (LH) dysregulation [43]. The presence of insulin receptors on the neurons producing Gonadotropin-Releasing Hormone (GnRH) in the hypothalamus could explain this phenomenon. Indeed, GnRH is a hormone that controls the development of ovarian follicle, ovulation and maintenance of the luteal body in the menstrual cycle in women, but also spermatogenesis in men. Studies have shown that insulin stimulates the release of GnRH both *in vitro* and *in vivo* [45, 162, 289].

Other insulin functions not related to metabolism and reproduction have been suggested to occur in the brain: neuroprotective qualities, stimulation of neuritic growth and synaptic plasticity, promotion of neuronal survival during development, stimulation of protein synthesis as well as a role in the neuronal activity (for review Blazquez et al. [33]).

Finally, numerous studies suggest that insulin may be involved in formation and storage of memory. The hypothesis linking insulin to memory is born from the abundant presence of insulin receptors in the hippocampus and the cortex, highly involved in reward and recognition systems as well as in memory. Insulin through GLUT-4 and GLUT-8 could allow that areas involved in cognition receive the necessary amount of fuel to function. Several studies in mice indicate that insulin contributes to changes in hippocampal synaptic plasticity by increasing the induction of long term potentiation (LTP) [202] or long-term depression (LTD) [300], two molecular mechanisms involved in hippocampal-dependent learning and memory. In accordance, impaired LTP and spatial learning have been reported in mice with a down-regulation of IRs in the hippocampus [109]. One of the mechanisms proposed is the regulation of cell membrane expression of N-methyl-D-aspartate (NMDA) receptors by insulin [269]. These changes are thought to involve insulin activation of ERK1/2 [315] or PI3K signaling [257]. Insulin signaling has been shown to contribute to synaptogenesis and synaptic remodeling in the rat brain and cultured hippocampal neurons [1]. In Humans, administration of intranasal insulin improved memory functions in healthy subjects [26, 27], with no effect on word recall and non-declarative memory but improvement of declarative, hippocampus-dependent memory contents [26, 148]. Besides, epidemiological studies have shown that patients with deregulation of insulin secretion and/or function (type 1 and type 2 diabetics) have cognitive impairment and an increased risk of AD [253].

Thus, insulin is a key hormone in the body, whether in the CNS or at the periphery. It is therefore not surprising that an alteration of the functions of this hormone leads to pathological conditions, the best known being diabetes mellitus.

Pathophysiology of Insulin Functions: Diabetes Mellitus

Diabetes mellitus, commonly referred to as diabetes, is a chronic condition characterized by hyperglycemia. Chronic hyperglycemia is caused by a lack or failure of insulin use. There are different forms of diabetes, the two major ones being type 1 diabetes (T1D) and type 2 diabetes (T2D) accounting for 5–10% and 90–95% of cases respectively. According to the WHO, 422 million people worldwide had diabetes in 2014. This pathology is in full expansion; by 2030, diabetes will be the seventh leading cause of death in the world [191].

Type 1 Diabetes

T1D is insulin-dependent diabetes, characterized by autoimmune destruction of insulin-producing β-cells of pancreatic islets. This destruction leads to very low or no insulin production resulting in hyperglycemia. This type of diabetes, whose diagnosis is made early in life (children and young adults), requires treatment with insulin accompanied by tight blood glucose control to maintain glycemic homeostasis in the body. Although the exact causes of this disease are still not well understood, it seems that a combination of genetic factors, mainly related to the major histocompatibility complex, and environmental

contribution, probably viral or toxic, are necessary for the development of the disease [13]. In terms of clinical symptoms, T1D results in weight loss despite an increased appetite, accompanied by polyuria (abundant urine) and polydipsia (feeling of intense thirst).

Type 2 Diabetes

T2D is a diabetes independent of insulin. In T2D, β-cells of pancreatic islets produce insulin correctly, but when secreted because of hyperglycemia, cells are unable to use it to initiate insulin signaling cascade to restore physiological blood glucose level: this is insulin resistance [6]. Insulin resistance results in decreased skeletal muscle uptake of glucose and increased production of free fatty acids by adipose tissue, as adipose cells are no longer able to use insulin to inhibit lipolysis. In most patients, persistent hyperglycemia due to insulin resistance in the cells causes the body to overproduce insulin in an attempt to restore circulating glucose homeostasis. This positive feedback from insulin-producing cells leads to hyperinsulinemia that accompanies hyperglycemia [80]. Ultimately, this overproduction of insulin can lead to depletion of β cells of pancreatic islets [64, 65]. Patients become then, as in T1D, hyperglycemic and hypoinsulinemic.

The exact causes of insulin resistance are not yet well understood. Rare cases have been explained by mutations in genes encoding insulin receptors or key players in its signaling pathway [291]. However, for most patients, no mutation has been found. The strongest hypothesis to explain insulin resistance is related to physical inactivity that could lead to chronic inflammation of the tissues and thus contribute to the development of insulin resistance through overproduction of lipids and metabolic damage. This hypothesis suggests that the accumulation of lipids in the tissues activates pro-inflammatory signaling pathways. This pro-inflammatory activation impairs insulin signal transduction by altering phosphorylation events and key protein-protein interactions (for review Schmitz-Peiffer [262], Turban and Hajduch [298], and Roberts et al. [251]).

There are several risk factors that promote the development of T2D, the three main ones being genetic factors, age and obesity [49, 129, 196]. Historically diagnosed in adults, T2D is a growing disease that is now increasingly present in children and adolescents mainly because of the raise of childhood obesity in recent decades. According to the WHO, T2D cases have almost quadrupled since 1980 in the world, mainly because of the growth of obesity and the aging population. More than one in ten people will be diabetic by 2025 if this trend persists. These results are worrying as T2D is an important risk factor for many diseases such as vascular diseases that can lead to heart problems, blindness or amputations [224, 316]. T2D is also a subsequent risk factor for the development of neurodegenerative diseases such as Alzheimer's disease [30, 31, 203].

Diabetes Mellitus, Tau Pathology and AD

T1D, Tau Pathology and AD

In Humans

There are currently no epidemiological data to link T1D to AD. These data are difficult to collect given the lower life expectancy of T1D patients [130, 220]. However, many studies have shown significant cognitive and intellectual impairments in patients with T1D, including memory and mental plasticity, as in AD [38, 83, 121]. The early onset of T1D appears to be an aggravating factor for cognitive impairment in patients [90, 176]. In addition, higher phosphorylated Tau levels were observed in the CSF of patients with T1D compared to healthy subjects, as in patients with AD [212]. Interestingly, the same study also revealed a rise of Aβ levels in the CSF of these patients, contrary to what is seen in AD (Fig. 21.1).

Several studies have observed Alzheimer-type neuropathologies in animal models of T1D,

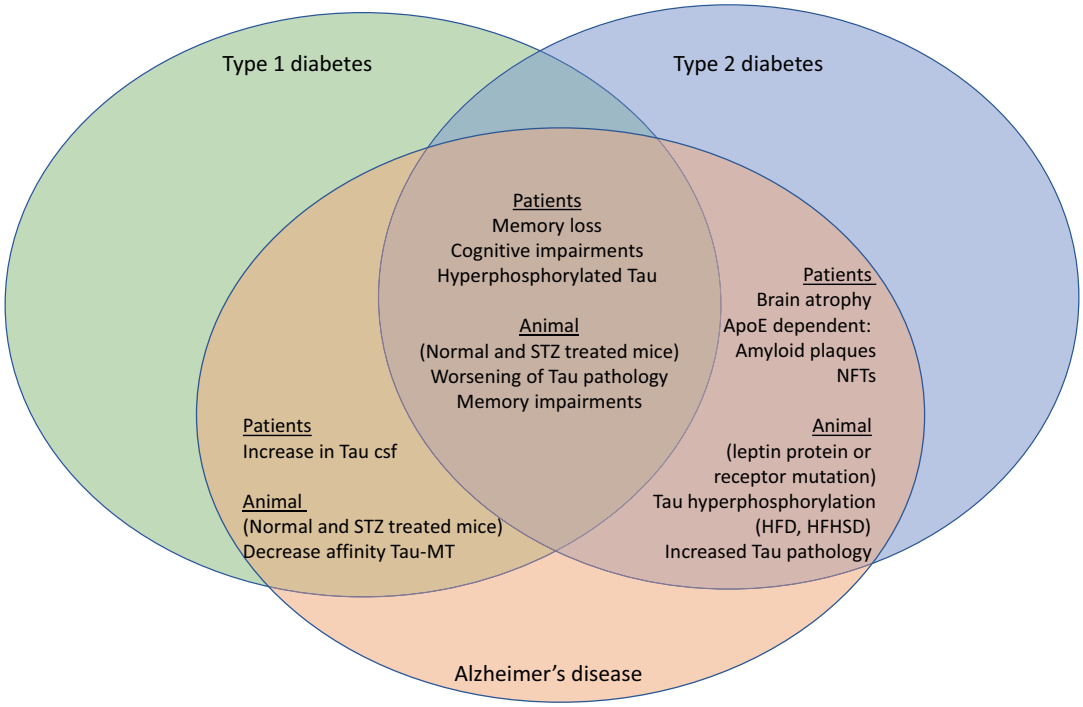

Fig. 21.1 Venn diagram of common symptoms between Alzheimer's disease and diabetes in patients and animal models. *STZ* streptozotocin, *CSF* cerebrospinal fluid, *MT* microtubules, *ApoE* apolipoprotein E, *NFT* neurofibrillary tangles, *HFD* high fat diet, *HFHSD* high fat high sucrose diet

including the development of Tau pathology. Although the link between T1D and AD is not clearly established in humans, these data provide valuable information because they allow to evaluate the effects of hyperglycemia and hypoinsulinemia on Tau pathology.

In Animal Models

One of the most commonly used methods to mimic T1D is based on intraperitoneal injection of streptozotocin or STZ (2-deoxy-2- (3- (methyl-3-nitrosoureido)-D-glucopyranose). This drug enters the β cells of pancreatic islets, by GLUT2 receptors, thanks to its analogy with glucose [283]. STZ then destroys these cells by methylation of the DNA, resulting in hypoinsulinemia accompanied by hyperglycemia, as in T1D. Hyperphosphorylation of Tau protein on many epitopes, a decrease in its affinity for microtubules and impaired memory have been observed in non-transgenic rodents following different STZ injections in many studies [56, 138, 151, 228, 239].

Although Tau hyperphosphorylation seems well characterized following STZ injections, the mechanisms proposed to explain this phenomenon differ from one study to another. Some suggest an increase in kinase activity, and more specifically GSK-3β, Tau major kinase, *via* a decrease of its inhibitory phosphorylation on serine 9 [138, 239]. Other studies attribute hyperphosphorylation of Tau following STZ injections to inhibition of Tau phosphatases, mainly PP2A, Tau's major phosphatase [228, 239]. According to Planel et al., the decrease of phosphatase activity leading to Tau hyperphosphorylation is the result of animal hypothermia caused by profound metabolic alterations after STZ injections [228]. The latter study demonstrated that hyperphosphorylation of Tau is greatly reduced in STZ-injected mice whose normothermia was restored. Indeed, the phosphorylation of Tau is very sensitive to temperature changes;

a degree below 37°C can induce an 80% increase of Tau phosphorylation in mice [227]. Hypothermia is a common consequence of diabetes in humans [204, 265] and in animal models [149, 207, 267]. Unfortunately, very few studies that observed hyperphosphorylation of Tau measured animal body temperature following STZ injection.

Several authors have induced T1D with STZ injections on transgenic models of AD. These studies evaluate whether metabolic disorders may exacerbate or interfere with the development of AD-like neuropathology in these models. In addition, the use of transgenic models makes it possible to observe markers that are very difficult to observe in non-transgenic animals such as Tau aggregates. Ke et al. analyzed the effects of STZ on Tau pathology in pR5 transgenic mice overexpressing mutated Tau protein (P301L) [146]. These mice, which spontaneously develop hyperphosphorylation of Tau and NFT, show an exacerbation of these 2 phenotypes following STZ injection. In hTau mice (overexpressing non-mutant human Tau), we did not detect any change in Tau solubility after STZ injection despite marked hyperphosphorylation [106]. The differences between the two models could be due to the fact that P301L Tau mutant is more prone to aggregation than non-mutant Tau. Indeed, the P301L mutation has been shown to accelerate the formation of paired helical filaments and promote β-sheet formation [21, 81, 308]. While hTau mice do develop Tau aggregates, they do it slower than pR5 mice. Interestingly, insulin injection 30 min before sacrifice partially restored physiological Tau phosphorylation levels in STZ-injected hTau mice [106], confirming a link between insulin homeostasis and Tau phosphorylation. Some animal models develop T1D spontaneously and therefore do not require drug injection. Phosphorylation of Tau was analyzed in two of these models, BB / Wor rats (for BioBreeding/Worcester) and NOD mice (for Non-Obese Diabetic). While no change in Tau phosphorylation was observed in BB/Wor rats [175], NOD mice showed hyperphosphorylation of Tau protein, accompanied by inhibition of PP2A activity [213], as in some STZ-injected models of T1D [228, 239].

T2D, Tau Pathology and AD

In Humans

Although early epidemiological studies failed to find a link between T2D and AD [120, 166], it is now well established that T2D is a major risk factor for AD. One of the first studies to clearly demonstrate this, is the Rotterdam study of over 6000 subjects [124, 210, 211]. The authors reported that T2D doubled the risk of developing AD, an even greater risk when diabetic patients were treated with insulin. Subsequently, many other epidemiological studies have confirmed these findings [12, 42, 170, 182, 216, 318]. A 2010 meta-analysis determined, based on available studies at that time, a relative risk of 1.54 to develop AD for a type 2 diabetic patient [235]. This study also notes that the presence of the ApoE4 isoform significantly increases the risk of AD in T2D patients compared to diabetics with ApoE3 or ApoE2 isoforms. Moreover, progression to AD appears to be more rapid in diabetic patients, both symptomatically [5], and neuropathologically [174, 299].

Finally, these data are reinforced by the 2014 Alzheimer Society report, which announces a comorbidity of 29% between AD and T2D [190]. Moreover, a study in a retirement home described dementia as the most common comorbidity in T2D patients [87]. These observations are not surprising, as one in ten cases of dementia may be linked to T2D [32].

Since the link between T2D and AD is well established at the epidemiological level, it was subsequently essential to understand the molecular mechanisms underlying this link, to better understand why and how T2D increases the risk of AD.

Studies in humans have shown that T2D patients display many neuropathological alterations similar to those of AD. Indeed, significant cognitive impairments including memory disorders have been observed in diabetics [15, 278], and mainly in the elderly [255]. These cognitive disorders could be caused by marked brain atrophy in diabetic patients [187]. Brain atrophy affects very similar regions to those of AD, such as the hippocampus [32, 200, 252]. Moreover,

brain loss, a normal process seen during aging, is much more pronounced in T2D patients than in healthy people of the same age [63, 78, 161, 301]. Several hypotheses exist to explain this brain atrophy. Among the most supported, the duration of diabetes, specifically insulin resistance correlating with atrophy, as well as cerebral infarctions related to vascular alterations in T2D seem quite plausible [186, 187]. The resulting vascular disorders of T2D are also present in the microvasculature of the brain and could contribute to the loss of brain matter and cognitive deficits. Cerebrovascular abnormalities, also present in AD, may explain, at least in part, the decline in glucose uptake and hypometabolism in some brain regions of T2D patients [16, 89], as in AD. A recent study using brain imaging with PET and ^{18}F-FDG biotracer showed an exacerbation of reduced cerebral glucose metabolism in patients with mild cognitive impairment when they were also diabetic compared to patients with mild cognitive impairment without diabetes [327]. No statistical difference was observed between healthy and diabetic patients despite a strong tendency to reduced glucose metabolism in the brain of diabetic patients. Nevertheless, other studies on larger cohorts of patients (only 31 diabetic patients in the study by Zhang et al. [327]) are needed to give a definitive answer on the impact of T2D on brain glucose metabolism.

Finally, amyloid and Tau pathologies have been observed in diabetic patients [134, 178, 195, 216]. Aβ deposits and hyperphosphorylated Tau proteins were detected in the pancreatic islets of patients with T2D [195]. Amyloid plaques as well as NFT were also found in greater numbers in the hippocampus of T2D patients with the ApoE4 allele compared to healthy patients [216]. On the other hand, another study shows that there is no increased incidence of amyloid deposits in patients with T2D [134]. However, when cerebral amyloid plaques are present, the extent of accumulation correlates with the duration of T2D [134]. Regarding the Tau protein, a study showed its hyperphosphorylation on many epitopes (Ser202, Thr217, Ser262 and Ser396) in the frontal cortex of T2D patients compared with healthy patients [178]. These results explain the presence of NFT at more advanced Braak stages in the brains of these patients with T2D.

However, post mortem data on humans are rare and have many limitations as to the mechanistic study linking the two pathologies. That is why many studies have turned to animal models of T2D, and combined models of T2D and AD.

In Animal Models

There are now many animal models that spontaneously develop a T2D (for review King [154]). These models are derived from animals with one or more genetic mutations transmitted from generation to generation, or by selective crossbreeding of individuals leading to diabetic lines such as Otsuka Long-Evans Tokushima Fatty (OLETF) in the rat. Studies have explored some Alzheimer-type pathologies to better understand the links between these diseases in many of these models.

The db/db mice, with a mutation in the leptin receptor gene, leptin being a satiety hormone, show an important hyperphosphorylation of Tau protein in their brains according to several studies [75, 151, 172, 243, 268]. However, the study by Jolivalt et al. observed no change in Tau phosphorylation in this model [138]. The mechanistic hypotheses of this hyperphosphorylation of Tau protein in db/db mice vary from one study to another; activation of some Tau kinases like GSK-3β [268] or JNK [172] has been suggested, but also an alteration of the BBB [243] or an increase of Tau cleavage [151]. Our study suggests that Tau hyperphosphorylation is mainly due to hypothermia caused by impaired thermoregulation in db/db since restoring normothermia recovered physiological phosphorylation of Tau similar to control mice [75]. It is interesting to note that some studies have observed a rise in levels of Aβ (40 and/or 42) in these mice [172, 272]. However, these results have not been confirmed by other teams [206, 241].

Ob/ob mice, which have a mutation in the gene encoding leptin, are also a common model of T2D in which hyperphosphorylated Tau protein has been observed in the brain [104, 150, 256]. This hyperphosphorylation of Tau is also

present in OLETF [142] and Bio-Breeding Zucker diabetic/ Worcester (BBZDR/Wor) [175] rats. These observations are often associated with increases in Aβ levels [175, 256]. Unfortunately, the mechanisms of Tau hyperphosphorylation in these models are still poorly understood.

Other pathological changes in Tau have been observed in these rodents, like an increase in Tau cleavage in db /db and ob/ob mice [150, 151], an alteration of Tau exon 10 splicing in favor of the Tau 3R form in OLETF rats [142] or a decrease of Tau O-glycosylation in ob/ob mice [104].

Some studies have crossed these spontaneous models of T2D with transgenic models of AD in order to better evaluate the interaction between T2D and AD pathologies. In contrast to the previous models, these crosses make it possible to determine if T2D can aggravate AD pathologies. The ob/ob and db/db mice were crossed with murine models of amyloid pathologies: APP23 mice [286] and APP/PS1 mice [241] respectively. Crossing these models of amyloid pathologies with T2D models worsens cognitive disorders and increases Aβ levels (but not aggregation). The study by Ramos-Rodriguez et al. even shows an exacerbation of Tau phosphorylation in db/APP/PS1 mice [241]. One team also transduced db/db mice with mutated P301L Tau protein using an adeno-associated virus, exacerbating Tau pathology (both phosphorylation and NFT), but the mechanisms involved remain unexplored [229].

Although all these models provide very valuable information on the impact of T2D on AD, they are quite far from human and physiological conditions. To get close to a human situation, studies have used hypercaloric diets in animal models to mimic a western diet, a common cause of T2D in humans. Most of these studies use a diet with a high calorie fat percentage. Unfortunately, despite a large number of studies, the results obtained vary widely from one study to another. For example, high-fat diets (between 54% and 60% kcal from fat) in wild-type mice induced hyperphosphorylation of Tau in two studies [136, 152], but no change in phosphorylation in two other studies [25, 243], and even a decrease of Tau phosphorylation in another study compared to mice under control diet [292]. Studies done on transgenic mice reproduced these inconsistent results. Indeed, while Leboucher et al. observe a hyperphosphorylation of soluble Tau protein without increasing its aggregation in THY-Tau 22 mice (Tauopathy model with two mutations on Tau (G272 V and P301S)) under a high-fat diet [167], Koga et al. report a hyperphosphorylation of Tau only in the insoluble fraction of PS19 mice (model of Tauopathy with P301S Tau mutation) under high fat diet [158]. In 3xTg-AD mice under high fat diet, Ma et al. observe abnormal phosphorylation of Tau on serine 422 [184] while others detect no change in Tau phosphorylation [23, 141, 157, 304]. Ramos-Rodriguez reported an increase in Tau phosphorylation, microglial activation and impaired memory in APPswe/PS1dE9 after high-fat diet [242]. Some research groups have combined these high-fat diets with high sugar and/or cholesterol diets to mimic a “western” diet, often accused of the worrisome growth of obesity and T2D [7, 29, 47, 123, 214, 218, 285]. Most observed hyperphosphorylation of Tau protein regardless of the animal model used, but significant variability in the results persists.

Given the variability of all these results, we can hypothesize that some confounding parameters vary from one study to another, such as the composition of diets (caloric source, percentage of vitamins or fatty acids saturated vs. unsaturated, omega 6/3 ratio, unsuitable diet control, etc.), duration of diet, animal model used or temperature of animals and use of anesthesia. In addition, many studies directly attribute the effects of diets observed in T2D while this type of diet can modulate certain factors such as obesity [167], leptin signaling [108] or cerebrovascularization and inflammation [203] that may also modify Tau phosphorylation.

With the main goal of clarifying the impact of hypercaloric diets on Tau pathogenesis, we published a study closer to human conditions [105]. We therefore chose hTau mice that express non-mutated human Tau protein as in AD and capable of developing NFT [8, 9]. We used diets high in fat, sugar and cholesterol (alone or combined), the three key components of western diet, at levels comparable to human diets (in pathological

conditions). Surprisingly, we found no effect of fat, sugar and cholesterol overconsumption, even when combined, on Tau phosphorylation, O-GlcNAcylation, splicing, cleavage and aggregation, suggesting that their overconsumption does not seem to aggravate Tau pathology in these mice [105]. While we have demonstrated that fat, cholesterol and/or sugar overconsumption did not exacerbate Tau pathology in this model, our study calls for further high quality investigations (controlling and reporting a maximum of parameters) in different mice models to have a definite answer on whether these diets can enhance Tau pathogenesis.

Together, the data summarized above indicate that a decrease of insulin production or sensitivity may exacerbate Tau pathology suggesting that brain insulin resistance could play a key role in AD and Tauopathies.

Brain Insulin Resistance and Tau Pathology: A Vicious Circle?

Brain Insulin Resistance in AD Patients

Considering the prominent role exerted by insulin towards plasticity and cognition, it is not surprising that the AD brain has been described to exhibit an insulin resistance state, the so-called "Type 3 Diabetes" [127, 274]. Indeed, CSF insulin levels have been described to be lower in AD patients than in healthy subjects [61, 93] although these data have not always been confirmed by others [86, 198]. Some explained reduced CSF insulin levels by a decreased transport of insulin across the BBB as suggested by Banks et al. who reported that chronic plasma hyperinsulinemia, often reported in AD patients [44, 61, 86, 183, 277], diminishes the passage of insulin to the brain [18–20]. However, whether all AD patients with reduced CSF insulin display hyperinsulinemia remained to be established and other mechanisms could be instrumental. Insulin receptor signaling pathway and ability to respond to insulin have been described to be strongly impaired in the brain of AD patients [199, 287]. A notable increase of inhibitory IRS-1 phosphorylation at Serine 616 and 636/639 has been particularly observed [287]. Tau pathology would likely contribute to the establishment of such brain insulin resistance. Indeed, a study by Yarchoan et al. found increased phosphorylation of Serine IRS-1 in primary Tauopathies, including Pick disease (PiD), corticobasal degeneration (CBD) and PSP, but to a lesser degree than in AD [323].

Consequences of Brain Insulin Resistance for Cognition, Longevity, and Metabolism

In accordance with the above-mentioned role of insulin towards the regulation of synaptic plasticity and memory, correlation between increased serine phosphorylation of IRS-1 and cognitive score in AD patients (episodic and working memory) sounds logical [287]. However, while brain insulin signaling finely tunes energy homeostasis and peripheral glucose homeostasis, much less is known about the potential impact of brain insulin resistance towards metabolic disturbances in AD.

Metabolic alterations have been reported in patients with AD with or without diabetes. Weight loss was already described by Aloïs Alzheimer [96, 288] and it is now recognized as a clinical feature of AD [194], impacting 20–45% of patients [94, 95, 111, 112, 305]. Weight loss in patients with dementia is associated with accelerated progression of AD, higher rate of institutionalization [10] and increased mortality [79, 88, 312]. Although dementia-associated weight loss often begins before the onset of the clinical syndrome and accelerates by the time of diagnosis, it is unclear if it is a cause or a consequence of AD pathology [131]. Metabolic deregulations in animal models of Tauopathies resemble those found in patients with Tau related dementia. Indeed, decreased body weight is found in mouse models of Tau deposition such as THY-22 [167] or Tg4510 [40, 41], sometimes despite increased feeding behavior [139]. Additionally, genetic deletion of Tau resulted in increased body weight [201]. Impaired satiation and increased feeding behavior [2], as well as changes in body temperature [156]

were also observed in a mouse model combining amyloid plaques and Tau pathology (3xTgAD). In Tg4510 mice, weight loss was specific to fat mass and co-occurred with deregulation of metabolic rate [139] as well as disturbances in circadian rhythm [276]. In both cases Tau pathology was found in the hypothalamus.

One could wonder if these metabolic alterations, appearing for most part before the detection of pathological deposits in the brain, could really be the result of early deregulation of central insulin signaling. However, only rare observations support that brain insulin resistance may play a role in the metabolism of AD patients. First, paradoxical overeating concomitant with weight loss has been observed in patients with AD [314] as well as in frontotemporal dementia (FTD) [36, 223, 271]. Second, an increased risk to develop T2D has been reported in AD patients (35% diabetics +46% with glucose intolerance) [134]. Furthermore, some sparse studies report that AD patients can exhibit hyperinsulinemia or alterations in glucose metabolism [44, 61, 86, 183, 245, 277].

What Is the Trigger for Brain Insulin Resistance in AD?

AD brain insulin resistance has been originally ascribed to the detrimental impact of Aβ oligomers. Aβ peptide in oligomeric form can promote insulin resistance by competitively binding and internalizing IRs [330] and by increasing the phosphorylation of IRS-1 and JNK [34] *in vitro*. *In vivo*, these results were confirmed in crabgrass macaques and APP/PS1 transgenic mice, which received intracerebroventricular (ICV) injections of Aβ oligomers [34]. Consistently, several rodent models of amyloid pathology develop metabolic alterations such as glucose intolerance [55, 137, 197, 302]. Particularly interesting is the observation of impaired glucose homeostasis following ICV injection of Aβ oligomers [34]. Recent data also pointed out that ApoE4 could be strongly related to the development of brain insulin resistance [329]. Notably, ApoE4 reduces insulin-IR interaction and impairs IR trafficking.

Despite early studies reporting co-localization and correlation between decrease of total IRS-1 and IRS-2 along with increased phosphorylated IRS-1 on $Ser^{636/639}$ and Ser^{616}in the brain of AD patients and the NFT deposition [184, 199, 287, 323] as well as the link between increased phosphorylated IRS-1 and primary Tauopathies [323], no study had any interest in the direct role of Tau protein on central insulin signaling until a recent work published by our laboratory [188]. In the study of Marciniak et al., we have demonstrated that Tau deletion induces a disruption of hippocampal response to insulin and impairs hypothalamic anorexigenic effect of insulin associated with energy metabolism alterations (enhanced food intake and body weight, increased adipose tissue mass, hyperinsulinemia and glucose intolerance). These findings strongly suggested a putative function of Tau protein as a regulator of insulin signaling in the brain. The potential mechanism by which Tau regulates hippocampal response to insulin seems to act through IRS-1 and PTEN. Indeed, we reported decreased activation of IRS-1 and AKT after insulin exposure in Tau KO mice, suggesting brain insulin resistance. This is consistent with altered IRS-1 activity in AD brains that correlates with NFT deposition [184, 199, 287] whereas a direct interaction between Tau and IRS-1 has never been reported. On the other hand, we found that Tau interacts with PTEN, already known to inhibit insulin signaling, and that human Tau is able to reduce PTEN activity [188]. Although at the current stage it is still impossible to determine which of IRS-1 or PTEN is the instrument of Tau to regulate brain insulin signaling, our study identified a new function of Tau protein as a modulator of brain insulin signaling and highlights potential mechanistic explanation whereby alteration of insulin signaling would occur in AD *via* pathological Tau loss of function. In addition, we have highlighted a link between Tau haplotype and peripheral metabolism using genome-wide association study (GWAS) data [236, 259]. Indeed, we reported that patients with H1 haplotype, previously shown with higher risk of Tauopathies [226], exhibited higher circulating glucose levels and lower insulin levels during an

oral glucose tolerance test, suggesting that Tau impacts peripheral metabolism in humans. These GWAS data contribute to recent evidences suggesting that Tau can regulate both brain insulin signaling and peripheral glucose metabolism.

Overall, these data open the possibility that cognitive and metabolic deficits seen in AD patients are the consequence of a Tau loss-of-function. For a long time, Tau aggregation into NFT was thought to be the only culprit of neurodegeneration and cognitive deficits. But obviously, previous observations supported this is not the case [258, 282]. More recent data ascribed Tau oligomers as detrimental species towards plasticity. Interestingly, as Tau oligomers [237], Tau deletion also impairs hippocampal plasticity and spatial behaviour [3, 4, 153, 154]. This raises the possibility that both "toxic gain of Tau function" and "loss of normal Tau function" play a role in the development of cognitive deficits in Tauopathies. Respective contribution of both phenomena will need to be further elucidated in the future.

Impact of Insulin Resistance on Tau Lesions

Interestingly, although the interest for the impact of Tau protein on insulin signaling in the brain is very recent, it has for many years been known that, conversely, insulin is capable of modulating Tau protein, mainly its phosphorylation state [74]. There are increasing data showing that insulin regulates Tau phosphorylation and could exacerbate NFT development in AD [53, 84, 126, 171, 260, 263]. *In vivo*, mice deficient for insulin receptors in neurons show inhibition of PI3K/Akt signalling pathway and an increase in phosphorylation of Tau [264]. Similarly, the deletion of IRS-2 gene causes inhibition of the PI3K/Akt pathway and therefore induces Tau hyperphosphorylation in IRS-2 knockout mice [263]. Overall, the pathological effect of brain insulin resistance on Tau has mostly been attributable to the modulation of several downstream pathways [74], involving some Tau kinases (GSK-3β, JNK, ERK, and AMPK) and Tau phosphatases (PP2A and PP1), known to play a pivotal role in the development of Tau pathology [14, 50, 54, 68, 99, 102, 103, 132, 219, 290, 297, 307, 332]. Therefore, chronic insulin signaling impairment seen in the brains of patients with AD and Tauopathies, is prone to favour the development of Tau pathology, through the disruption of the balance between Tau kinases and phosphatases. Moreover, altered brain insulin signaling would also promote Tau pathology by favoring Tau cleavage [150, 151] and deregulating Tau alternative splicing [285].

Therapeutic Considerations

Intranasal Insulin

Insulin treatment is a promising therapeutic strategy for treating AD. Intravenous insulin injection has already shown numerous positive effects on cognition and, in particular, on memory in healthy subjects [148] and in patients with AD [60]. Because of hypoglycaemic side effects [58], researchers turned to intranasal insulin delivery, allowing rapid delivery of insulin to the brain with no peripheral hypoglycaemic effect [35, 147, 217]. A first study showed that administration of intranasal insulin (20 IU twice daily for 21 days) improved memory and attention in patients with AD [248]. Another study using doses of 20 IU or 40 IU for 4 months confirmed memory and cognitive improvements as well as beneficial effects on quality of life [59]. This same study demonstrated that both doses improve glucose uptake in many brain areas, using brain imaging techniques with PET and 18F-FDG biotracer compared to placebo [59]. Although optimistic, these results need to be confirmed over longer periods and larger cohorts of patients. This will allow for large-scale analysis of the effect of intranasal insulin by considering ApoE ε4 allele, since one study reported cognitive improvements 45 min after insulin therapy only in patients without ε4 allele, and negative effects of insulin in ε4 allele carriers with the highest dose [247].

In vivo, Chen et al. demonstrated that 7-day intranasal insulin does not alter Tau phosphorylation in 9-month 3xTg-AD mice, while observing a reduction in amyloid pathology and neuroinflammation [51]. Conversely, another study observed in the same murine model a decrease in anesthesia-induced Tau hyperphosphorylation when mice were pretreated with intranasal insulin for 1 week [52]. More recently, intranasal insulin has been shown to reduce Tau hyperphosphorylation as well as neurodegeneration, neuroinflammation and cognitive impairments in ICV streptozotocin-injected rats [113] and diabetic mother offspring mice [244]. Although intranasal insulin appears to be an interesting therapeutic issue, further studies are needed to understand how insulin affects memory and whether it is able to decrease, or at least slow down, the evolution of Tau pathology and neurodegeneration in AD and other Tauopathies.

Diabetic Drugs: Peroxisome Proliferator-Activated Receptor-γ Agonists, Metformin, GLP-1, Amylin and Future Drugs

Several diabetic drugs that belong to thiazolidinediones have also been tested in AD including rosiglitazone, which has reached phase 3 of clinical trials. Thiazolidinediones are Peroxisome Proliferator-Activated Receptor (PPARγ) agonists used in the treatment of T2D by improving the sensitivity of tissues to insulin. Following encouraging results in preclinical studies on amyloid, Tau and inflammatory pathologies and on cognitive impairments [77, 208, 215, 293, 317, 325], a dozen clinical studies have evaluated the effects of rosiglitazone in AD patients. Although a pilot study has observed encouraging results on memory and Aβ levels in the CSF [309], the subsequent phase 3 clinical studies showed no beneficial effect regardless of the dose used or the ApoE genotype [98, 116].

Metformin, that is a first-line drug for the treatment of diabetes, reduces blood glucose by improving glucose uptake by peripheral tissues and reducing gluconeogenesis by the liver. Although metformin has been suggested as an interesting therapeutic issue for AD in the past, an *in vivo* study has recently reported that metformin is able to promote Tau aggregation and abnormal behaviour in a mouse model of Tauopathy [22]. These results could explain, at least in part, recent data reporting that the risk of developing AD was 2.13 times as high in type 2 diabetic patients who took metformin as in those who did not [165]. Results of a 12-month pilot trial of metformin in MCI patients have shown no change in glucose uptake or plasma amyloid levels but an increase in verbal memory scores [181].

GLP-1 receptor agonists, commonly used in diabetic patients, have shown encouraging results. GLP-1 is an incretin that increases glucose-dependent insulin secretion and decreases glucagon secretion, contributing thus to better insulin sensitivity. In animal models of AD, exendin-4 and liraglutide, two GLP-1 receptor agonists, restored altered insulin signaling, decreased amyloid burden, and improved cognitive deficits [34, 192]. A pilot clinical trial testing liraglutide in AD patients has demonstrated that it was able to prevent decline of brain glucose metabolism [91]. Two clinical studies are underway to evaluate the effects of these two drugs in Alzheimer's patients or with mild cognitive decline.

Another strategy recently tested in AD patients is pramlintide, an amylin analog [331]. While authors suggested that a single injection of pramlintide into AD reduced the amyloid burden and lowered the concentrations of Aβ, this pilot study used a too small number of patients to draw definitive conclusions. Moreover, no effect on Tau has been found.

Although diabetic drugs provide a therapeutic alternative for Alzheimer's disease and other Tauopathies, an important consideration must be given to the adverse effects that these treatments may have on periphery by affecting circulating concentrations of glucose and insulin. Moreover, other potential future diabetic drugs may represent attractive therapeutic approaches to correct aberrant insulin signaling pathways in AD like PTP1B inhibitors [306]. Indeed, PTP1B inhibition has been shown as an effective approach to

rescue neuronal insulin signaling in non-AD models with neuronal insulin resistance [164, 221, 238, 328].

Could Anti-Tau Immunotherapy Impact on Brain Insulin Sensitivity?

In recent years, Tau immunotherapy has become a rapidly growing area of drug development in clinical trials. A recent study suggests that Tau passive immunization reduced not only total and hyperphosphorylated Tau in the brain, while improving memory, but also nudged down levels of the APP and cleared Aβ plaques [62]. These data lead us to wonder whether anti-Tau immunotherapy impacts on brain insulin sensitivity. According to Marciniak et al. study, a reduction of Tau protein itself, by immunotherapy in this case, could lead to exacerbate brain insulin signaling impairments in AD and Tauopathies. However, C2N 8E12, an antibody currently in phase 2 trial in AD patients might be interesting here since it recognizes an aggregated, extracellular form of pathological Tau. In P301S mice, it reduced brain neurofibrillary pathology, insoluble Tau, microgliosis, seeding and brain atrophy without affecting soluble total Tau levels [320, 321]. This antibody could therefore be an interesting alternative to eliminate pathological forms of Tau (potentially less able to regulate insulin signaling) while preserving the soluble Tau protein and its physiological role on insulin signaling.

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Part V

Tau Aggregation, and Propagation

22 Top-Down Projections Direct the Gradual Progression of Alzheimer-Related Tau Pathology Throughout the Neocortex

Heiko Braak and Kelly Del Tredici

Introduction

Sporadic Alzheimer's disease (sAD) is a disorder that is not known to occur in non-human primates or non-primate vertebrates [9, 108]. Involved in the disease-associated pathological process are nerve cells of the central nervous system (CNS) and, among the many different neuronal types, only a few show a pronounced proclivity to become involved. Nearly all of the vulnerable types belong to the class of postnatally-maturing projection neurons that generate in relation to the size of their cell body a long and sparsely myelinated axon [25, 65].

The relentlessly progressive process associated with the disease requires an extended period of time – nearly a lifetime – to reach its full extent [29]. The reasons for this extremely slow progression are presently unknown, and it is likewise unclear why sAD consistently fails to go into remission [25]. Abnormal changes in the protein tau begin early in life [24, 29, 48] and, after their appearance, the tau lesions gradually increase in severity, systematically progressing throughout the brain and spinal cord and leaving behind a characteristic regional distribution pattern (Fig. 22.1) [10, 19, 25, 45, 66]. Only the late phases of the pathological process cause readily recognizable symptoms and correlate with the clinical picture of sAD [4, 26, 82, 98, 102]. However, the frequency of clinically overt cases increases considerably with age and imposes a major socio-economic burden on societies with advancing life expectancies [31, 39, 50, 90].

As pointed out above, one of the typical features of the sAD-associated process is that vulnerable brain regions become involved according to a predictable topographical sequence (Figs. 22.1 and 22.2, *red arrow at left*). Whereas the pace of inter-individual disease progression varies considerably, the degree of inter-individual variability with respect to the sequence of the pathological tau changes is so minimal that the distribution pattern of the lesions can serve to distinguish different neuropathological stages in asymptomatic and symptomatic individuals [19, 25, 28, 47].

Crucial to the pathological process are alterations of the protein tau [3, 8, 11, 54, 57, 67, 74]. In the cerebral cortex, the first abnormal tau aggregates develop in the transentorhinal region, a phylogenetically late-appearing transition cortex intercalated between allocortical regions of the temporal lobe (entorhinal region, hippocampal formation) and the neocortex

In commemoration of Korbinian Brodmann (November 17, 1868 – August 22, 1918).

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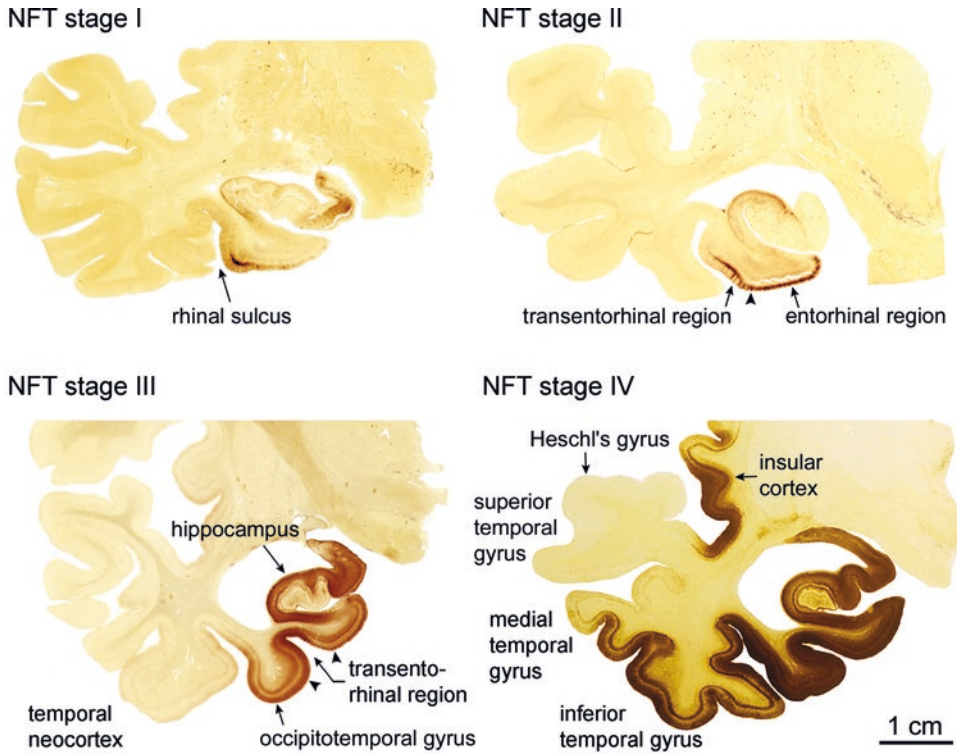

Fig. 22.1 Overview of intraneuronal AT8-immunopositive pathology (NFT stages I–IV) in frontal sections (100 µm) through the temporal lobe. The tau pathology is macroscopically visible in anteromedial portions of the temporal lobe (brown chromogen DAB). The density of the lesions is highest in the transentorhinal and entorhinal regions, decreases from there, and gradually tapers off. In **NFT stage I** (female, 80 years of age) abnormal tau pathology is confined to the allocortical transentorhinal region. During **NFT stage II** (male, 80 years of age), not only the transentorhinal region but also the entorhinal region proper and a portion of the hippocampal formation display clear involvement. In a case assigned to **NFT stage III** (female, 90 years of age), neurofibrillary changes extend beyond the transentorhinal region (*arrowheads* indicate its borders) into the laterally adjoining neocortex of the occipitotemporal gyrus, whereas the remaining areas of the temporal lobe, particularly the superior temporal gyrus, appear to be uninvolved. During **NFT stage IV** (female, 82 years of age), neurofibrillary pathology advances more widely into high order association areas of the temporal lobe up to (seldom beyond) the medial temporal gyrus and into the adjoining frontal fields. However, at NFT stages I–IV, isolated AT8-immunoreactive pyramidal cells are found in the superior temporal gyrus, possibly representing an initial 'reaction' to the neuron-to-neuron progression of the pathological process originating in earlier-involved cortical regions (see [27, 70]). (Reproduced with permission from Ref. [25])

(Fig. 22.1, NFT stage I). The transentorhinal region exists in higher primates and achieves its largest extent in the human brain [18]. From there, the pathological process enters both the entorhinal region and hippocampal formation (Fig. 22.1, NFT stage II) and the temporal neocortex of the occipitotemporal gyrus accompanying the transentorhinal region as well as adjoining prefrontal fields (Fig. 22.1, NFT stage III). Subsequently, this pattern of tau involvement is complemented by the development of tau aggregates within additional sensory high order association fields and prefrontal areas (Fig. 22.1, NFT stage IV), followed by sensory first order association fields and premotor areas (NFT stage V), and, finally, the primary areas of the neocortex (NFT stage VI). Notably, all of the regions that gradually become affected during sAD-process are anatomically interconnected (Fig. 22.2) [2, 25, 52].

Fundamental Organization of the Neocortex and of Cortico-Cortical Connections

The various fields of the neocortex evolve, phylo- and ontogenetically, in an orderly sequence and they also mature consecutively. This phenomenon is traceable in the ontogenetic gradually progressive process of myelination as well as in the functional maturation of the neocortex that is linked to myelination [58, 88, 109] (Fig. 22.2, *blue arrow at center*). In general, the neocortical myelination commences late, progresses slowly during childhood, and, after puberty, persists well into adulthood. Notably, the myelination of the human neocortex is influenced by environmental and social interactions [15, 21, 43, 58, 86, 88, 109, 112].

The neocortical primary sensory and motor areas are the first to emerge (Fig. 22.2**A**). A girdle of first order sensory association areas and premotor are as follows (Fig. 22.2**B**). These again are complemented by a profusion of high order sensory association areas and prefrontal fields (Fig. 22.2**C**). The last region is the transitional cortex between the temporal neo- and allocortex: the transentorhinal region (Fig. 22.2**D**) [25, 27].

One of the essential tasks of the cerebral cortex is the continuous reception and processing of large units of input from the sensory organs and the visceral organs together with the transformation of these data into steering impulses that control movements of skeletal muscles and functions of other organs as part of the whole organism's interaction with the world outside [5, 83, 116] (Fig. 22.2). In the context of the present chapter, it is important to emphasize that nerve cells of the CNS that are primarily and directly occupied with these tasks remain intact during sAD and, if at all, only become involved at end-stage disease.

A frequently underestimated requirement for the effective functioning of the neocortex is the tight functional linkage on the part of the different fields (Fig. 22.2). This is accomplished and ensured by means of a multitude of association fibers that guarantee a precise and rapid exchange of data between the different cortical areas [40, 83]. Notably, a considerable proportion of the neurons that generate such cortico-cortical fibers is highly susceptible to the sAD-associated process and becomes severely affected in the early phase of the disease [23, 25, 27, 34, 42, 62].

Cortico-Cortical Bottom-Up and Top-Down Connectivities in the Neocortex

Connectivities exist that establish links between the primary sensory and motor fields to the sensory first order association areas and premotor fields (Fig. 22.2**A**, **B**), and, similarly, links from these fields to the sensory high order association areas and prefrontal fields (Fig. 22.2**B**, **C**), and from these fields to the transentorhinal region (Fig. 22.2**C**, **D**, *thin black arrows* between boxes). All of these pathways constitute bottom-up or 'upstream' connectivities, whereas other pathways running in the opposite direction constitute top-down or 'downstream' connectivities (Fig. 22.2 from **D** to **C**, from **C** to **B**, and from **B** to **A**, marked by *thick red arrows* between the boxes) [14, 49, 52, 61, 95].

The top-down connectivities are presently the focus of experimental studies seeking to explain the mechanisms of impaired conscious perception during anesthesia [33, 61, 78, 79]. Bottom-up connectivities terminate preferentially on small projection neurons of the granular layer (IV), namely, the spiny stellate cells [41]. These cells establish contacts via their short radially-oriented axon to the proximal segments of the basal dendrites of their target pyramidal cells in neocortical layers III and V [76, 77]. In this manner, the spiny stellate cells establish a point-to-point transsynaptic transmission and disseminate data in a highly ordered manner to select projection neurons belonging to a single cortical column [12, 13].

In terms of functional maturation, bottom-up connectivities from **A** to **B** mature earlier than those from **B** to **C**, and these connectivities also mature earlier that those from **C** to **D** (Fig. 22.2).

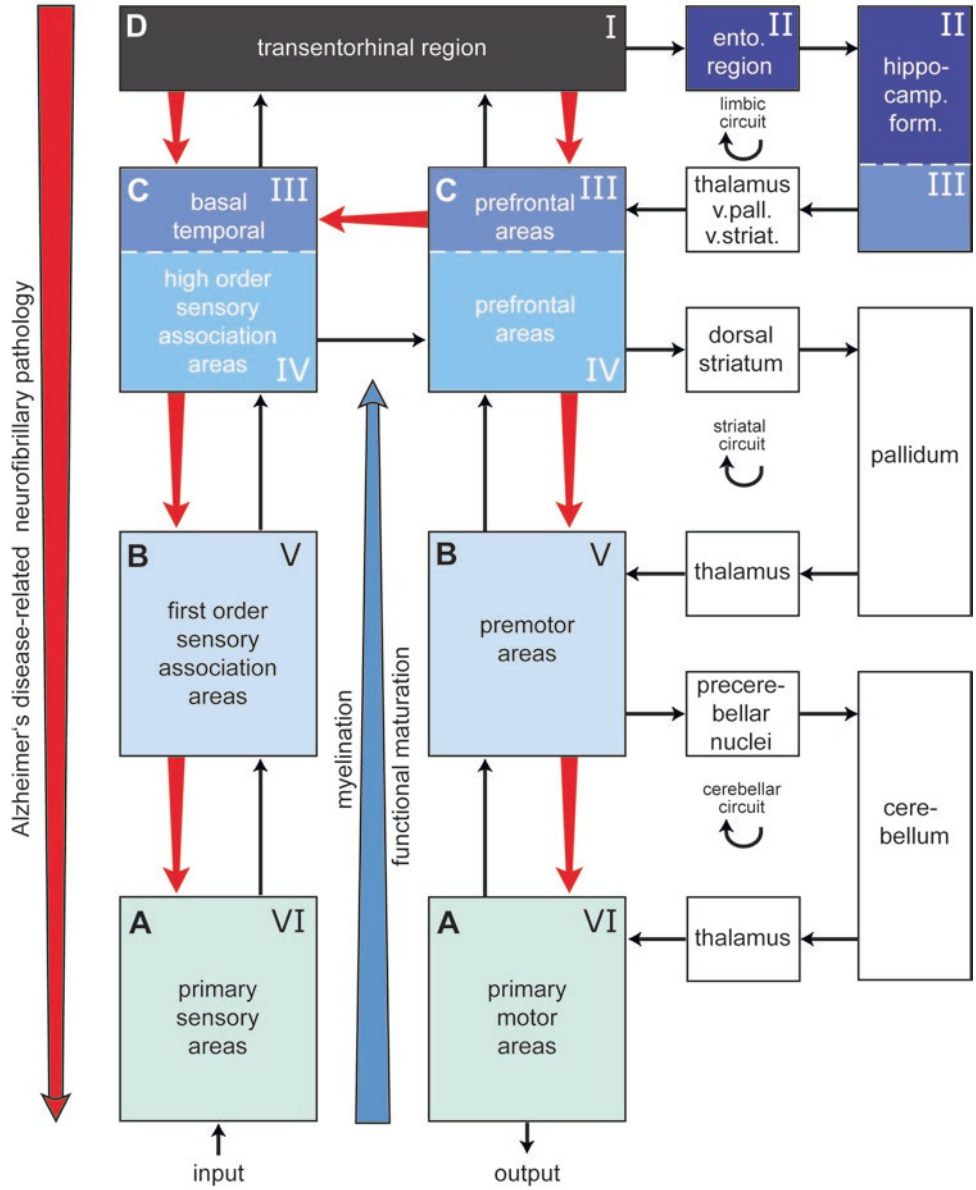

Fig. 22.2 Diagram depicting the top-down progression of sAD-related tau pathology along cortico-cortical connections. The degrees of shading from black to aquamarine represent the growing severity and the proposed spread of the lesions. (**D**) Cortical tau lesions begin in the transentorhinal region, a transitional cortex between allocortical and neocortical regions of the temporal lobe (Roman numerals indicate the first macroscopically visible lesions in NFT stages, here stage I, black). From the transentorhinal region, the tau pathology progresses into the entorhinal region proper and into portions of the hippocampal formation (e.g., CA1 sector) (NFT stage II, navy blue). The lesions then encroach upon additional sectors of the Ammon's horn (NFT stage III, dark blue) (**C**) From the transentorhinal region, the lesions enter into high order sensory association areas of the basal temporal neocortex, at first those covering the occipitotemporal gyrus, as well as into adjoining prefrontal fields (NFT stage III, dark blue). From neocortical fields that become involved at NFT stage III, tau pathology develops in the remaining high order association fields of the temporal, parietal, and occipital cortices as well as in additional prefrontal areas (NFT stage IV, blue). (**B**) From there, tau lesions progress into the first order sensory association fields and into premotor areas (NFT stage V, light blue). (**A**) Finally, the pathological process arrives at the primary fields of the neocortex (NFT stage VI, aquamarine). The *red arrow* at the far left indicates the systematic

Top-down connectivities lag behind all of their corresponding bottom-up connections. Those from **B** to **A** mature only after the connections from **A** to **B** have attained sufficient maturation. Similarly, the top-down connectivities from **C** to **B** and from **D** to **C** develop later than their bottom-up counterparts [27] (Fig. 22.2). After axons of spiny stellate cells belonging to the bottom-up pathway find their contacts to the proximal dendrites of their target pyramidal neurons, the dendrites continue to sprout and to produce distal tips with new sites for synapses of top-down axons that grow into the cortex during this developmental phase. Immediately after birth, the axons of top-down projections from **C** to **B** and from **D** to **C** are unmyelinated and subsequently acquire their myelin sheathes during childhood and adolescence in an activity-dependent manner organized by external stimuli [23].

Involvement of Cortico-Cortical Projection Neurons and Their Target Cells in the sAD Process

The sAD process progresses from **D** to **C**, from **C** to **B** and, finally, from **B** to **A** (Fig. 22.2, *red arrow at left margin*) – i.e., it advances in the reverse sequence and opposite direction to cortical myelination and the functional maturation of the neocortex (retrogenesis) [20, 93, 94, 97] (Fig. 22.2, *blue arrow at center*). An explanation for the pronounced order in the trajectory of the tau pathology is still lacking, but it remains clear that the vulnerable nerve cell types in sAD that are subjected to early abnormal tau changes emerged, for the most part, during the later phases of primate evolution and also mature late during ontogenesis [6, 7, 9, 20, 91–93]. From the standpoint of evolutionary biology, this neuronal vulnerability is probably inconsequential because it does not threaten the preservation of the human species [23].

The uniform regional distribution pattern of the tau lesions is consistent with the concept put forth by many researchers that spreading of tau pathology within the human brain might proceed along axons of involved nerve cells within a given field to hitherto uninvolved nerve cells in other, including more distant, fields [22, 89, 99]. Experiments show that pathogenic forms of tau, including human tau, are capable of seeding and can be transmitted over distances axonally and transsynaptically [2, 32, 35, 37, 38, 46, 55, 59, 70, 73, 81].

Projections from subcortical nuclei probably do not cause the early cortical lesions because the only subcortical source involved during NFT stage I that could possibly transmit abnormal tau seeds to neocortical projection neurons is the group of non-thalamic nuclei (locus coeruleus, upper raphe nuclei, magnocellular nuclei of the basal forebrain) with diffuse projections to all areas of the cortex [53, 84, 96]. However, highly ramified axonal projections from these subcortical nuclei exert their influence upon the expansive fields of the cortex chiefly via non-junctional varicosities and volume transmission rather than via classical synapses with pre- and postsynaptic densities [1, 85, 87]. In addition, non-junctional varicosities (incomplete synapses) are not suited for the seeding-induced transmission of tau pathology [70]. It also means that the ascending diffuse projections from the subcortical non-

Fig. 22.2 (continued) progression of the disease-related tau pathology, whereas the *blue arrow* indicates the gradual progression of myelination in the diverse cortical regions and, at the same time, represents the increasing degree of the functional maturation of their neuronal constituents. The *short red arrows* between involved regions represent the top-down cortico-cortical connections that direct the pathological tau process. By contrast, the bottom-up projections and their target neurons (*thin black arrows*) refrain from developing neurofibrillary pathology in sAD. The efferent side of the diagram (at the right) also includes three important circuits. These incorporate subcortical CNS components into the regulation of cortical output. Foremost among these is the 'limbic' circuit that carries output data from the hippocampal CA1 sector and subiculum via the ventral striatum (v.striat.), ventral pallidum (v.pall.), and mediodorsal thalamus to the prefrontal fields. The 'striatal' circuit follows, guiding data through the dorsal striatum, pallidum, and thalamus to chiefly the premotor areas, and these, again, influence the group of precerebellar nuclei/cerebellum that provide via the thalamus important input to the primary motor cortex. **Abbreviations:** *CA1* first sector of the Ammon's horn, *ento* entorhinal, *hippocamp **form*** hippocampal formation

thalamic nuclei would be unlikely to transport seed-containing tau via axons to distant cortical sites in a highly selective manner because a generalized spreading pattern is inconsistent with the specific regional distribution pattern of abnormal tau in NFT stages I–VI.

Remarkably, the sAD-associated process from beginning to end leaves the spiny stellate cells completely intact [25]. Whether projection neurons receiving chiefly bottom-up connectivities become involved at all during sAD is presently unknown. The spiny stellate neurons are not specifically vulnerable and, thus, it is improbable that they would produce tau seeds or transport them via their axons (Fig. 22.2, *thin black arrows* between boxes). The question then emerges as to how the parent cells of the top-down projections and their target cells fare (Fig. 22.2, *thick red arrows* between boxes). Our recent findings in the target cells are summarized in section "Morphological and abnormal tau changes in target cells of top-down connectivities" below and indicate that it is the targets of the top-down projections which bear the brunt of the sAD neurofibrillary pathology.

Early NFT stages (e.g., stages I–III) display obvious AT8-immunoreactive neurofibrillary pathology limited to portions of the anteromedial temporal lobe (Fig. 22.1). In thick tissue sections (100–300 μm), the existence of the lesions is visible to the unaided eye. Heavily involved regions can be easily distinguished from uninvolved ones and those with less pathology (Fig. 22.1). If one follows an imaginary line between the transentorhinal region and the primary areas of the neocortex corresponding to that of the red arrows connecting the boxes in Fig. 22.2, one only has to keep looking at the areas of the cortex, beginning with the transentorhinal region, in the direction of the less involved or apparently uninvolved regions and check them for the presence or absence of abnormal tau. This would include the regions in the occipitotemporal, inferior, medial, and superior temporal gyri, including the transverse gyrus of Heschl (Fig. 22.1). The sAD process proceeds uniformly in this direction with a delay upon reaching the border between the medial and superior gyri of the temporal lobe, and it enters portions of the superior temporal gyrus only in end-phase disease stages [23, 25].

The reason for the delay is that the cortex of the superior temporal gyrus in the human adult brain is characterized, compared to other temporal lobe regions, by a stronger degree of myelination [64, 86]. When looking for potentially very early pathological tau lesions within the target neurons of top-down connectivities, one could begin with portions of Brodmann area 22 in the superior temporal gyrus. In fact, although the first macroscopically detectable lesions routinely develop in Brodmann area 22 in NFT stage V, a few isolated AT8-immunoreactive pyramidal cells mostly limited to layers III and V already occur there at lower NFT stages I–III.

Morphological and Abnormal Tau Changes in Target Cells of Top-Down Connectivities

Abnormal tau in neocortical pyramidal cells is either confined to specific compartments of the involved neuron or fills it completely. Sections of 100 μm thickness facilitate recognition of the entire dendritic tree of involved projection cells, and we recently found there four distinct groups of sequential morphological and pathological changes [27].

The first group displays swollen AT8-positive distal segments of the basal dendrites and of the side branches of the apical dendrite belonging to involved pyramidal cells (Fig. 22.3a, b). These skirt-like distal tips point to a central immunonegative area (Fig. 22.3a, b). A second group displays additional thread-like trailing structures ('spokes') surrounding the immunonegative area (Fig. 22.3c, d). A third group reveals for the first time the presence of abnormal tau in the cell soma as well as in the stem of the apical dendrite (Fig. 22.3e, f). In the last group, the pyramidal cells display pathological tau even within their axons (Fig. 22.3g, h). Cells of all four groups can occur in one and the same individual, and the four groups possibly represent phases in a developmental sequence [27] (Fig. 22.4). The four phases may be part of an initial 'reaction' by a

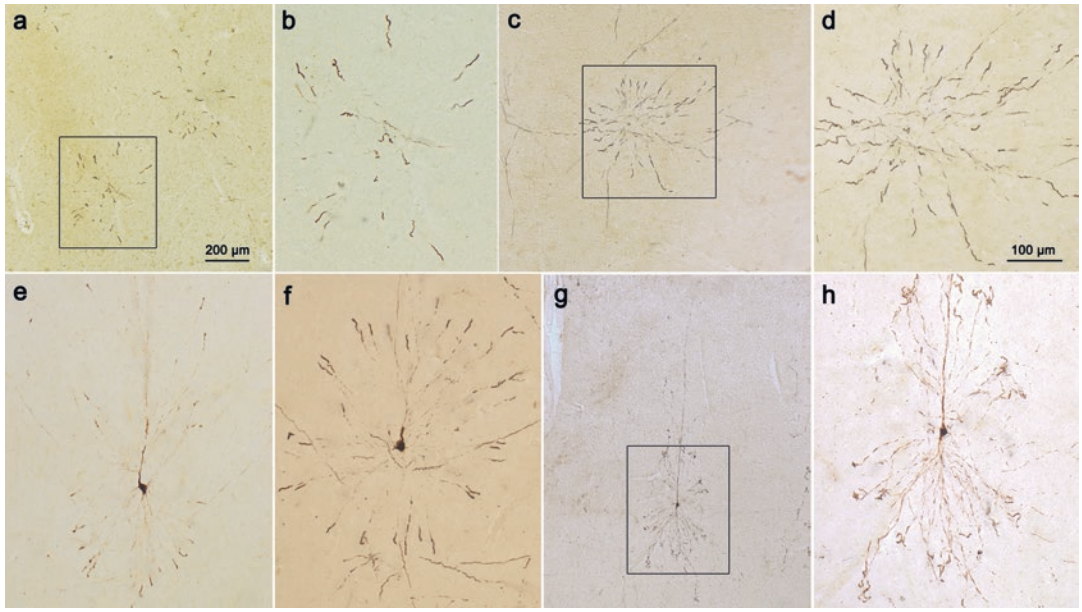

Fig. 22.3 AT8-immunoreactive abnormal tau during early NFT stages develops in isolated pyramidal cells in neocortical areas that routinely become involved only in late NFT stages (100 µm sections). (**a**) Two AT8-immunoreactive neurons assigned to group 1 (NFT stage III, superior temporal gyrus, male 72 years of age). Framed area indicates a nerve cell that is shown at higher magnification in (**b**). Its distal dendritic segments are bent, thickened, and filled with abnormal tau. The skirt of basal dendrites surrounds a central immunonegative area. (**c**) A group 2 neuron (NFT stage I, located in the medial temporal gyrus, male, 57 years of age), which, in addition to thickened distal segments of the basal dendrites, exhibits thread-like extensions from there into the proximal segments that also head towards an AT8-immunonegative center. Framed area indicates the same cell shown at higher magnification in (**d**). (**e** and **f**) Micrographs of two AT8-positive neurons assigned to group 3 (NFT stage II, superior temporal gyrus, male, 77 years of age in (**e**), and NFT stage I, superior temporal gyrus, male 57 years of age in (**f**)). In addition to the dendritic changes, the pathological material also occurs in the cell soma and in proximal portions of the apical dendrite. (**g**) A group 4 neuron displays in addition to the entirely AT8-immunopositive dendritic tree an AT8-immunoreactive axon (NFT stage III, superior temporal gyrus, male, 57 years of age). The framed area indicates the same neuron reproduced at higher magnification in (**h**). There are no detectable signs of severe axonal pathology nor are there varicosities or visible morphological changes at the junctions of the axon collaterals. (Reproduced, in part, with permission from Ref. [27])

previously uninvolved neocortical field to a progressive neuron-to-neuron and top-down transaxonal spread of pathological tau originating from cortical fields with a previous (and thereby greater) degree of involvement. All newly involved neocortical fields react similarly: The same four phases develop regardless of age, gender, or the presence/absence of amyloid-β plaques. Taken together, the four phases repeat, in reverse order, the phylo- and ontogenetic development of neocortical pyramidal cells [27, 75] (Fig. 22.4).

The existence of such an initial 'reaction' by hitherto uninvolved regions of the neocortex is in line with a recent study, in which tau seeding activity at low NFT stages (I–III) anticipates tau pathology in, e.g., the superior temporal gyrus or high order association fields of the occipital cortex [70]. As such, it can be postulated that the progression of the sAD-associated pathological process may take place chiefly by means of an anterograde transport of tau seeds along top-down connectivities [27].

Several implications emerge from the description and theory of the four phases. One is that abnormal tau seeds would only be transmitted in the first phase and solely by means of the distal dendritic segments, where the seeds trigger a renewed pathological response in the recipient neuron (Fig. 22.3a, b). It is known that, following

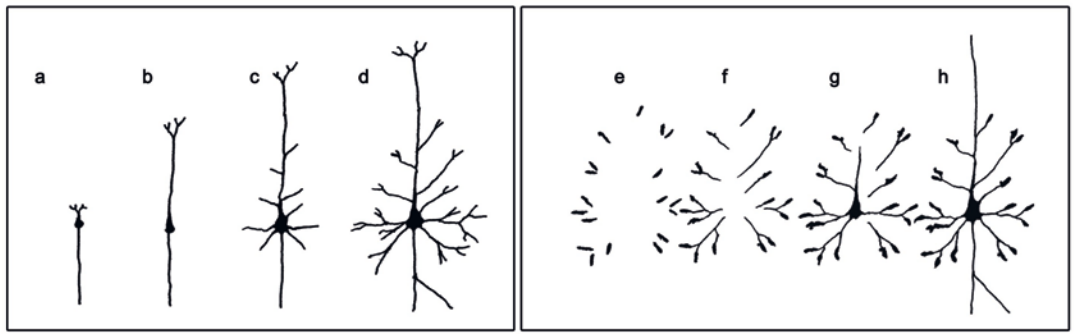

Fig. 22.4 Ontogenetic development of neocortical pyramidal cells (a–d) Generation of the axon (**a**) is followed by the development of a radially oriented apical dendrite, including its tuft of terminal twigs (**b**). Next (**c**), short basal dendrites (later the proximal segments) develop, which are complemented by a skirt (**d**) of later-maturing distal segments of the basal dendrites. **Early development of AT8 lesions in solitary pyramidal cells located in regions of the neocortex that typically become involved only in late NFT stages (e–h)** Initially, distal dendritic segments become filled with AT8-immunoreactive tau aggregates (**e**) This is followed by the development of immunopositive thread-like structures that emanate from the distal segments into the proximal dendrites leading towards an AT8- immunonegative soma (**f**). The soma of the involved cell together with proximal portions of the apical dendrite become AT8-immunopositive (**g**) Finally, the entire pyramidal cell, including its axon, becomes AT8-immunoreactive (**h**) The sequence of pathological tau changes shown in **e**–**h** repeat, in reverse order, the phylo- and ontogenetic development of neocortical pyramidal cells in **a**–**d**. (Reproduced with permission from Ref. [27])

its production in the soma, some of the native protein tau undergoes resorting into the axon [67, 111], where it stabilizes microtubules [3, 44, 101, 106, 114]. It is not likely that part of the axonal tau protein, induced by signaling in the dendrites, peels away from the axonal microtubules and is rapidly transferred without a trace from the axonal compartment into the dendritic tips [26, 27]. Moreover, none of the nerve cells in the first phase displays the slightest traces of abnormal tau protein in their axonal compartments (Fig. 22.3a, b). Abnormal tau first develops there during the fourth phase (Fig. 22.3g, h). Our findings are consonant with earlier reports, which show that normal tau is not confined to axons but is also consistently present in dendrites [63, 68, 69, 71, 77, 104, 105]. This in turn implies that, following the potential transsynaptic transmission of tau-containing seeds, it is dendritic tau that perpetuates the pathological cascade in sAD [77, 104].

During the second phase, abnormal tau aggregates progress with thin protrusions from the distal tips into the proximal dendrites (Fig. 22.3c, d), thereby emphasizing the distinction between distal and proximal dendritic segments [30, 56, 60, 77]. Apparently, synaptic contacts on proximal dendrites are not essential for the disease propagation. During the last two phases, the cell soma, including the stem of the apical dendrite (Fig. 22.3e, f), and the axon become filled with abnormal tau (Fig. 22.3g, h). The axonal cytoskeleton remains without light microscopically detectable injury, i.e., abnormal tau changes do not have their origin in the axon. Initially, the axonal cytoskeleton remains intact and thereby would make the propagation of tau seeds possible. The pathological "dendritic" tau is soluble and uniformly distributed within the axoplasm [72]. Gradually, presumably less soluble and spindle-like tau aggregates develop in the axon and these are interspersed by immunonegative segments [25, 110]. Therefore, the transmission of tau seeds only appears possible for a brief period, a constraint that could contribute to the interminably protracted progression of sAD. Most of the abnormal tau-containing nerve cells survive for decades [17, 25, 80], and their longevity may be attributable to the circumstance that the soluble pathological tau material is primarily generated within the dendritic compartment while leaving the axonal cytoskeleton virtually unimpaired.

It is seldom that one finds a lone AT8-immunopositive dendrite in the neuropil. Instead, as a rule, all of the distal dendrites of an initially involved dendritic tree belonging to an affected projection neuron are AT8-immunoreactive (Fig. 22.3a, b). This finding supports the assumption that a *single* seed-bearing top-down axon, including its numerous terminal collaterals, synapses on *all* dendrites of a *single* target neuron and refrains from contacting other nerve cells in the immediate vicinity. The mechanisms of this one-to-one selectivity require more study; nevertheless, it is conceivable that axons of late-maturing pyramidal cells establish connections with late-emerging distal dendrites of neocortical pyramidal cells in distant cortical regions in a highly ordered rather than haphazard manner [113].

This top-down one-to-one neuronal selectivity may underlie a capacity for a point-to-point transmission of data. Traditionally, top-down neurons are thought to send their axons in a diffuse manner to numerous and different neuronal constituents of their target areas, with the exception of the layer IV spiny stellate cells [13, 79]. Our findings, by contrast, point to the existence of a highly selective involvement limited only to the distal dendrites of a single target cell (Figs. 22.3a, b and 22.4e). This selectivity could increase the toxic effects of transmitted abnormal tau seeds and might explain in part why the abnormal tau protein spreads so effectively from neuron to neuron and why the progressive pathological process associated with sAD fails to go into remission.

Late-Maturing Top-Down Neurons and Their Target Cells Develop the Earliest Tau Changes. Do They Deliver Directives for Slowing the Pathological Process?

Top-down neurons and their target cells become involved sequentially during sAD, beginning with those that establish connections from **D** to **C** and followed by those from **C** to **B** and from **B** to **A**, i.e., neocortical pyramidal cells that mature latest are the first to develop tau pathology [27] (Fig. 22.2). Connections from **D** to **C** and **C** to **B** (Fig. 22.2) achieve functional maturity along with their developing myelin sheath only postnatally under the influence of factors that reach the human organism from the outside world and enhance the activity of the top-down projection cells [23].

The sAD-related pathological process as a whole clearly reflects the phylo- and ontogenetic development of the human brain. As such, it is understandable why projection neurons that commence axonal myelination early and develop a sturdy myelin sheath prenatally are spared [25]. By contrast, projection cells that begin their axonal myelination late (postnatally) – such as the majority of the top-down cells and their target neurons – are highly susceptible. The molecular backgrounds of these relationships are not fully understood. It should be pointed out, however, that it is also the activity of a projection neuron that provides the physiological stimulus for oligodendroglia cells to produce and sustain its myelin sheath [16, 107, 109]. The greater the degree of neuronal activity and the thicker the caliber of the myelin sheath during neuronal development, the better the nerve cell is protected against the sAD process.

The question arises whether the ultimate maturation of the top-down connectivities (Fig. 22.2, from **D** to **C** and **C** to **B**) can be effectively promoted by placing greater functional demands on them in childhood and early adulthood during the postnatal myelination phase [16, 23, 36, 51, 100, 103, 107, 115]. When toddlers and young children acquire their first language, the cytoarchitectural areas of the superior temporal gyrus are especially challenged functionally. These possibly manifest themselves in the strikingly robust myelination of the superior temporal gyrus compared to that of the remaining regions of the temporal lobe [64, 86]. As pointed out already above, the predictable progression of the pathological process is subject to a noticeable delay at the border between the medial and superior temporal gyri. The more intensively the late-maturing neurons are used, the more successful their myelination and, perhaps, also the chances of delaying the sAD-related process, so that the onset of the clinical phase could be postponed.

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Tau Prion-Like Propagation: State of the Art and Current Challenges

23

Simon Dujardin and Bradley T. Hyman

Tau Prion-Like Propagation, State of the Art

In Alzheimer's disease, tau deposition generally starts in the entorhinal cortex and then, following neuronal connections, progresses through the hippocampus, the limbic and association cortices (Fig. 23.1a) [7, 12, 13, 26, 33]. This stereotypical "staging scheme" was recently confirmed by tau PET scan studies [60, 71, 131, 132] and strongly correlates with cognitive decline [51]. Interestingly, although affecting different circuits, similar progression patterns through connected neuronal circuits have been identified in other tauopathies including Progressive Supranuclear Palsy (PSP) (Fig. 23.1b) [149, 155], Argyrophilic grain disease (Fig. 23.1c) [126], or more recently Pick's disease [67].

The similarities between these pathologies as well as the clear neuron-to-neuron progression led to the hypothesis that cerebral proteinopathies share similar cellular and molecular pathways of pathological protein propagation. Other types of well-characterized cerebral proteinopathies that spreads through neuronal networks are prion-diseases. Therefore, it was postulated that all these pathologies could be gathered and that all these proteins could be considered as prion-like protein or prionoids (for review: Aguzzi and Rajendran [3], Clavaguera et al. [22], Frost and Diamond [45], Goedert et al. [53], Hall and Patuto [56], Jucker and Walker [72], Walker et al. [151], and Dujardin [29]).

The designation "prion-like" is subject to an intense semantic debate among researchers because, although it is evident that these proteins share similar pathological principles, major differences can also be pointed out [102]. Prion diseases are rare neurodegenerative pathologies characterized by the accumulation of misfolded prion proteins. In 1982, Prusiner and collaborators ventured the hypothesis that the protein prion itself would be an infectious agent, and the unique responsible of the pathology spreading in the body and between individuals [119]. This hypothesis was rapidly confirmed, even if the term "infectious" is still controversial [87], showing that abnormal prion proteins are able to convert normal prion proteins into the pathological form. The latter form soluble oligomers and consequently amyloid fibers. The mechanism of formation of the fibers by recruitment of monomers by an initial oligomer (or nucleus) is called seeding (Fig. 23.2) [72]. Interestingly, different strains of prion exist with dissimilar, and transmissible, fibril conformations [145]. Moreover, the pathological prions can be

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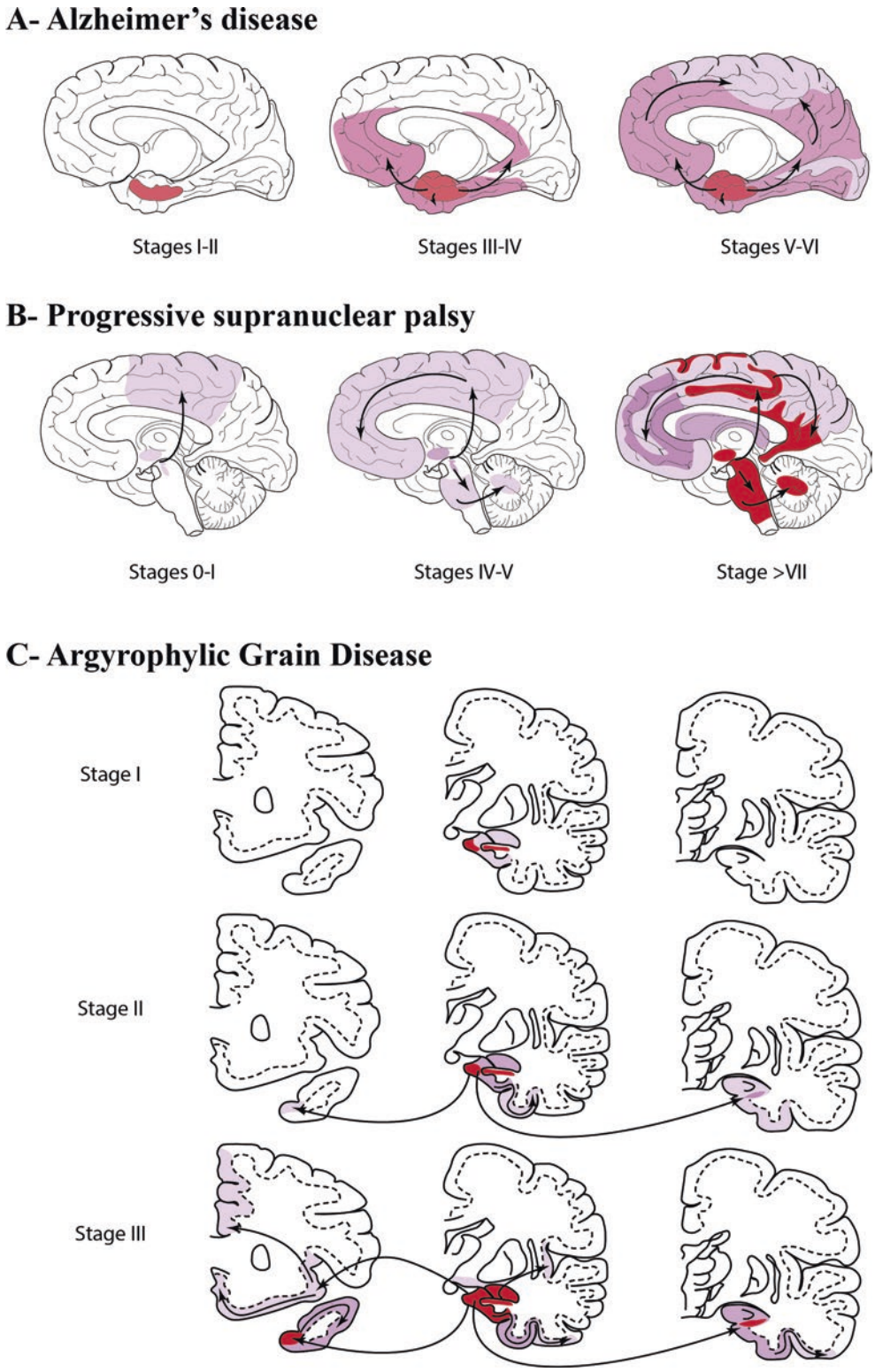

Fig. 23.1 Staging in tauopathies. Progressive appearance of tau pathology lesions in the brain of AD (**a**), PSP (**b**) and Argyrophilic grain disease (**c**). Light purple, purple and red respectively represents a small, moderate or

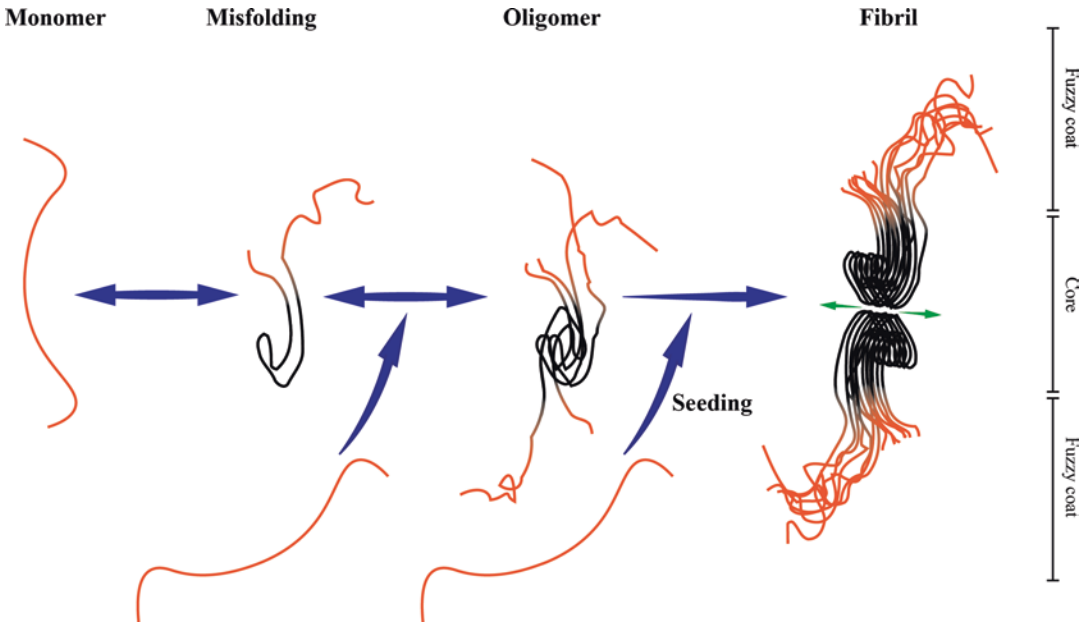

Fig. 23.2 Tau seeding. Tau monomers are naturally soluble, unfolded and flexible (orange). In pathophysiology context, some transient-to-stable folding can appear (black). In tauopathies, misfolding leads to the recruitment of several monomers into an unstable multimeric forms or oligomers. Through the addition of monomers in a seeding mechanism, misfolded tau proteins transmit their folding properties to a large number of naïve monomers and recruit them into a higher order stable fibril. The black structures represent the core of the fibril as shown by Fitzpatrick and collaborators [39]. Not to scale

transferred from cell-to-cell, spreading the pathology in several brain areas.

This was the first description of a protein with such pathological properties but recently, the hypothesis that other proteins like the amyloid peptide (Aβ), alpha synuclein and Tau could share these properties has been raised. For the amyloid peptide and synuclein, the lesions are transmissible by a seeding mechanism [27, 63, 70, 73, 89, 90, 93, 100, 101, 139, 150]. Similarly to prions, different strains with different functional and physicochemical properties seem to co-exist [11, 109, 111, 161]. Lesions also appear to actively propagate across neural circuits for these two pathological proteins [43, 58, 90, 150].

These studies led the way to the hypothesis that tau could actively propagate transsynaptically. Tau propagation (or spreading) is a broad term including several cellular pathways including active neuron-to-neuron transfer and the contamination of secondary cells potentially through a seeding mechanism (Fig. 23.3).

Fig. 23.1 (continued) severe amount tau pathology in the affected brain region. (**a**) In Alzheimer's disease, tau deposition generally starts in the entorhinal cortex and then, following neuronal connections, progresses through the hippocampus, the limbic and association cortices (sketch inspired by Braak and Del Tredici [13]). (**b**) In PSP, tau deposition progresses from the system pallidus – subthalamic nucleus – substantia nigra to the pedonculopontine nucleus and premotor cortex. The whole basal ganglia and dentate nucleus are then affected followed by affection of cerebellum, putamen, caudate nucleus and all neocortical regions at the exception of temporal areas (sketch inspired by Williams et al. [155] and Braak and Del Tredici [13]). (**c**) In Argyrophilic grain disease, tau deposition starts in the ambiens gyrus and CA1 region of the hippocampus before affecting amygdala, transentorhinal cortex, subiculum and temporal lobe. In stage III, frontal lobe is affected (sketch inspired by Saito et al. [126])

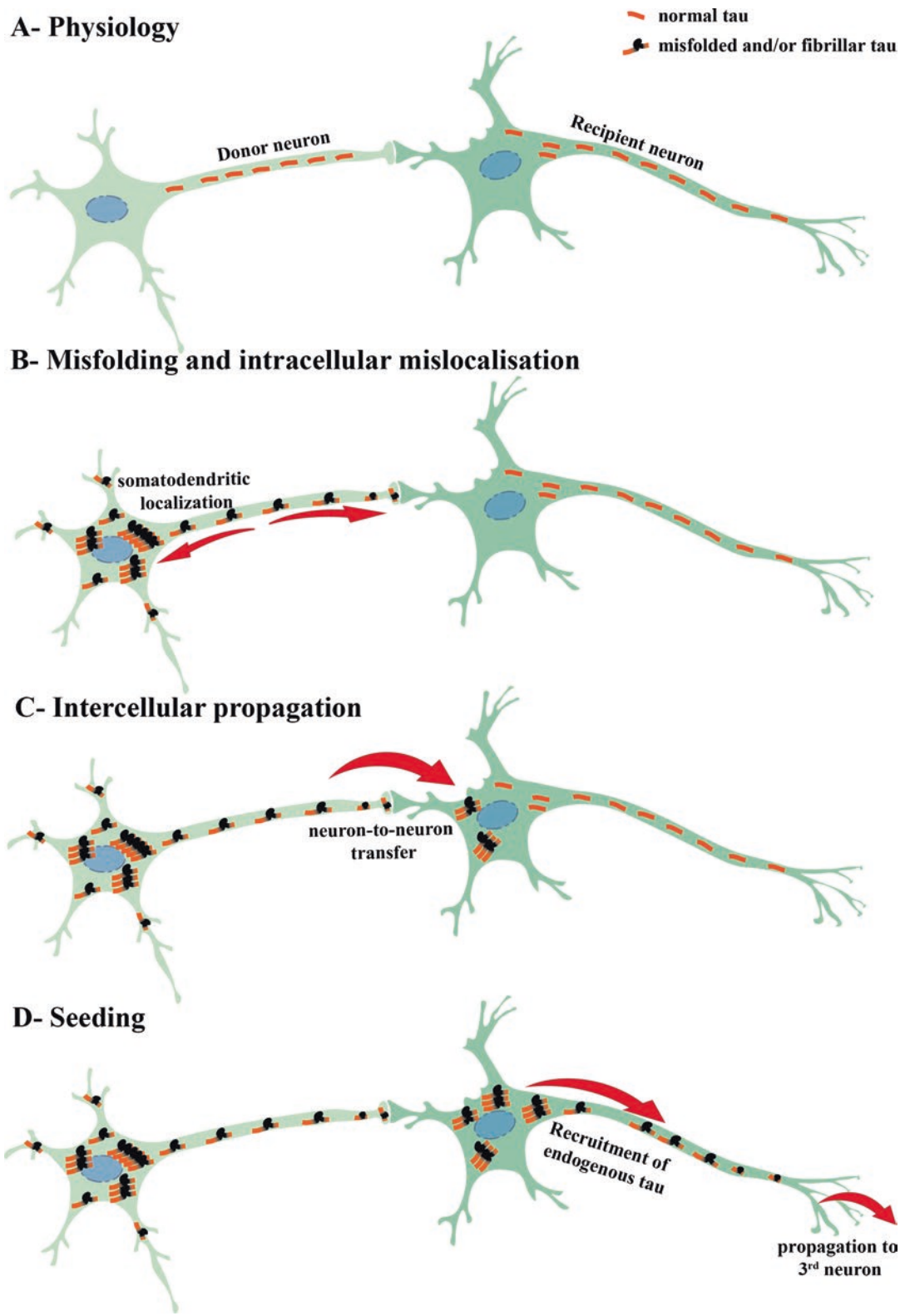

Fig. 23.3 Tau prion-like propagation model. (**a**) In physiology normal tau proteins (orange) are predominantly located into the axonal part of neurons. (**b**) Tau propagation starts with an early misfolded event that

Transmissibility of Tau Pathology, Tau Seeding?

Several studies have investigated the transmissibility and seeding of Tau pathology (Fig. 23.2) confirming this concept *in vitro* [44], *in cellulo* [46] or *in vivo* [20]. After these three studies, many have confirmed and reproduced this data. Briefly, Clavaguera and coworkers showed that injecting Tau aggregates extracted from mice overexpressing mutated Tau to mice overexpressing wild-type Tau was sufficient to induce Tau pathology [20]. It is interesting to note that when a Tau-immunodepleted extract is injected, no pathology can be detected showing that Tau is the responsible factor. The same group has also shown similar results injecting human brain lysates of several tauopathies and reproducing the morphology of the lesions seen in the human disease [19]. It is also worth noting- even if not reproduced so far in the literature- that they reported the formation of tau pathology in the brain of transgenic mice injected intraperitoneally with Tau aggregates [21]. Many following studies have shown injections of cerebral lysates or synthetic fibers in tau transgenic animals, potentiating the transmissibility [4, 24, 52, 65, 66, 68, 77, 79, 110, 128, 135, 138]. Several of these studies show the presence of murine Tau in the aggregates, suggesting that aggregates are able to recruit endogenous Tau proteins converting them to a pathological form [20, 25, 65, 104]. This is also supported by the observation that tau pathology is transmissible to wild type (WT) animals that don't usually get any sign of tau pathology [20, 104], and by early observations that endogenous mouse tau was incorporated into the NFT that develop in transgenic P301L human tau overexpressing mice [130]. An interesting recent study show strong tau seeding *in vivo* when tau aggregates are injected into transgenic animals developing β-amyloid plaques [59]. Tau seeding in this study seems to be highly dependent on the formation of tau aggregation in neuritic processes around plaques. We showed that in human AD patients, tau seeding at the synapses seems to precede tau pathology [28] and that tau seeding was markedly increased when amyloid pathology is present in both mouse models and human brain [9]. These studies could show that amyloid pathology potentiates tau propagation likely at the synaptic terminals and actively triggers its progression outside of the hippocampal formation in the cortical regions where both pathologies ultimately "meet" in Alzheimer's disease.

In parallel with data on animal models, *in cellulo*, several authors showed that after the incubation of aggregates, these were internalized and were able to promote aggregation of overexpressed Tau in cell lines [46, 55, 62, 107, 129, 141, 147, 156] but also in primary neurons or human iPS cells overexpressing human tau [106, 124, 142]. These studies showed, using truncated Tau species, that the microtubule-binding domain is necessary to promote aggregation. There is, however, a debate on what is the needed species of tau that is needed to promote tau aggregation or seeding. In a 2015 study, Mirbaha and coworkers observed that Tau trimer is the minimal unit with which a seeding mechanism could be observed [97]. The same authors in 2018 have identified a monomeric tau specie could be converted to a seeding competent form [96]. Although the minimal unit for seed-competency is still subject to debate, most of the studies agree to the idea that soluble oligomeric tau are the most seeding prone species [68, 79, 82, 83, 142].

Neuron-to-Neuron Transfer of Tau

Is Tau Transferred from One Cell to the Other?

Most of the models cited above analyzed the propagation of Tau pathology looking for cell-to-cell transfer of Tau pathology. In cell models,

Fig. 23.3 (continued) spreads through a neuron and a large number of tau proteins accumulate into the somatodendritic as well as the synaptic terminals of the neurons. (**c**) Misfolded tau proteins are transferred from neuron-to-neuron through the synapses and (**d**) in the recipient neuron, recruit endogenous tau, transmit their misfolding properties in a seeding mechanism and spread the pathology. Not to scale

both the (presumably misfolded or posttranslationally modified) WT proteins [32] and aggregated truncated Tau proteins seem to transfer to secondary cells [15, 46, 78, 160]. Models of microfluidic axonal isolations strongly suggest that Tau is trans-synaptically transferred and that synapses are required for neuron-to-neuron transfer [15, 32, 142, 152]. In other cell models, transfer of tau-containing conditioned medium seems to be sufficient to transfer tau to secondary cells suggesting that cell-to-cell contact or the close synaptic environment might not be a prerequisite for tau uptake by cells [15, 78, 160]. We hypothesize that the synaptic uptake therefore is at least in part due to the relative proximity of the pre and post synaptic elements as well as the potentially high local concentrations of any tau released into the extremely small perisynaptic space.

Cell-to-cell transfer of tau was also suggested in *in vivo* models in which Tau pathology evolves from the injection site to closely connected areas [4, 20, 65, 138]. However, it is difficult from these particular models to firmly conclude and determine the part of active propagation of aggregates versus diffusion area of the injection. In order to answer this question, in two independent studies, a transgenic model using the neuropsin promoter allowing for the overexpression of mutated Tau specifically in the entorhinal cortex has been developed and used to convincingly show the propagation of tau pathology from the entorhinal cortex to recipient neurons in the hippocampus [25, 88].

In addition, viral vector-mediated focal overexpression of tau proteins either in the hippocampus of rats or the entorhinal cortex of mice clearly and undoubtfully show that tau proteins can be cell-to-cell transported across long distances in accord with connectional neuroanatomical connections [30, 32, 153, 154]. The advantage of viral vector-mediated systems is that we can easily test different constructs and species of tau. Indeed, we could compare tau propagation of both mutant and wild-type tau as well as 3R versus 4R tau. We found that if the pathological conversion was faster for mutant tau, the long-distance propagation of tau pathological species was more efficient when 4R wild-type tau was used as compared to 3R and mutants [14, 30, 32]. Interestingly, we showed in two different models that the presence of endogenous tau was not required for tau cell-to-cell transfer. However, mouse tau was needed to observe pathological changes in secondary neurons [154] again arguing for the importance of endogenous tau in the pathological recruitment of tau. Worth noting, Tau cell-to-cell transfer was also observed *in vivo* in one model of lamprey [80, 85]. All together these studies demonstrate that tau can be transferred neuron-to-neuron (and, more generally, in some instances cell-to-cell) which leads to experiments aimed at understanding the cellular pathways implicated in this process (Fig. 23.4).

Routes of Cell-to-Cell Transfer

Different routes of cell-to-cell transfer of tau have been proposed. In principle, cell to cell transfer of an intracellular protein like Tau could occur either through release into the extracellular space, or via direct cell-to-cell contact. Regarding the extracellular space, it necessitates two distinct mechanisms: Tau secretion and tau uptake (Fig. 23.4).

(a) Tau secretion

Tau protein is a cytosolic protein thus its secretion via the conventional exocytosis implicating the Golgi apparatus is hardly conceivable. Therefore, several studies examined different routes of secretion and notably via extracellular vesicles. Two types of extracellular vesicles have particularly been shown to be involved in tau secretion: microvesicles (also called microparticles or ectosomes) and exosomes. The generation of microvesicles is still poorly understood but they are large vesicles (between 100 nm to 1 μm diameter) directly shed from plasma membrane. The release of exosomes is better characterized: during endocytosis, invagination of the plasma membrane leads to the formation of the primary endosome. Other invaginations occur in the membrane of this endosome leading to the formation of a multivesicular body. During this step, the intraluminal vesicles trap cytoplasmic material. When the multivesicular body fuses with the

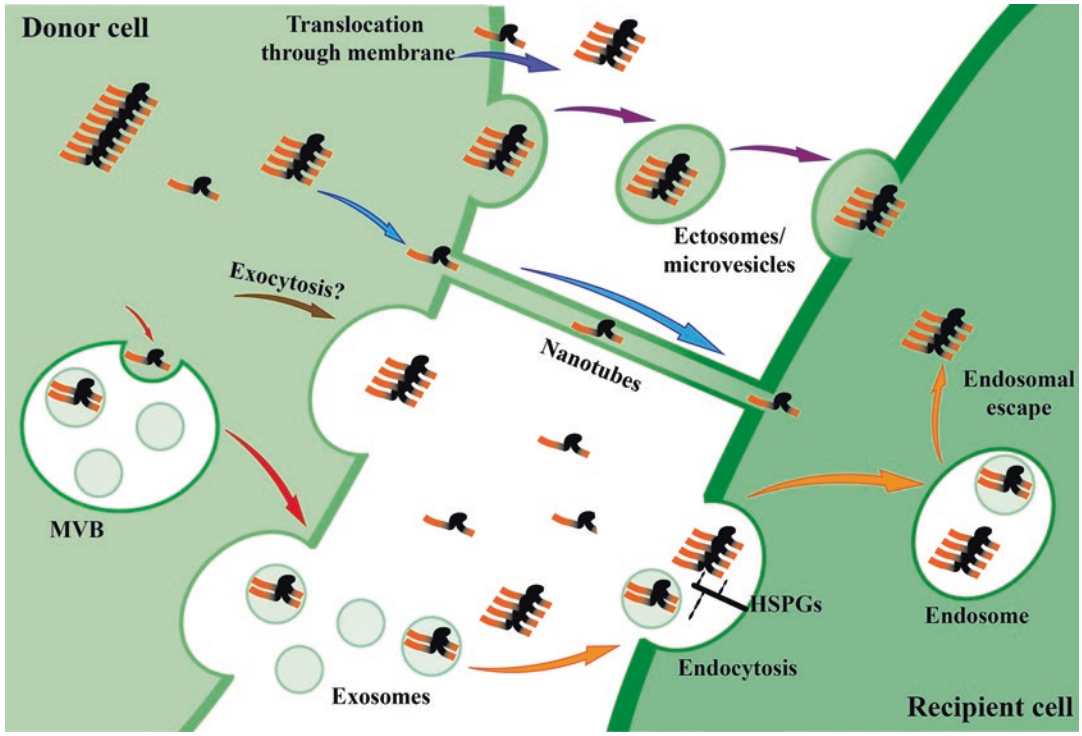

Fig. 23.4 Mechanisms of cell-to-cell transfer. Different pathways of neuron-to-neuron transfer have been proposed and observed. Tau is secreted into the extracellular space via translocation through the plasma membrane (Purple), extracellular vesicles (Ectosomes – pink or exosomes – red originating from the fusion of multivesicular bodies (MVB) with the plasma membrane). Regular exocytosis (brown) has also been a proposed mechanism but is largely questionable. Tau can also be transferred between cells via nanotubes (light blue). Uptake of tau largely happens through endocytosis (clathrin-mediated or clathrin-independent) and regulated by heparan sulfate proteoglycans (HSPGs) (orange). Tau aggregates are able to escape the endosome to reach the cytoplasm. (Sketch inspired by Dujardin [29] and Mudher et al. [102])

plasma membrane, the intraluminal vesicles are released and called exosomes. Exosomes are largely characterized (for review: Kowal et al. [81] and Rajendran et al. [120]) and were shown to carry prion [38], Aβ peptide [121] and synuclein [5, 84]. Moreover, it was shown that exosomes convey some lipids which potentiate fibrillogenesis and accelerates the formation of amyloid structures [162]. In the case of tauopathies, the implication of extracellular vesicles remains somewhat dependent on techniques and model systems used. Indeed, we and other authors describe the presence of Tau in both microvesicles [31] and exosomes purified from cells overexpressing Tau and from patient-derived cerebrospinal fluid [8, 31, 127, 134, 152], whereas other studies failed to reproduce these results in different models [37, 75, 116, 129, 143]. These discrepancies probably rely on the models used but also on the intracellular concentration of tau as it seems that tau is re-directed to exosomes when there is an accumulation in the cytoplasm. Contrariwise, tau seems to be present physiologically in larger microvesicles [31] (Fig. 23.4).

Regardless of whether tau is present in extracellular vesicles, several studies showed that extracellular tau is mostly found free in the medium, not -associated with vesicles [31, 76]. Several studies have proposed different secretory pathways by which tau reaches the extracellular space free. Katsinelos and coworkers found that phosphorylated tau can potentially be translocated across the plasma membrane via a type I unconventional protein secretion mechanism

(Fig. 23.4) [76]. Interestingly, chaperone-mediated translocation through membranes seems to lead to secretion as well in another type of unconventional secretion mechanism [41]. Another study by Tang and colleagues showed that in case of overexpression, Tau seems to relocalize, thanks to the mTor kinase, in autophagic vesicles and in the endoplasmic reticulum, which could suggest also the existence of a mechanism of exocytosis [143]. Interestingly, *in cellulo,* Rab GTPase regulates tau secretion in the extracellular space arguing for an active tau secretion mechanism rather than a potential passive diffusion through the plasma membrane [98, 125]. Additionally, the presence of tau in the extracellular media is stimulated by neuronal activity [116, 158, 159]. Interestingly, Tau proteins seem to be mainly dephosphorylated and truncated in their carboxy-terminal part when secreted [74, 80, 99, 105, 113, 116, 136]. However, truncation of Tau is not retrieved in every model [18]. The mechanisms of secretion described above were mainly identified *in vitro* and it is likely that part of them represent a physiological secretion rather than pathological processes [18, 116]. In human CSF, where tau is used as a biomarker of Alzheimer disease, both freely soluble and exosome containing pools have been described [10, 108, 127, 152].

(b) Tau uptake

Several studies have shown that tau placed in the extracellular medium is internalized into the soma of secondary cells arguing for the existence of one or several mechanism(s) of uptake [15, 46, 78, 106, 142, 157, 160]. Uptake of tau has also been observed *in vivo* [17, 142]. Tau monomers and oligomers are also efficiently internalized by human iPSC-derived neurons [146] and by astrocytes in culture [91, 112]. Interestingly, Piacentini and coworkers see a toxic effect of this uptake of oligomeric tau inside the astrocytes on the synaptic activity of neurons. Martini-Stoica and colleagues see that an increased uptake in astrocyte can markedly reduce tau propagation [91, 112]. Several studies showed that uptake of tau happens through endocytosis either clathrin-independent, likely through macropinocytosis, or clathrin-mediated [16, 35, 61, 94]. The uptake of free tau aggregates seems to be mediated by heparan sulfate [61, 122, 140] even if it is likely that other receptors are also implicated in these mechanisms (Figs. 23.4 and 23.5).

This uptake mechanism through the endocytic pathway might actively participate in the pathological propagation mechanism (Fig. 23.5). Indeed, three independent studies demonstrated that tau aggregates can induce a rupture of the endomembrane [16, 35, 40] and reach the cytoplasm. Calafate and coworkers report that BIN1, a genetic risk factor for late-onset Alzheimer's disease, negatively regulates this endocytic pathway and that a loss of function of BIN1 subsequently results in more uptake and more underlying tau seeding of aggregates [16]. When the endosomal membrane is ruptured autophagy pathways are activated through the galectin-8 and NDP52 receptors [35]. Stress-mediated or pharmacological inhibition of autophagy also potentiate tau aggregation arguing for a key role of autophagy in tau pathology propagation [35, 133]. Michel and co-workers even suggest that tau aggregation from monomers can happen directly inside the endocytosis vesicles and thereby favor tau pathology spreading [94] (Fig. 23.5). Worth noting, exosomes containing tau proteins are also endocytosed and hijacking the endosomal pathway are transferred to several connected neurons [114] (Fig. 23.5).

(c) Nanotubes

Although a secretory pathway is likely to take part in the propagation of Tau pathology, another route has been identified: nanotubes [1, 144] (Fig. 23.4). Nanotubes are filamentous-actin-containing membranous structures with a diameter of 50 to 800 nm forming bridges that connect remote cells. They have been shown to carry prion proteins as well as viruses [54, 137]. Tardivel and co-authors showed that tau could spread through these tunneling nanotubes and that interestingly extracellular tau can induce the formation of these structures.

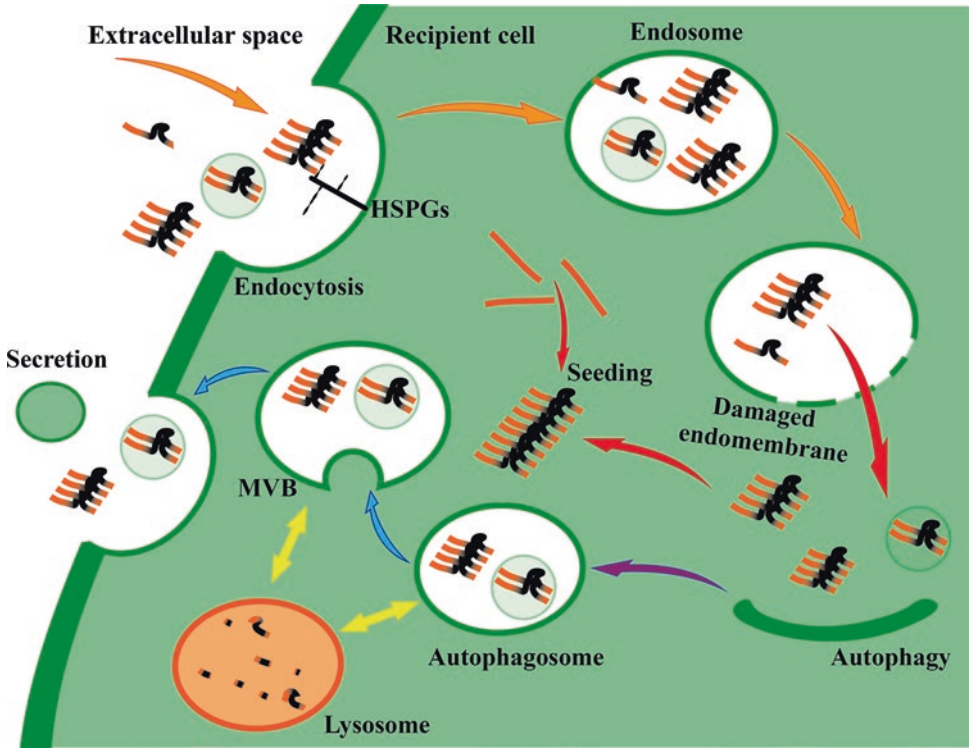

Fig. 23.5 Model of uptake and subsequent pathology spreading in the recipient neuron. Tau is released by cells probably from synaptic terminals mostly freely, but also via extracellular vesicles and taken up by secondary cells through a clathrin-independent mechanism of endocytosis or micropinocytosis (orange). Once in the endosome, the endosomal membrane is ruptured, and tau gets into the cytoplasm (red). Once in the cytoplasm misfolded tau induces seeding and spread of the pathology (red). However, autophagic mechanisms are activated to clear tau out causing tau to be internalized inside autophagosomes (purple). Once there, the autophagosome fusion with the lysosome leads to degradation of its content (yellow) or secretion via multivesicular bodies (MVB) (light blue)

Existence of "Strains" of Tau Aggregates

The question that actual strains of tau aggregates exist, parallel to the clinical strains observed in prion diseases, has been highly investigated and the answers are not yet definitive. What "strain" means for prion diseases is, based on the same protein template, there exist different but stable conformations of misfolding that are transmissible to native non-misfolded proteins. In prion disease, these different conformers convey different biological properties, including their clinical course and aggressiveness of disease.

In support of this idea, there is substantial evidence for tau that different conformers exist and have various behaviors in pathology. Indeed, different species were described with distinct behaviors in term of tau propagation phenotypes. Differences in different tau preparations ability to undergo cell to cell transfer and seeded misfolding, differences in structure of tau aggregates in different diseases, and differences in the patterns of tau in varied neuropathological diseases like AD compared to Pick's disease or PSP all point to the existence of something analogous to strains for tau. Firstly, comparing mutant and WT tau gives some insight. In fact, mutated tau proteins seem to bear different fiber morphologies and biochemical characteristics that influence the ability of seeding [6, 34, 47]. It is interesting to observe that the specific fibers' conformation of mutant tau can be transmitted and conserved by WT tau [34, 47]. *In vivo,* the propagation and

conformations of mutant vs WT tau is also different [30, 32]. Secondly, the different isoforms of tau – particularly the presence or absence of the exon 10 in the respectively called 4R or 3R isoforms – impacts tau behavior. It is of note that fibers made of various Tau isoforms bear variable properties [2, 36, 49, 86, 107], and 3R and 4R tau proteins differ in behavior in in vivo propagation [30]. These properties could explain the different morphologies of lesions observed in human pathologies and transmissible to animal or cell models [19, 128] and the differences in spatio-temporal evolution of Tau pathology between so-called 3R (Pick's disease) and 4R (PSP, Corticobasal degeneration) tauopathies.

Comparing tau fibers from different sporadic tauopathies and their capacity to seed led to the identification of potential strains [19, 77, 128]. The Diamond laboratory used a stable cell reporter assay [48, 62] to model seeding using brain lysate from several human cases with various tauopathies. They show that the shapes of resulting intracellular aggregates differ from case to case and that this could be transmitted to mice. Different regional pattern of progression in the brain are also observed consistent with the existence in sporadic tauopathies of different tau strains [77, 128].

Structural analysis of tau are difficult to carry out due to the biophysical properties of tau. Nevertheless, recent studies of Cryogenic Electron Microscopy have clearly identified the structure of aggregated tau, either in Alzheimer's disease [39] or in Pick's disease [36]. As the content in isoforms differs, the structures of the fibrils are different as well explaining the shapes of fibers as previously observed with electron microscopy. Mirbaha and colleagues also recently suggested that even a single monomer can bear a stable and transmissible conformation that seed aggregation [96]. This result also argues for the presence of specific strains but contradicts previous results showing that monomeric tau in solution has only transient folding [103], raising the question of what stabilizes the monomeric tau into a stable conformation under some circumstances but not others. Whether monomeric tau can have stable structures with a seeding potential in AD and other tauopathies is therefore really intriguing but must be currently viewed as uncertain.

In conclusion, tau species share with prion strains different behavior in tau pathology propagation (fibrils structures; lesions structures; cell types affected; brain regional patterns etc.). Structural evidence shows at least three stable conformations (straight filaments, paired helical filaments, and wide filaments in Pick bodies) but much more structural work in underway [36]. It remains unknown if these stable conformational changes can be reliably transmitted in a human disease setting. Techniques used in the research and diagnosis for prion such as protein misfolding cyclic amplification are also not available for use for tau proteins mostly because tau needs the addition of a polyanion such as heparin to aggregate *in vitro* [92]. This is one of the challenges for future studies of tau propagation.

Tau Prion-Like Propagation, Challenges and Future Directions

Extensive studies have been conducted in the past decade to understand tau propagation and the underlying cellular and molecular pathways, researchers are still facing several challenges.

Using More Sporadic Tauopathies Models

Tau pathological changes such as hyperphosphorylation or aggregation are fairly frequent in the human brain of even non-demented people. However, for diverse and mostly unknown reasons, in cell and animal models, tau pathology is fairly hard to model. Therefore, most of the time, researchers have to use artifices such as mutating the tau gene (MAPT gene) and/or artificially cleaving tau protein in order to obtain markers of tau pathology. Mutations of the MAPT gene are rare events that only occur in rare genetic familial frontotemporal dementia cases and don't occur in all the other sporadic tauopathies such as AD, PSP, Pick's Disease etc. [42, 50]. Tau propagation

studies are no exception to this limitation. Some examples: the main cell reporter assay for the study of tau seeding is a truncated and mutated version of tau protein [62]; the main animal model used for the study of tau cell-to-cell transfer is a mutant version of tau [25, 88]; the main animal model used to study tau transmission and seeding is the PS19 mouse model that bears a P301S mutation [65]. Using these tau versions is indeed convenient but one could ask their relevance for sporadic tauopathies especially knowing the previously described structural differences between mutant and WT tau [6, 34, 47]. Some studies tried to use more "sporadic" models of tau propagation. For example, Clavaguera and collaborators were able to induce transmission/seeding of tau pathology into a mouse model overexpressing a WT form of human tau protein [20] or even into WT mice [20, 104]. We used WT rats and overexpressed WT 3R or 4R tau into their hippocampus using lentiviral vectors resulting in the long-distance transfer of tau proteins and also propagation of pathological epitopes [30, 32]. These results are promising but they have limitations as well because (1) of the very long kinetic needed to analyze them (analysis 8–24 months after pathology transduction), (2) you hardly get the full tau pathology phenotypes with only weak aggregation in neurofibrillary tangles, neurodegeneration, or synaptic and behavioral deficits). These limitations of the models probably rely on fundamental kinetic differences in the rodents brain (month of pathological development at max) compared to the human brains (years of pathological evolution). Metabolism, brain clearance and neuroinflammation also seem to occur faster in rodents than in humans. In addition to that, fundamental molecular differences cannot be ignored as mice only express the 4R isoforms of tau when humans express both 3R and 4R. The use of more humanized models such as tau Knock-In rodents, human iPS cells and/or organoids might give us more insights in the future. In conclusion, it would be interesting to invent efficient tools to study tau pathological propagation in a "sporadic" environment to tackle the specific mechanisms of tau release uptake and instructive misfolding.

Tau Propagation, Physiological Versus Pathological Mechanisms

As stated just above, one drawback of some models of tau propagation is that the pathological phenotypes such as tau phosphorylation, misfolding and/or aggregation can be limited. This leads us to hypothesize that at least part of the described mechanisms of tau propagation are happening physiologically. Indeed, for example, regarding the trans-synaptic transfer of tau, in a recent *in vivo* study we showed that this transfer could happen in the absence of epitopes of tau hyperphosphorylation and misfolding [30]. This result suggests that the trans-synaptic transfer of tau can be a physiological mechanism. This idea is also supported by the fact that tau propagation still occurs in absence of any pathological conversion in a mouse lacking endogenous tau [154]. However, several questions remain: is this physiological mechanism conserved in a diseased brain? Are the routes of cell-to-cell transfer the same in physiology versus pathology? Is the kinetic of this transfer impacted? Some elements of response are present in the literature. Firstly, the routes seem to differ: tau secretion via exosomes, for instance, seem to happen only when tau accumulate intracellularly and not physiologically [31]. Secondly, the kinetic of nanotubes formation is impacted by the extracellular presence of tau aggregates [144]. Finally, numerous studies all show that secreted tau proteins are mostly non-phosphorylated, monomeric, soluble and truncated [18, 31, 41, 75, 76, 99, 113, 115, 116, 127, 129, 134, 143, 159]. This goes in line with a physiological secretion of tau and could explain the presence of tau in the cerebrospinal fluid of every individuals independently of neuronal death [57]. However, on the other side, tau uptake seems to be more efficient for aggregated species than monomeric tau [97, 142]. Understanding this discrepancy between secretion and uptake may be a key point for understanding the exact mechanisms of tau propagation. Whether this is due to different kinetic of uptake, route of uptake or intracellular stability after uptake is difficult to understand at that time.

Precisely Identify the Kinetics of Tau Life Cycle Events

Understanding tau propagation also comes by deciphering the kinetic of events in the brain. This notion is largely unknown and very dependent on the models used but the current literature can inform us about the slow versus fast points of this process. Firstly, it seems that the neuron-to-neuron transfer of tau is a slow point both *in vitro* and *in vivo* where we can observe it only after respectively days or weeks/months [15, 25, 32, 88]. It is interesting and counterintuitive to notice that both secretion and uptake are fast points as they can be observed in hours mostly *in vitro* [15, 31, 46, 78, 106, 116, 142, 157–160]. The discrepancy between transfer and secretion/uptake probably rely on (1) the sensitivity of the histology techniques used to see tau transfer and (2) the mostly non-physiologic amount of tau used to observe fast secretion and fast uptake. Lastly, tau seeding seems to be a process regulated by a sigmoidal growth with a long lag phase, an exponential growth phase and a plateau phase [62]. *In vitro*, when the right conditions are reunited (high intracellular content of tau, presence of tau truncation/mutation favoring misfolding etc.), the whole seeding process can happen in less than 24 h [28] when *in vivo* the lag phase takes probably weeks and also relies on the amount and quality of tau in the recipient brain. Indeed, inducing seeding and aggregates formation in a tau transgenic animal with a mutation (as soon as 2 weeks post induction in animals such as PS19 mice (see Ahmed et al. [4] and Iba et al. [65, 66]) is faster than for a tau transgenic animal without a mutation (months post-injection in animals such as alz17 or hTau mice (see Castillo-Carranza et al. [17] and Clavaguera et al. [20] or in WT animals [20])). The fast/exponential appearance of neurofibrillary tangle after the lag phase *in vivo* is supported by observations using 2-photon microscopy [25]. Thus both transfer and seeding have their fast and slow points. It is also evident that factors such as the presence of amyloid beta [9, 59, 69, 117, 148] or brain activity [116, 158, 159] can also accelerate these slow points.

Identify Good Therapeutic Strategies

Identifying tau propagation as a key mechanism of tauopathies and particularly AD has raised some hope to find a disease-modifying treatment tackling the progression of the disease. An obvious target is the extracellular species of tau. This led to intense research by academic and industry teams around the development of immunotherapies. Most of the studies find a high potential for reducing tau propagation in diverse models. Examples include the work of Holtzman's team showing a reduction of tau uptake and seeding *in vitro* in addition to reduction of tau pathology in a transgenic mouse model of genetic Frontotemporal Lobar Dementia [160]. Antibodies against oligomeric tau (TOMA antibodies) showed reduction of seeding after injection of oligomers in the brain of hTau mouse model [17]. We more recently showed diverse efficacies of different antibodies raised against total tau and also phosphorylated tau or truncated tau onto uptake and seeding of high molecular weight tau [105, 106]. Recent studies confirm these data in other models and trying to assess the potential of different epitopes [23, 24]. Beside the use of immunotherapy, tau propagation research could lead to additional efforts to develop therapies by targeting the pathway by which tau propagates. This includes the mediators of tau propagation like targeting the mechanisms of secretion (chaperones, exosome formations), the mechanisms of tau uptake (the endosomal pathway), stimulating the autophagy pathways to favor clearance, stimulation of deacetylase [95] etc. To our knowledge, this research is still preliminary but the understanding of mechanisms of tau propagation might lead to more potential therapies in the future.

Understand the Regional Patterns, the Cell Vulnerabilities

It is striking to notice the differences between the pattern of progression of the different tauopathies in the human brain. While they are all patterns of

tau aggregation, they are quite heterogenous. Why are some pathologies very focal (Pick's disease or Argyrophilic Grain Disease) and some more global (PSP and AD)? Why tau progression leads to asymmetry in some diseases and global symmetry in others? Interestingly, the human data show for example that in AD the neurons receiving the neurofibrillary tangles are strongly interconnected but dentate gyrus granule cells, which receive the bulk of the perforant pathway projection from the early affected entorhinal cortex, are relatively resistant [64]. On the other hand, Pick bodies, another type of lesion made of tau aggregates are predominantly present in these particular granule cells in the dentate gyrus that are resistant to the alternatively folded form of tau found in neurofibrillary tangles [118]. These differences are thought to be due to different cell populations vulnerabilities to tau pathology [123]. This also suggests unique relationships between specific misfolded tau species and host cell characteristics. It would be really important to understand why different brain areas and neuronal subtypes are selectively vulnerable for different forms of inclusions? Beyond the observation that propagation is therefore dependent on both the anatomy and the properties of the recipient cell, these concepts are largely unexplored so far.

Conclusions

Tau propagation, this sequence of events leading to the progression of the disease in the brain of tauopathies (Fig. 23.2), has now been experimentally described. We can distinguish several pathways leading to tau propagation: (1) the transfer from cell-to-cell of both pathological and non-pathological species of tau (Fig. 23.4) and (2) the transmission of tau pathology in a second neuron via a seeding mechanism (Fig. 23.1). The most plausible route of tau transfer is through the extracellular space and particularly via its secretion (mostly freely in the medium but also through extracellular vesicles) and uptake into recipient neurons (Fig. 23.4). This uptake likely occurs via an endocytic mechanism. Once in the endosome, tau aggregates cause the endosomal membrane to rupture and reach the cytoplasm. It has been demonstrated that when they reach the cytoplasm, tau aggregates induce recruit endogenous naïve tau proteins in a seeding mechanism (Fig. 23.5). The similarities between these mechanisms and the prion protein spreading mechanism has opened a discussion between researchers over the idea that we can reunite tau, prion and other proteins under the banner of prion-like proteins. This semantic debate is still open but we clearly, here, brought arguments for the existence of strong convergence points but as well pointed out differences. Many other questions remain to be answered. Why would different brain areas and neuronal subtypes be selectively vulnerable for different forms of inclusions? What causes the initial misfolding events that are then propagated across neural systems? Why distinct and specific neural systems would respond differently to tau "strains"? What causes toxicity of neural networks? What is the impact of known molecular differences (isoforms and posttranslational modifications) onto these mechanisms? And, perhaps most importantly, can these insights help in the design and application of tau as a therapeutic target to slow progressive neurodegeneration in AD and other tauopathies?

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Tau Condensates

24

Kenneth S. Kosik and Songi Han

Introduction

The tau protein embodies a deep paradox. Under normal conditions it is one of the most soluble proteins known, but once it aggregates in a variety of different diseases, it becomes one of the most insoluble proteins known. How this transition occurs has remained baffling. Recently we and other groups have identified a novel state of tau in which the protein becomes phase separated within the cytoplasm [1–5]. The core unresolved question addressed in this chapter is whether LLPS (liquid liquid phase separation) of tau in brain cells is on-pathway to pathological tau aggregates. Two streams of prior work have brought the field to this question: (1) discoveries of membraneless organelles and biomolecular condensates by cell biologists and (2) discoveries by chemists of coacervation, which usually refers to an electrostatically driven liquid-liquid phase separation resulting from weak association of oppositely charged, but typically fully hydrated, macro-ions in the form of polyelectrolytes. Membraneless organelles are a set of cellular entities recognizable microscopically by immunostaining of specific marker proteins. Biomolecular condensates imply a more general biophysical structure that draws upon principles of polymer chemistry and soft matter physics to describe membraneless entities with an emphasis on the condensation of proteins and nucleic acids as a "crucible" for localized molecular reactions [6]. We will discuss these membraneless organelles and coacervation separately and then conjoin them toward the problem of tau aggregation in the tauopathies. The question is whether physico-chemical knowledge of coacervation processes can inform the cell biological problem: when do physico-chemical principles apply and when do the specific biological interactions take over and dictate the relevant mechanisms.

Discoveries in this domain related to other proteins involved in neurodegenerative conditions have greatly informed the work on tau. Among the most well-studied and informative of these proteins are FUS [7–10], hnRNPA2B1 and hnRNPA1 [11], TDP-43 [11, 12], C9ORF72 [13–15], Ddx4 [16] all of which are genetically

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associated with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration. As noted above, the tau protein now joins this list of amyloidogenic proteins undergoing LLPS [1–5]. The results of studies on FUS (fused in sarcoma/translocated in liposarcoma), a FET family member, exemplify a number of principles that have guided recent thinking about aggregation prone proteins in neurodegeneration. The protein has a prion-like low complexity domain near its carboxy terminus, which is RGG-rich (Arg-Gly-Gly-rich) and where most of the disease mutations reside. Interestingly, an arginine motif also shows up in C9ORF72. This region can polymerize into fibrous amyloid-like assemblies [11, 17, 18], which exhibit the macroscopic behavior of hydrogels. Extrapolating these observations to living cells and assessing whether liquid condensates are on pathway to fibers represents a considerable challenge. In the case of FUS, liquid-like droplets can form in living cells, FUS droplets have the appearance of stress granules, the liquid-like state can convert into a solid state and disease-associated mutations enhance this conversion [10]. These observations and others have led to the speculation that phase transitions are a central pathobiological phenomenon in some neurodegenerative diseases.

One can ask how well the observations concerning FUS generalize to other proteins that undergo aggregation. This remains an open question that is asked by many in the field. It starts with the very basic challenge of knowing what criteria are necessary to conclude that a condensate in cells has droplet properties. Among the properties assigned to droplets are (a) dynamic protein exchange as measured by fluorescence recovery after photobleaching (FRAP); (b) condensation of fully hydrated proteins in high concentration within a droplet that can have a viscosity of 10- to 100-fold that of water; (c) a spherical shape that balances interfacial tension to its medium; (d) the interference with 1,6-hexanediol of weak hydrophobic protein-protein or protein-RNA interactions that are responsible for the formation of dynamic, liquid-like assemblies; and/or (e) droplet fusion into a single droplet [19]. It is our belief that criteria (a)–(c) should underlie all biomolecular condensates that can be deemed droplets, (d) weak interactions should be foundational for biomolecular condensates originating from and constituting droplets, while (e) droplet fusion is a stringent criteria only met by rapid dynamics, and possibly apply only to nascent droplets upon formation, before solidification processes have occurred.

Membraneless Organelles

To contextualize tau in this context, some consideration of the recognized organelles that derive from liquid liquid phase separation is useful. Cells contain a class of organelles in which cellular material is condensed and moves cohesively through the cell, but are devoid of any limiting membrane. The condensed constituents are retained within the volume of the organelle, presumably held together by electrostatic interactions, while water can be freely exchanged. Liquid-like behavior is observed by FRAP of their protein components as well as flowing, fusing, and rapid reorganization of internal components [20, 21]. The liquid-liquid phase separation (LLPS) often arises from binding sites on RNA-binding proteins that interact with themselves or other RNA-binding proteins to create a larger multivalent assembly [10, 16, 22–26]. Organelles, which meet these biophysical criteria are diverse and include RNA granules, germline P granules [20], P-bodies, stress granules that mostly tend to reside in the cytoplasm and nucleoli, Cajal bodies, nuclear speckles, paraspeckles, histone-locus bodies, and promyelocytic leukemia bodies, which are all present in the nucleus [27–30]. Synapsin can form a distinct liquid phase in an aqueous environment and capture small lipid vesicles [31]. The presence of RNA is one unifying, but not absolute feature of these structures. The phase separated states are often rapidly disassembled upon phosphorylation. In the case of synapsin, by calcium/calmodulin-dependent protein kinase II, which could serve as a mechanism for clustering synaptic vesicles at synapses. Ribonucleoprotein (RNP) granules such as the nucleolus and centrosomes are also phase

separated entities. Furthermore, macromolecular complexes such as super enhancer-enriched transcriptional coactivators have properties of phase separation [19, 32, 33]. Proteins that contribute to the formation of these organelles often have low complexity domains [34] or more generally have regions of intrinsic disorder, a common feature found in RNA binding proteins. In fact, tau is an intrinsically disordered protein that binds RNA, but is not classified as an RNA binding protein. Clearly, a taxonomy based upon organizational principles of their diverse elements is sorely needed.

When turning to the pathobiology of proteins known to form inclusions in neurodegeneration, such as FUS, TDP-43, c9orf72, hnRNPA2B1 and hnRNPA1, TIA-1 and tau several biophysical facets begin to converge. These proteins can undergo LLPS *in vitro*, appear to form biomolecular condensates in living cells, have low complexity domains (or more generally intrinsically disordered regions) with a resemblance to yeast prion proteins, often bind to RNA and can be associated with membraneless organelles. Yeast prion proteins have low complexity domains, and can be driven to interconvert from a liquid state to fibers [35]. This, however, does not mean that the liquid state is an intermediate state towards, or is facilitating, fibril formation. Rather, the current state of observation merely tells us that there is a pathway for proteins in the LLPS state to transform to fibrils. For some proteins and environments, this path may be a highly likely pathway under pathological conditions, and for other proteins this might not be the case.

Neuronal RNA Granules Among the earliest sightings of a membraneless organelle in neurons were reports beginning in 1996 of "RNA granules" [36–38] that appear to have a role in synaptic plasticity. Historical precedents for "RNA granules" are rooted in developmental biology beginning with Metschnikoff [39], who observed dark staining granules at one pole in the Miastor metraloas (fly) larvae and subsequent research identified "polar granules" involved in primordial germ cell differentiation from a variety of insect species [40, 41]. A remarkable feature of neuronal RNA granules was their translational silence despite a dense collection of ribosomes and co-localization with poly(A+) mRNA. Consistent with their translational incompetence is their failure to incorporate radioactive amino acids and the absence of eIF4E, 4G, and tRNAs. Silencing may be due to the inadequacy of translation initiation factors, incomplete ribosomal subunit composition, inadequate tRNA populations, the presence of FMRP or steric hindrance due to their dense packing. When visualized with SYTO14 in living neurons they demonstrated motility—often small bidirectional displacements—around synapses. These features suggested that RNA granules transported mRNAs to dendritic locales in a translationally silent state that could then be de-repressed by synaptic depolarization. With depolarization, many mRNAs, including those involved in plasticity, rapidly shift from the RNA granule fraction to polysomes where they can function as the building blocks for the translational needs of a synapse. Among the mRNAs detected are *CaMKIIα*, *MAP 2,* and *β-actin*, and the proteins Staufen, RNA helicases of the DDX family and other RNA binding proteins [37, 42]. A key component is RNG105/caprin1, an RNA-binding protein [43]. Knockdown and knockout of RNG105 in cultured neurons reduces the synaptic connections on dendrites and the density of neural network [44, 45]. By establishing an exposed charge surface by virtue of the intrinsic disorder of RNA binding proteins, this class of proteins is crucial to the partitioning of many phase-separated structures. Depolarization reorganizes granules and induces a less compact organization of their ribosomes. Thus, RNA granules serve as a local storage compartment for mRNAs under translational arrest, but are poised for release to actively translated pools. The local release of mRNAs and ribosomes from granules may link RNA localization to translation and synaptic plasticity.

Germinal Granules Among various membraneless organelles, RNA granules in neurons bear the closest resemblance to germinal granules as described in Xenopus laevis, polar

granules in Drosophila melanogaster, and P granules in Caenorhabditis elegans. This category of granule is collectively referred to as germ cell granules [46, 47]. In Caenorhabditis elegans, when the first germ cell is established, P granules localize to the posterior of the one-cell embryo and undergo rapid dissolution and condensation. When condensation was biased to the posterior, localization occurred as a classic phase transition, in which polarity proteins vary the condensation point across the cell and potentially structure the cytoplasm [20]. By silencing and then releasing mRNA translation, they direct the timing of maternal translation to promote germ cell specification in the early embryo.

P Bodies One year after the description of RNA granules, P bodies (processing bodies) were discovered as small discrete cytoplasmic foci that functioned in RNA turnover via the 5'-3'exoribonuclease, mXRN1p [48]. At this time they were not recognized as membraneless organelles, nor had they been named. Many more proteins were discovered in these bodies that mediated decapping of mRNAs, nonsense-mediated mRNA decay, adenylate-uridylate-rich element mediated mRNA decay, RNA storage and translational repression by miRNAs (microRNAs) [48–51]. These diverse functions generated different names for the structures, but P bodies have become a consensus term for the localization of most of these RNA processing functions [52] especially after their extensive characterization in yeast [52, 53].

Stress Granules Stress granules serve as triage centers that sort, remodel, and export specific mRNA transcripts for reinitiation, decay, or storage [54]. The response of the cell to stress by the formation of cytoplasmic foci or granules was reported in 1983 in tomato cells [55], in 1988 using chicken embryo fibroblasts [56] and HeLa cells [57]. Cultured Peruvian tomato cells demonstrated that untranslated mRNAs accumulate within these discrete foci with heat stress [58, 59]; once returned to ambient temperature, the mRNAs rapidly move from the granules to polyribosomes and begin translating. Heat shock mRNAs that are selectively translated during stress are excluded from the granules [58]. Thus, stress moved many "housekeeping" mRNAs to a translationally incompetent state, while the translation of mRNAs encoding molecular chaperones and enzymes involved in damage repair was enhanced. Anderson [60] showed that the assembly of TIA-1 positive stress granules is initiated by the phosphorylation of eIF-2α. A TIA-1 mutant lacking its RNA-binding domains functioned as a transdominant inhibitor of stress granule assembly and therefore first suggested the role of RNA-protein complex sequestration in the assembly of stress granules [60]. An interesting link to neurodegeneration is the finding that stress granules are cleared by autophagy [61]. Most recently Roy Parker showed that RNA homopolymers as well as RNA binding protein–protein interactions contribute to stress granule formation [62]. The importance of this finding is its implication of a role for the neurotoxic arginine-containing dipeptides, which are among the translation products that derive from the C9ORF72 expansion associated with ALS (amyotrophic lateral sclerosis) and FTD (frontotemporal dementia). These dipeptides stabilize the RNA-RNA interactions and thereby reveal a possible step in the longstanding question: What initiates pathological aggregation?

Coacervation

The physico-chemical basis for the many biological phenomena enumerated above is complex or simple coacervation. The term, coacervation, and the concept were first introduced by Bungenberg de Jong in an under cited publication [63]. A complex coacervate refers to a phase-separated fluid, typically upon weak association of two oppositely charged polyelectrolytes in solution. When chemists apply the term "complex" the complexity pales in comparison to the analogous chemistry in a living biological system. The term "complex" in complex coacervation refers to multiple polymer types involved in the

complexation; often it is just two oppositely charged proteins or components compared to the thousands of proteins associated with membraneless organelles.

Historically, "simple" coacervation refers to a related process resulting in LLPS, however, only involving a single biopolymer type. The mechanism behind condensation for simple coacervation can be similar to that of complex coacervation if an intrinsically disordered biopolymer has spatially separated charges, and when modulation of pH and salt concentration can achieve effective local charge neutralization for polymer chain condensation to LLPS. An adhesive foot mussel protein from *Mytilus californianus* provides one illustrative example [64]. The formation of a bifluidic sponge-like nanostructured network undergoing water exchange is thought to be driven by tuning the electrostatic interactions between the polyelectrolyte constituents or their complexes. However, simple coacervation can instead also be driven by other forces, such as hydrophobic or π-cation interactions. Broadly, the field of coacervation has recently been invigorated by the realization that LLPS occurs *in vivo*, in particular involving proteins involved in neurodegenerative diseases, suggesting a possible physiological or pathophysiologic role for these assemblies. Specifically in the case of tau, both complex coacervation between tau and RNA or simple coacervation of hyperphosphorylated tau have been reported on in the literature.

Importantly, *in vitro* studies can validate the isolated properties of the components within a biological system to undergo LLPS. The liquid phase consists of a dense polyelectrolyte-rich phase, which form droplets and retain liquid-like mobility, as shown by NMR measurements [65, 66] and a dilute polyelectrolyte-depleted phase. The concentrated liquid phase is termed the coacervate phase and the dilute phase the equilibrium phase or solution phase, within which the coacervate characteristically appears as droplets of submicron to several micron dimensions that may further coalesce to larger droplets, even macroscopic phase separation. Complex coacervate fluids critically rely on their low interfacial tension with respect to water, and thereby can wet or engulf macromolecular surfaces under aqueous conditions, provided a weakly attractive interaction between the polyelectrolyte constituents and the surface. The engulfment properties may account for recent observations concerning possible A-beta engulfment and fibrilization pathways as an antimicrobial response [67]. Surface wetting of solid structures in the biological context and in cells by coacervate-like liquid condensates is a relatively unexplored area in cell biology, and may present yet another mechanism facilitated by LLPS of how biomolecules interact and cellular constituents associate. Taken together, the process of complex coacervation spans an enormous breadth of biological systems and has even been attributed a role in the origin of life [68].

Tau Droplets

With this foundational framework we now pose the question whether tau can undergo LLPS *in vitro* and *in vivo,* whether cofactors are required and whether in a condensed state, tau can transition to fibers. This thinking led to consideration of LLPS as an intermediate regulatory state, which could redissolve into a soluble state or transition to irreversible aggregation/amyloid fibrils. Intracellular aggregates consisting of the tau protein occur in many neurological conditions with Alzheimer's disease the most prominent among them. A variety of mutations in the tau gene lead to another neurodegenerative condition labeled frontotemporal dementia. Tau has been regarded as in a dynamic equilibrium between a microtubule-bound state and the solution state; however phase separation studies of tau suggest this two compartment model is simplistic. Tau exists in multiple phase states, as a solid when it polymerizes into fibrils and in liquid liquid phase separated state. Transitions across these states are central to understanding how tau contributes to disease. Under disease conditions tau self-assembles into solid fibrils that eventually lead to highly insoluble polymeric inclusions known as neurofibrillary tangles. The underlying biophysical process by which tau

transitions to an insoluble fibril is unknown. An early clue about the nature of this transition came from the observation that polyanions, such as heparin, promote tau fibrillization [69]. Although less effectively, RNA can also induce tau fibrillization [70, 71], and unlike heparin, RNA is present intracellularly, making it accessible to interact with tau. These early observations showed that certain types of tau interactions drive fibril formation and the fact that these interactions involve polyanions suggested the possibility of a phase separation due to their tendency to decrease solubility and favor entropy driven effects [72]. Coupling of assembly and phase separation of multivalent macromolecules is an important organizing principle for biomolecular condensates.

Our experiments extended the *in vitro* data of tau-polyanion binding to prove that tau can bind RNA in living cells. These experiments utilized a protein-RNA crosslinking method followed by antibody pull down of tau crosslinked to RNAs followed by RNAseq. Interestingly, tau-RNA binding showed some enhancement for tRNAs even after normalizing for their relative abundance. Among the tRNAs, $tRNA^{Arg}$ topped the list as differentially selected. Full-length recombinant human tau (4R2N) induced a gel shift in unacetylated $tRNA^{Lys}$ to yield a dissociation constant (Kd) of 460 ± 47 nM. The derived Hill coefficient was 2.8, implying cooperative binding of multiple tau proteins to tRNA. Isothermal titration calorimetry experiments independently confirmed the affinity of tau binding to tRNA (Kd = 735 ± 217 nM). The dissociation constant for 4R2N tau binding to a random 43 nucleotide RNA sequence was similar, suggesting that under *in vitro* conditions, tau effectively and non-specifically binds RNA. The gel shift assay showed multiple bands corresponding to high molecular weight protein-RNA complexes present at different tau:tRNA stoichiometries. The fraction of bound tRNA to 4R2N tau from the low and high molecular weight bands, plotted as a function of tau:tRNA molar ratios represent a range of stoichiometries from 1:1 to 6:1. Thus, multiple tau molecules bind tRNA with increasing tau concentrations and multiple populations with different tau:tRNA ratios coexist. The higher order multimeric structures are likely held together by weak interactions between multiple tau proteins and tRNA.

Along with the recognition of tau as intrinsically disordered, tau shares many properties with other RNA-binding proteins involved in neurodegeneration. These proteins include FUS [10, 65, 73] TDP-43 [11], C9ORF72 [14, 74], TIA1 [75], hnRNPA2B1 and hnRNPA1 [7, 76, 77], all of which can undergo liquid-liquid phase-separation (LLPS) from the surrounding aqueous medium to form droplets *in vitro*.

That tau can bind to RNA in living cells prompted a more detailed quantitative analysis of the *in vitro* conditions under which tau phase separates. We showed that tau-RNA complexation can lead to complex coacervation [1], and we also know from literature and our own experiments that tau can undergo simple coacervation, either when tau is phosphorylated or under high salt conditions. In the complex coacervation process, multiple tau molecules weakly bind RNA, and when overall charge matching is achieved between the polycation, tau, and polyanion, RNA, tau undergoes reversible condensation and liquid-liquid phase separation into micrometer-sized droplets detected as a turbid solution. The spontaneous and reversible droplet formation suggests that tau is held in a fluid state with low energy-barrier between the dilute solution and the complex coacervate condensate, as well as with a modest free energy difference between them, toggled by interactions mediated by ions and hydration water. Key experimentally accessible parameters that tune LLPS were found to be the biopolymer concentration, salt concentration, pH and temperature, crowding pressure, post-translational charge modification of the protein and the presence of biopolymer constituents that engage in multivalent electrostatic interactions, including RNA, single-stranded DNA and intrinsically disordered proteins (IDPs). Droplet formation followed spontaneously from the mixing of two oppositely charged biopolymers, tau and RNA, within a given range of pH, salt and protein concentration, as well as temperature. This process is fully reversible and reproducible,

characteristic of equilibrium states. Complex coacervation of tau as a function of temperature verified the process to be entropy-driven and likely toggled by the release of hydration water—not counterions, as commonly assumed in the literature—that reduces the net excluded volume of the hydrated biopolymer constituents. [78]. Under physiological temperature conditions (37 °C), droplet formation was observed at protein concentrations as low as 2.5–5 μM, in the presence of salt concentrations as high as 100 mM when glycerol was added as a crowding reagent to mimic the intracellular environment. All of the relevant parameters interact to maintain the electrostatic charge ratio at approximately 1:1. Importantly, tau coacervation can occur under physiologic conditions. Tau droplets also demonstrate a low interfacial tension that promotes fusion and coating. This phenomenon is associated with low cohesive energy between hydrated polyelectrolyte complexes and weakly bound water constituents, consistent with high internal fluid dynamics [79, 80]. Tau droplets were clearly visible with a highly spherical boundary, and two droplets frequently merged into one with no visible boundary at the fusion interface left, indicating that the droplets are fluidic with a relatively low interfacial tension. Confocal microscopy images of fluorescence-labeled tau verified that tau was predominantly contained within the droplet.

Within a condensed complex coacervate fluid, held together by non-specific and weak electrostatic interactions, the polyelectrolyte constituents must maintain their hydration layer and remain dynamic which we proved by labeling a tau truncation known as Δtau187 (4R tau 255–441) with fluorescence or spin labels at cysteine site 322. Confocal fluorescence imaging showed that fluorescence labeled Δtau187 was predominantly localized within the droplet. Continuous wave electron paramagnetic resonance (cw EPR) spectral line shape analysis of spin labeled Δtau187 revealed that the protein side chain dynamics and degrees of freedom of the tethered spin label of Δtau187, associated with poly(A) RNA or tRNA, in the dense liquid coacervate phase was like that of spin labeled tau in dilute solution state. In fact, the result of this experiment was quite remarkable, in that the EPR lineshape of labeled Δtau187 within the droplet phase was indistinguishable from that of Δtau187 in the dilute solution state when coacervated with poly(A) RNA or tRNA [1]. In contrast, the cw EPR line shape of spin label at site 322 dramatically broadened within minutes of adding heparin—a highly effective aggregation inducer of tau. Therefore, the condensation of Δtau187 to high protein concentration and formation of long-range associations with RNA within droplets, does not induce dehydration and aggregation of tau as indicated by the retention of the solution state dynamical properties. These results were further supported by doubly spin labeling at tau sites 272 and 285 to access the distances across the PHF6 and PHF6∗ hexapeptide region by double electron-electron resonance (DEER) spectroscopy [82]. This PHF6(∗) region of the tau sequence packs into the β-sheet core once fibrils are formed of tau [81], and hence undergoes a dramatic opening from a compact to a fully extended local conformation, well before fibril formation is observed, and remains extended as they pack into β-sheet structures as part of insoluble fibrils [82]. Dipolar EPR spectroscopy, widely known as dipolar electron electron resonance (DEER), showed that the mean distance flanking the PHF6∗ region of Δtau187 remained unchanged from dilute solution state to when tau was condensed into a concentrated complex coacervate phase in association with poly(A) RNA or tRNA, unless aggregation-inducing factors were added or present. This is in contrast to the conformational change that Δtau187 undergoes, within minutes upon the addition of heparin, of dramatic extension of the mean distance between the labels flanking the PHF6(∗) segment, and hence signifying the opening of the PHF6(∗) region of Δtau187 followed by neat stacking into β-sheets [82].

These studies revealed that tau in the phase separated state can maintain the same conformation as in its solution state, depending on the cofactor and tau variant used, despite the constraints of very high tau concentration and strong interaction and electrostatic association with

RNA. The same studies raise the question whether tau in its droplet state is on pathway to fibril formation. We observed a low-level Thioflavin T (ThT) fluorescence under tau-poly(U) RNA droplet forming conditions that gradually increased over 15 h [1]. When the salt concentration was increased to conditions under which tau-RNA associations are weakened and droplets disperse, the ThT fluorescence was nearly eliminated. However, even after 15 h of incubation, the ThT fluorescence intensity from the tau-RNA droplet samples was significantly less than occurred in the presence of heparin, under similar charge ratio and mass concentration and following a brief incubation time of less than 20 min. ThT fluorescence is commonly used as an assay to detect β-sheet content, suggesting that droplet formation of tau increases its aggregation propensity. Prolonged residence in this phase state may induce β-sheet formation, suggesting that the highly condensed phase state of tau can be a precursor to fibril formation. This observation is consistent with experiments that show liquid compartments of FUS convert with time into an aberrant aggregated state [10]. In the cited study, FUS droplets carrying FUS mutations or aged droplets increased their fusion time when single droplets were manipulated by laser tweezers. Over time some of the droplets had short fibers and others had longer fibers that extended beyond the boundary of the droplet eventually taking on the appearance of a "starburst."

Having observed several tunable conditions in which tau/RNA transitions towards a homogeneous phase or an LLPS state, we characterized the phase diagram of tau/RNA LLPS using experiment and simulation [78]. Using an N-terminus truncated version of the longest isoform of human 4R tau *in vitro*, we showed that tau/RNA complexation is reversible, and tau remains dynamic without a persistent structure within the dense phase. The phase coexistence curve separating a supernatant phase from a condensate phase is determined by the concentration, temperature, salt, and the nature of the interaction strength between the various solution constituents, including the solvent. This work offers a map of the free energy balance between the condensed LLPS and dilute phase, constituting the phase diagram. By constructing the phase diagram from cloud-point measurements at the onset of complex coacervation under varying conditions of temperature, salt, and polymer concentrations we established the features of phase coexistence boundaries which were modeled using theory and simulation. The resulting model supported the experimental observations that the equilibrium window for the complex coacervation of tau and RNA under cellular conditions is narrow. Extrapolating from the empirically determined phase diagram computed from *in vitro* experiments and validated by simulation, we conclude that the narrow window of tau-RNA LLPS likely resides within or near physiologic conditions of 37 °C, 150 mM salt concentration and cellular crowding pressure. The narrow window for LLPS may account for pathological potential of the tau splice site mutations which subtly increase the 4r to 3R ratio, and therefore alter one of the finely tunable elements that control phase separation. Likewise, the extended projection of MAP 2 may prevent its microtubule-binding domain, which is highly homologous to tau from forming condensates vulnerable to fibrillization.

Post translational modifications involve changes in the charge state of tau, again altering the propensity for LLPS, while cellular processes that alter the local pH would similarly affect the LLPS propensity of tau. Fragmentation of tau, or changes in the local concentration of RNA species will also shift the LLPS equilibrium. Thus, our finding that LLPS formation occurs within a narrow range of conditions close to or within cellular conditions imply that there can be a myriad of possible biological conditions that can regulate the formation, dissolution, or in fact solidification and transformation, of the tau droplet state.

The Necessity for Cofactors in Tau Droplet Formation

In vitro tau fibrillation of tau is typically triggered with cofactors, most commonly heparin [69], but also other cofactors such as RNA [70] or arachidonic acid [83]. Seeding tau aggregation utilizes premade fibrils added to fresh monomers, but the seeding process is improved by the presence of cofactors (RNA or heparin) in solution [84, 85]. Heparin is a limiting factor in fibril formation [86, 87], but whether heparin is part of [88] or not part of mature fibrils [87, 89] is unclear. In our work we addressed whether cofactors are crucial constituents of mature fibrils and contribute to their stability, or whether they only catalyze aggregation toward a self-sustained protein assembly [90]. Because seeding is presumed to convert naïve tau monomers into aggregates [91, 92], the hypothesis emerged that *in vivo*, monomeric tau can spontaneously polymerize into filaments when an appropriate seed template is provided. Therefore, in searching for the basis of aggregation, the focus has been on the properties of tau itself, such as hyperphosphorylation, cleavage, high local concentrations, and alternative splicing, but only marginally in abnormal interactions with cofactors.

We have recently shown that mature fibrils made of 4R tau variants, prepared with heparin or RNA, spontaneously depolymerize and release monomers when their cofactors are removed [90]. We next asked if the tau monomers released from the digested fibrils retained the conformation assumed in the fibril. If so, one would expect a much higher seeding capacity of the tau monomers; however the seeding propensity of heparin and RNA fibrils did not significantly differ between digested and nondigested fibrils, suggesting that the monomers released from fibrils are not seeding-active. Therefore, the loss of seeding capacity is due to the loss of fibril content after cofactor digestion and the monomers derived from fibrils have no memory of the fibril state, neither in conformation nor in seeding capacity. Tau may adopt seeding competent, or even seeding active, conformers in solution or oligomer state, but there must be other promoting factors available, not merely its dissolution from tau fibrils. Furthermore the high seeding capacity of transgenic tauopathy mouse brain extracted tau fibrils is exhausted after one generation, while supplementation with RNA cofactors resulted in sustained seeding over multiple generations. We suggest that a polyelectrolyte cofactor is essential for the stability of fibrils prepared in vitro. Targeting of assembly-inducing cofactors represents a unique approach to therapeutics.

Remarkably, it is the same type of cofactor that appears to stabilize tau fibrils and promote the seeding activity of tau fibrils by overcoming the seeding barrier, as well as induce LLPS of tau. We have shown that in the presence of aggregation promoting factors or aggregation-prone tau variants can transform tau from a droplet state to a fibril state. This implies that there is a pathway from tau in solution to the fibril state via the droplet state. However, whether the droplet is an obligatory intermediate state, or even whether the droplet path is a biologically relevant avenue is an open and important question.

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25 Liquid-Liquid Phase Separation of Tau Protein in Neurobiology and Pathology

Susanne Wegmann

States of Tau in the Cell

The microtubule associated protein tau (MAPT) is expressed in neuron of the central nervous system, where is involved in microtubule regulation in the axon. In the adult human brain, 6 isoforms of tau coexist and are generated by alternative splicing of one precursor mRNA. Tau is an intrinsically disordered protein (IDP) with very few transient secondary structural elements, mainly in its microtubule binding region (MTBR) (Fig. 25.1a–c). Interestingly, although tau is one of the most abundant proteins in the CNS, murine knock-out models suggest only minor effects on brain function upon tau ablation. In contrast, the removal of tau appears to have a large positive effect on neuronal health and survival in different diseases associated stress situations.

In a number of neurodegenerative diseases, such as Alzheimer's disease (AD), Frontotemporal dementia (FTD), Pick's disease, and supranuclear palsy, Tau owns a very negative prominence as a mediator of neurotoxicity (likely in its oligomeric state), and the intracellular aggregation of tau is a hallmark of these diseases. Until now it is not clear, how and why the highly soluble intrinsically disordered tau protein transitions into aggregates that often have a highly ordered structure.

Only recently, a new class of dynamic macromolecular tau assemblies has been identified: Liquid condensed phases of tau, which form spontaneously in aqueous solution and have liquid-like fluid properties. These liquid tau clusters contain high concentrations of tau, and they differ from monomeric and from aggregated tau in their biochemical and biophysical properties, and therefore provide a new mode of interaction with other biomolecules and organelles (Fig. 25.1d).

Monomeric (or Dimeric) Soluble Tau

Soluble monomeric tau is intrinsically disordered, which reflects in a floppy molecular structure that is guided by transient intramolecular interactions [1, 2]. The intrinsic disorder implicates that tau is likely involved in a plethora of interactions with other proteins and organelles [3]. Most prominently, tau binds microtubules [4–6], but also actin binding [7, 8], membrane association [9–11], kinase interactions through SH3 and SH2 domains [12, 13], and nuclear pore interactions [14] have been reported.

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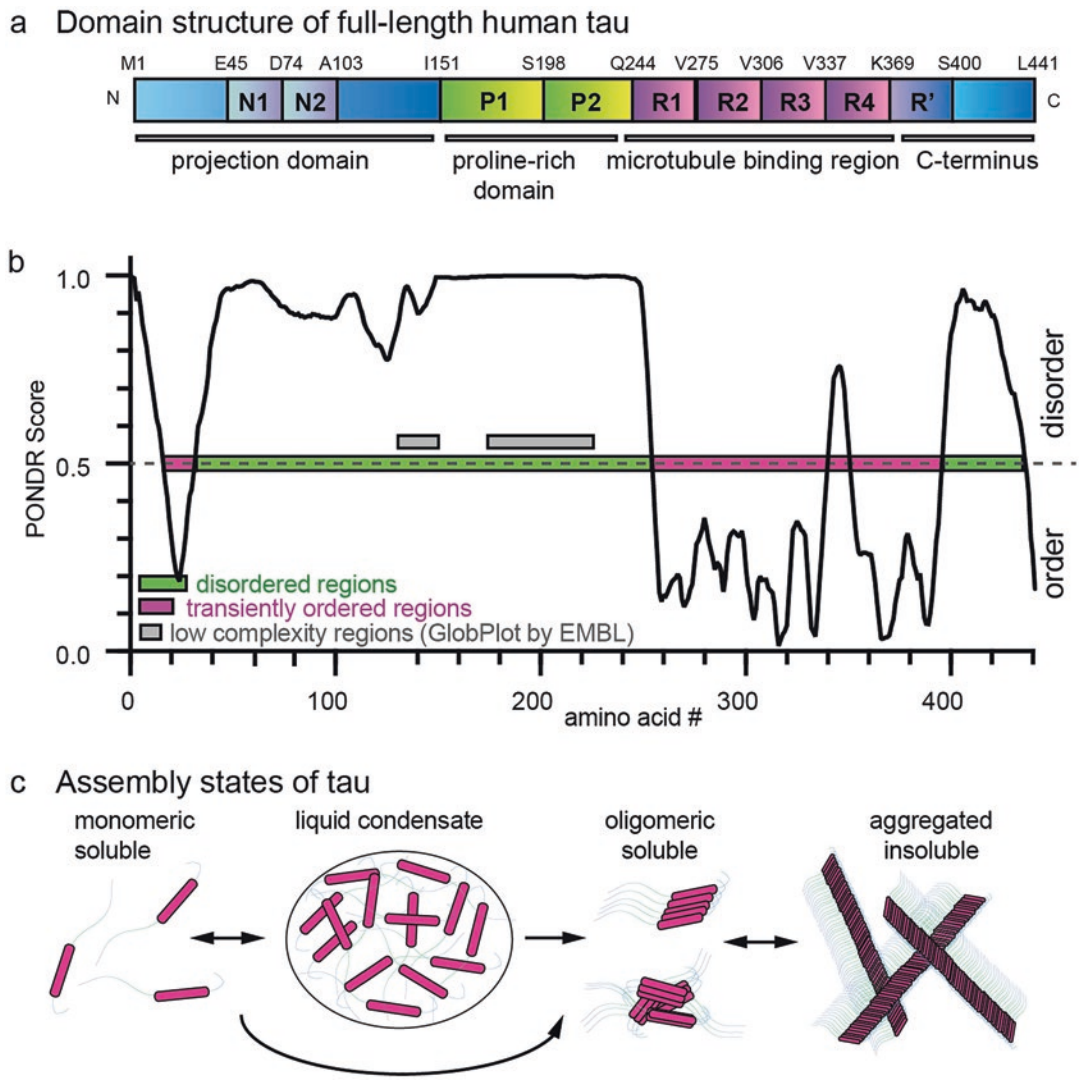

Fig. 25.1 Human tau domains, disorder, and condensation states. (**a**) Sequence and its domain structure of the longest brain isoform of human tau having two inserts (N1 and N2) in the N-terminal projection domain and four pseudo-repeats (R1-4) in the C-terminal half (2N4R; 441 amino acids). Repeats R1-4, and small parts of the proline-rich region P2 and the region R', are involved in the microtubule binding of tau, whereas the unstructured N-terminal half projects from the microtubule surface. (**b**) A protein disorder prediction for full-length tau (2N4R; http://www.pondr.com/) shows that the projection domain, the proline-rich domain (P1+P2), and the C-terminus are mostly disordered (green regions in middle horizontal line), whereas some secondary structure is predicted for the repeat domain (R1 to R4 and R′; pink regions in middle line). The predicted low complexity regions (grey bars; http://globplot.embl.de/) result mostly from high proline content. (**c**) Tau can exist in different assembly states that have different physicochemical and interaction properties: soluble tau monomers (or dimers) can reversibly condensate into liquid dense phases, from which small soluble oligomeric tau assemblies can form; oligomers could also assemble directly from monomers/dimers, and can evolve further into larger fibrillary aggregates

Oligomeric and Aggregated States of Tau

In AD and primary tauopathies, the aggregation of tau into neurofibrillary tangles (NFTs) represents a pathological hallmark. Tau aggregation also occurs in other neurodegenerative diseases such as dementia with Lewy bodies [15], chronic traumatic brain encephalopathy [16], and prion diseases [17]. NFTs contain highly ordered, amyloid-like twisted or straight filamentous tau aggregates, whose structure has been described by electron [18] and atomic force [19] microscopy, and recently was solved by Cryo-EM [20, 21]. The core of these fibrils is assembled from stacks of tau microtubule binding domain (Fig. 25.1a; more details on tau domains in section "Tau protein domains in LLPS"). The *in vitro* assembly of tau aggregates seems to involve oligomeric intermediates [22], and tau oligomers have also been suggested as the toxic species in neurons and *in vivo* [23–26]. As of now, it is not clear if toxic oligomers are *on-path* with larger ordered aggregates of tau *in vivo*. In any case, the mechanism, by which monomeric tau converts into oligomers and/or amyloid-like aggregates is unclear as well; the liquid condensates of tau described in this chapter may provide a possible mechanism for this transition.

Liquid and Gel-Like Phases of Tau

In addition to the monomeric, oligomeric and aggregated phases of tau, another assembly state of tau in the cell has very recently been discovered [27–30]: dense liquid-like tau protein phases, formed by liquid-liquid phase separation (LLPS) of tau from the surrounding aqueous solution, can form spontaneously in a tau concentration dependent manner, or upon interaction with polyanions such as heparin, the microtubule surface, or RNA (references).

These phases can be seen as an intermediate between monomeric/dimeric and oligomeric/aggregated tau species for two reasons: (1) they seem to facilitate the transition between the two mentioned states in that the formation of tau liquid droplets out of monomeric tau solutions can initiate the aggregation of tau, and (2) condensed tau phases (liquid or gel-like) can establish both molecular interactions with single molecules in the inner body and the outer surface of the condensates, and macromolecular interactions with other polymer phases and cellular structures (organelles, filaments, etc.) at their surfaces.

In the following, we will describe the formation and interactions of these condensed tau phases – as far as is known to date – with a focus on these two aspects. The mechanism and biological implication of tau phase separation by coacervation with RNA molecules is covered in another chapter of this book, authored by Kenneth Kosik and colleagues.

Drivers of Tau Liquid-Liquid Phase Separation

Introduction to Protein Phase Separation

The *adhoc* liquid phase of proteins together with RNA in living cells has been first described for p-granules in germline cells of *Caenorhabditis elegans* [31]. In this system, the condensation of PGL-1 protein together with RNA enables the establishment of a transcriptional gradient in the cell, which is essential to break the symmetry during development; similar mechanism exists also for other proteins involved in development of the nematode *C. elegans* [32]. The biophysical process, in which aqueous solutes in a solution spontaneous condensate into separate aqueous phases, is also called liquid-liquid phase separation (LLPS). In the cell, the condensation of proteins (and RNA) into liquid dense phases is used to efficiently and reversibly generate membraneless organelles with a high inner protein concentration that can function, for example, as little nanoreactors to catalyze translation, transcription, or other reactions [33]. A large number of the proteins that are known to undergo LLPS are RNA binding proteins that co-condensate with RNA into liquid droplets under stress conditions to form reversible intracellular stress granules

[34, 35]. The aberrant phase separation and transition into aggregates have been observed for different RNA binding proteins, such as FUS [36, 37], hnRNPA1 [38], and TDP-43 [39] in the context of disease-associated mutations and conditions in amyotrophic lateral sclerosis (ALS). Another ALS condition, the nuclear accumulation of di-peptide repeat proteins derived from C9orf72 hexanucleotide repeat expansions, has been shown to be driven by LLPS and at the same time also to interfere with LLPS of RNA binding proteins [40, 41].

But also proteins with other functions have been shown to undergo LLPS under physiological model conditions *in vitro* and in cells. This includes the synaptic structural proteins synGAP together with PSD-95 [42] and synapsin-1 [43], and the tumor suppressor SPOP [44]. And the assembly of nucleoli, paraspeckles, and heterochromatin, which organize RNA and chromatin in the nucleus and thereby regulate gene transcription, is likely based on LLPS of proteins as well [45–47]. Interestingly, LLPS of another microtubule binding protein, BugZ, has been shown to be important for spindle assembly during mitosis [48].

The molecular base of protein condensation into liquid droplets seems to be encoded in the primary amino acid sequence of LLPS proteins: intrinsically unfolded protein stretches of low amino acid diversity, so called low complexity regions (LCRs), can facilitate the condensation of proteins into liquid dense phases [38]. The basic principle behind the phase separation is governed by the preference of the LCRs to interact with other proteins of its own kind, rather than with the surrounding solvent and biomolecules; thereby, certain protein conformations, or complex formation with other molecules like RNA, may be necessary to expose the LCD in a protein to enable LLPS. The molecular interactions established within the liquid dense phase are thought to be weak (to ensure reversibility and liquid behavior of the phase) and can rely for example on electrostatic or pi-pi, or weak hydrophobic interactions [49].

In many RNA binding proteins, LCRs coincide with prion-like domains (PrLDs or PDLs) that contain aggregation prone sequences and have the ability to self-propagate certain protein conformations and misfolding, similar to yeast prion proteins [46]; for example, PrLDs facilitate both the LLPS and the misfolding of the RNA binding proteins FUS, TDP43 and hnRNPA1/2. Note that lateral infectivity between individuals and species, a hallmark of prion diseases, does usually not occur in proteins with PrLDs.

The amino acid sequence in LCRs of LLPS proteins is enriched in polar amino acids such as serine, glutamine, asparagine, and glycine, with interspersed charged (e.g. lysine, arginine) or aromatic residues (e.g. tyrosine, phenylalanine) [38, 50]. The residue composition determines the phase behavior of LCRs, and with that regulates their function. Accordingly, changes in the apparent sequence or the biochemical character of an LDR, e.g. by mutations [30, 36, 39, 44, 51] or post-translational modification [30, 37, 52], can largely alter the phase behavior of LLPS proteins and either promote or inhibit the formation and stability of the liquid dense phases. External factors that change the interactions involved in the formation of liquid dense protein phases are for example protein concentration, molecular crowding (which changes the apparent protein concentration through excluded volume effects), electrolyte concentration, pH, and temperature.

Tau Protein Domains in LLPS

Tau is an axonal neuronal microtubule binding protein that is very conserved across mammalian species, and has analogues in *C. elegans* [53] and birds [54]. In the human brain, six isoforms of tau co-exist [55] with varying abundance depending on developmental stage [56, 57] and brain region [58]; an exceptional large variant of tau (often called “big tau”) exists in the peripheral nervous system. The isoforms in the central nervous system are generated by alternative splicing of mRNA from a single gene, *MAPT*, on chromosome 17, and vary in the presence of pseudo-repeats (each ~30aa long) in the N-terminal end and the C-terminal half of the protein (Fig. 25.1a): two N-terminal insets (N1+2) encoded by Exon 2

and Exon 3, and four central pseudo-repeats (R1-4), of which R2 (Exon 10) can be alternatively spliced-out to generate the 3-repeat isoforms of tau. The longest human tau variant (2N4R) consists of 441 amino acids, large parts of which have no fixed secondary or tertiary structure, assigning tau to the group of intrinsically disordered proteins (IDPs) [1, 59] (Fig. 25.1a).

Functionally, the sequence of tau can be divided into protein domains according to their function in MT binding: the pseudo-repeats R1-4 build the microtubule binding region (MTBR; aa 244–368); this domain harbors some transient beta-structure with two hydrophobic hexapeptides that mediate tau's aggregation into fibrillar aggregates [60, 61]. The N-terminal projections domain of tau (aa 1–150) and the proline-rich domain (P1+P2; aa 151–243) are unstructured and protrude from the surface of the microtubules. In fibrillar aggregates assembled from full-length tau, the N-terminal half builds a polymer brush around the fibril core [62].

In most LLPS proteins, low complexity or prion-like domains drive their condensation into liquid phases. Tau does not have a clear LCR that is particularly enriched in polar and charged residues [38, 63, 64] - low complexity predictors mostly point out the proline-rich regions (P1+P2)). The aggregation prone region of tau, which could maybe be compared to PrLDs in RNA binding proteins because of its aggregation propensity, is located in the transiently structured MBTR. In fact, tau is generally very rich in charged residues, with regions of positive and negative net charges in the protein that create multivalence along the primary sequence of tau (Fig. 25.1a). Low amino acid complexity (high proline content) exists only in the proline-rich regions.

In the absence of a LCR or PrLD in the tau sequence, predicting which part of tau can undergo LLPS becomes difficult. In fact, different tau protein domains have now been shown to individually be able to condensate into liquid droplets. As of now, it has been shown that full-length tau [29, 30], but also the N-terminal half (aa1–256; [28, 30]) and the MTBR [27, 28]) can undergo LLPS in absence of the other protein domains. It appears that different regions of tau can contribute to the process of liquid-liquid phase separation in an individual as well as in a combined fashion, making the phase separation of tau a complex process.

In addition, the LLPS conditions and characteristics for the different tau domains seem to be very different. For example, the condensation of phosphorylated full-length tau is inhibited by aliphatic alcohols like hexanediol but largely resistant to high ionic strength of the buffer, whereas LLPS of the phosphorylated N-terminal half is inhibited under such conditions [30]. These data suggest that hydrophobic interactions – presumably by two short beta-strands in repeats R2 and R3 of the tau repeat region – may stabilize the liquid phase of phospho-tau, whereby charge interactions in the N-terminal half may help to initiate condensation or have an unknown additional function in the process of LLPS. Un-phosphorylated tau (from E. coli) appears generally more sensitive to high salt conditions. In summary, these different LLPS characteristics indicate a distinct and complementary role of the protein domains in tau LLPS behavior and function, which need to be further explored.

Protein Concentration Critical for Tau LLPS

Tau is one of the most abundant proteins in the brain and is physiological intracellular concentration was suggested to be ~2 μM [30, 65]. This, however, does not take into account local differences in tau concentration that occur in the neuron: tau is almost exclusively found in the axon, where its local concentration is likely much higher, but it has been suggested that at least 50% of tau molecules are bound to microtubules [66]. Due to tau's high solubility, the tau concentrations in cells can reach up to ~100 μM (for example during recombinant protein expression in insect cells) while remaining in a free soluble state. Immunohistological labeling of neurons in culture and *in vitro* microtubule binding experiments with fluorescently labeled recombinant tau show that there are even "hot spots" of locally

enriched tau in the axon, which recently have been suggested to consist of liquid condensed tau phases as well (more information below).

Different independently performed studies reported the condensation of tau into liquid droplets *in vitro* at concentrations between 1–25 μM in the presence of crowding agents [29, 30], which were added to mimic the molecular crowding (~100–200 mg/ml proteins) present in the intracellular environment. Typical crowding agents used in *in vitro* protein condensation studies are for example PEG-8000, Ficoll-400, or dextrans in concentration of 5–15% (w/vol); they increase the apparent protein concentration through excluded volume effects. In the absence of such molecular crowders, the critical LLPS concentration of tau is about one magnitude higher (10–100 μM; [28, 30]). It remains unclear, however, what is the critical tau concentration for intracellular tau condensation, which may in fact be highly dependent on the local subcellular environment (electrolytes, pH, biomolecules, binding partners etc.). In addition, one has to keep in mind that the concentration of tau (and other molecules) in highly differentiated cell like neurons is strongly dependent on the neuronal compartment (e.g. soma, dendrites, axon, nucleus, synapse) (Fig. 25.2a).

Phosphorylation of Tau

An important finding is that tau phosphorylation reduces the critical LLPS concentration of tau by an order of magnitude. Phosphorylation (and other PTMs) may thus be an important regulator not only of tau microtubule binding and aggregation, but also of tau condensation. For example, minor phosphorylation by the kinase MARK2 at 4 sites in the MTBR, which has been shown to catalyzes the detachment of tau from microtubules [67, 68], is sufficient to induce condensation at tau concentrations of 1–2 μM [28, 30] (Fig. 25.2b). Interestingly, the phosphorylation of tau by MARK2 was shown to increase the local beta-structure content in the tau repeat domain; these data support the idea that beta-strand interactions may enhance tau condensation. Higher levels of phosphorylation at multiple sites in tau (~12 phosphates per tau molecule) from insect cells increases the LLPS rate and decreases the critical tau protein concentration even more [30]. On the other hand, the addition of 17 negative charges in a phospho-mimetic of tau, which mimics the charge changes introduced by phosphorylation of tau, can also facilitate tau LLPS [30]. This shows that changes in the molecular conformation, as well as charge compensation or addition, are favoring the condensation of tau into liquid phases. Charge compensation through phosphorylation followed by conformational changes/misfolding have previously been implicated in the oligomerization of tau [69]. Liquid tau condensates may thus be resemble a macromolecular state between soluble and aggregated forms of tau.

In healthy cells, phosphorylation by multiple kinases (e.g. MARK2, GSK3b, Fyn, CDK5, PKA, and JNK) is the main regulator of tau's functions such as microtubule binding. In fact, tau in neurons is mostly phosphorylated. Accordingly, liquid-like tau condensates can also be found in primary neurons expressing GFP-tau [30], and several conditions that are known to trigger tau phosphorylation may also cause tau condensation. For example, several neuronal stress situations associated with neurodegenerative diseases, such as mechanical injury [70], chronical stress enhancing glucocorticoids and ROS [71], ER stress [72], and the exposure to Amyloid-beta [73], acutely enhance tau phosphorylation. Interestingly, tau LLPS – at least *in vitro* – seems to be inhibited at low temperatures [27, 28], whereas tau phosphorylation has been shown to increase in hibernating animals during torpor [74]. These data suggest a complex relationship between tau phosphorylation and condensation, in which functional and dysfunctional LLPS might be differentially regulated. The regulation of tau LLPS by phosphorylation (and other PTMs) could thus be an important switch between function and disease.

At the moment, however, *in vivo* evidence for tau condensates in rodent models or human post-mortem tissue is still very sparse, mainly because of technical difficulties in characterizing the liquid state in living animals or fixed tissue. As a first approach to this challenge, we recently were

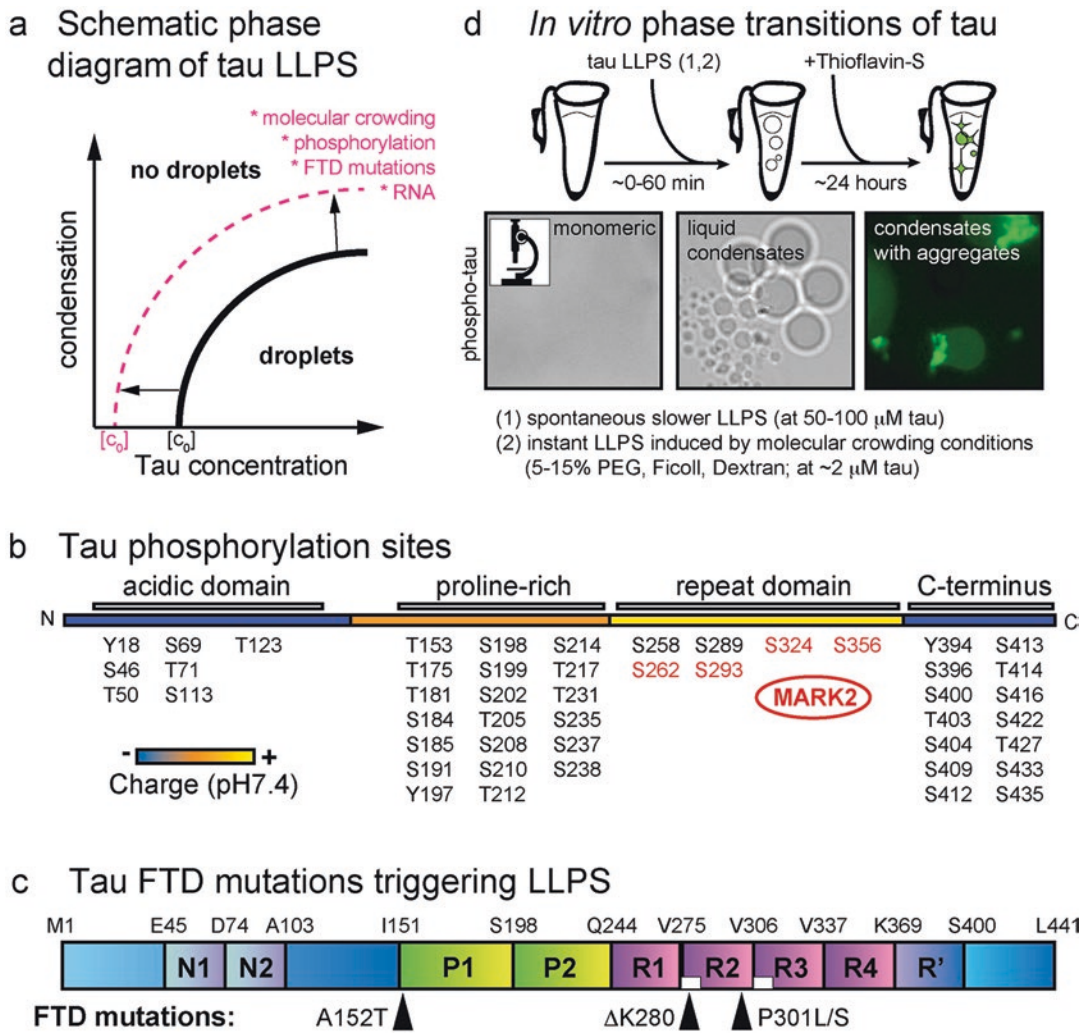

Fig. 25.2 Conditions enhancing tau liquid-liquid phase transition. (**a**) Schematic phase diagram of tau LLPS: the phase separation of tau, which leads to the formation of liquid "droplets", depends on the concentration of tau protein and other soluents (e.g. salts, solvents, biomolecules, crowding agent) in the solution. Phosphorylation, FTD mutations, RNA, and molecular crowding lower the critical tau LLPS concentration [c0] and enhance tau condensation into liquid droplets (pink line). (**b**) Phosphorylation sites are distributed along the sequence of tau but enriched in the proline-rich region and in the C-terminus. In contrast to most kinases (e.g. GSK-3beta, CDK5, Fyn), kinase MARK2, which catalyzes the detachment of tau from microtubules [68], phosphorylates tau at four sites in the repeat region (red sites). This minor phosphorylation by MARK2 is sufficient to enable tau LLPS at a physiological concentration of 2 μM [30]. (**c**) FTD mutations, which are known to increase the aggregation and oligomerization propensity of tau, significantly decrease the critical concentration necessary for tau LLPS to physiological concentrations (~2 μM). The small white rectangles represent two hexapeptide motifs that mediate the aggregation of tau; their beta-structure propensity and hydrophobicity is enhanced by FTD mutations in this region [60]. (**d**) Phase transition of tau in vitro, from monomeric to liquid condensates to aggregates, can occur spontaneously or can be initiated at ~50-fold lower, physiological tau concentrations by using polymeric crowding agents. Over time, liquid droplets of phospho-tau form small aggregates on their surface that can be labeled with Thioflavine-S, suggesting beta-sheet content of the aggregates

able to detect first signs of droplet-shaped tau accumulations in the mouse cortex by *in vivo* two-photon imaging, and could initiate condensation of tau isolated from AD brains *in vitro* [30].

Frontotemporal Dementia Mutations

In Alzheimer's disease, tau aggregation is associated with hyperphosphorylation and likely occurs due to changes in the intramolecular interactions and protein conformation caused by aberrant post-translational modification. In contrast, in frontotemporal dementias (FTDs), mutations in the tau gene (*MAPT*) drive the aggregation of tau, and several point mutations have been identified in patients that trigger the aggregation in tau *in vitro* [60, 75]. The molecular mechanism for some of these mutations has been identified: Aggregation-prone mutations in the tau repeat domain – such as P301L, P301S, and DeltaK280 – seem to increase the beta-structure propensity of a hexapeptide motif in R2 (*PHF6**), which increases the local hydrophobicity and favors the assembly of tau into amyloid-like fibrils [76].

Interestingly, the same mutations also enhance the condensation of tau into liquid phases. More precisely, they substantially lower the critical concentration of non-phosphorylated tau to undergo LLPS *in vitro* (down to <2 μM; [30]). Another FTD mutation located in the N-terminal projection domain of human tau, A152T, which has been shown to enhance oligomerization but not aggregation of tau *in vitro* [77] and cause functional deficits *in vivo* [78–80], also facilitates tau LLPS at low concentrations. These findings provide further intriguing evidence that tau LLPS may be "on-path" with tau oligomerization; tau oligomers are currently considered most responsible for tau induced neurotoxicity (Fig. 25.2c).

RNA and Polyanions

A class of very potent inducers of tau LLPS are polyanionic molecules: tau condensates form spontaneously in the presence of RNA [27, 30] and heparin [28, 30], without the need of molecular crowding agents. RNA and heparin are commonly used as polyanionic cofactors to induce the aggregation of recombinant tau, connecting the concepts of tau liquid phase separation with tau aggregation. Notably, the binding of RNA to tau and the RNA-induced tau LLPS may have functions in the regulation of RNA transcription that are not fully understood yet (see Chap. 26 by Benjamin Wolozin and Chap. 24 by Kenneth Kosik in this book for more details).

Phase Transition from Tau Liquid Condensates into Aggregates

Several RNA-binding proteins that transiently condensate into liquid phases under certain conditions have been suggested to also be involved in the pathobiology of motor neurons in ALS. In the presence of disease-associated mutations or aberrant PTMs, liquid-like protein condensates of FUS [36], TDP-43 [39] and hnRNP [38] can transition into aggregates. Such phase transition from a liquid into an aggregated state has also been described for tau [28, 30].

Maturation of Condensed Liquid Tau Phases

The spontaneous formation of liquid-like condensates of recombinant tau in the presence of crowding agents is an instant process. "Young" droplets (~0–15 min old) show several liquid-like characteristics, for example they readily fuse into larger ones and show surface wetting behavior. However, already after ~15 min, the fusion of individual tau condensates seems to be hindered, and after ~1 h, the condensates start to lose their perfect spherical symmetry and spots of deformed solid-like material start to appear on the surface of the condensates [30].

Fluorescence recovery after photo bleaching (FRAP) shows that this "maturation" of liquid tau condensates is accompanied by a rapid (in minutes) decrease in the exchange of tau molecules between the droplet phase and the surrounding buffer, as well as with a decrease of tau

diffusion rate in the droplets [30]. In fact, after a few hours, the condensed phase of tau can be enriched by centrifugation. Stiffness and viscoelasticity of the tau droplets during maturation could for example be quantified using atomic force microscopy and spectroscopy; from such first measurements (unpublished data) it seems that, with time, the liquid state of tau condensates autonomously evolves into a viscoelastic hydrogel-like state, similar to what has been described for the polymerization process of gels. Investigating the dynamic molecular rearrangements of tau condensates during maturation is one of the current aims in the field and will be needed to understand the molecular mechanisms of phase evolution from liquid-like tau phases into aggregates.

Emerging of Tau Aggregates from Condensates

The condensation into liquid droplets leads to a high local concentration of tau in the condensates. Calibrated fluorescence imaging of fluorescently labeled recombinant phospho-tau in the presence of crowding agents and the measurement of tau protein concentration in the condensed phase (after centrifugation) suggest a tau concentration of >30 μM in the liquid dense phases [30]. This concentration is similar to concentrations commonly used in *in vitro* tau aggregation assays (20–50 μM). It is not surprising that protein aggregates emerge from the condensates already 1 h after their formation, and that more and more spherical condensates transition into amorphous aggregates over time. Interestingly, although these tau aggregates do not show the well-described ordered structure of tau paired-helical filaments (PHFs), they harbor the potential to seed tau aggregation in cells [30]. The formation of seeding-competent tau aggregates from tau condensates implicates an intriguing connection between tau phase separation and pathological aggregation. However, at the moment it remains unclear to what extend tau LLPS plays a role in neurodegenerative diseases with pathological tau aggregation (Fig. 25.2d).

Potential Roles for Tau LLPS in Neurodegenerative Diseases

Ignition of Tau Aggregation by Liquid-Liquid Phase Separation

Tau aggregation is one of the main pathological changes in AD and tauopathy brains. Until now, the role of tau liquid dense phases for tau neuropathological changes observed in AD and tauopathies is uncertain. However, there is strong evidence that tau LLPS – at least *in vitro* – can be *on-path* with tau aggregation (Fig. 25.1d), suggesting a possible connection of these two processes. In fact, multiple observations related to tau in neurodegenerative diseases could be explained by dysfunctional phase separation in these diseases. For example, intracellular and/or physiological differences between brain areas and neuronal subpopulations could lead to differential tau condensation, and thereby explain the observation that tau aggregation occurs in distinct brain areas in AD. In other tauopathies with a less "organized" tau aggregation pathology, such as FTD, the condensation of tau, which is enhanced by tau mutations that lower the threshold concentration for LLPS [30], could occurs more spontaneously and independently of the neuronal population. This could explain the different pattern in tau aggregation between AD and other tauopathies.

On the cellular level, intraneuronal tau condensation may, for example, occur as a consequence of tau mislocalization into the somato-dendritic compartment, where it encounters a different local environment that may favor its LLPS. Another possibility is that aberrant post-translational modifications of tau (hyperphosphorylation, acetylation, ubiquitination, SUMO-ylation, truncation etc.) increases its tendency for tau LLPS in the axon.

Tau has also been shown to be associated with neuronal stress granules, which are reversibly assembled, membrane-less organelles formed by LLPS of RNA-binding proteins and RNA molecules under stress conditions in neurons [81]. A co-partitioning of tau in these granules has been suggested to be involved in tau-mediated toxicity

in transgenic mice [82], however, independent of tau aggregation.

Tau Protein Propagation Through Condensation in the Brain?

The progressive deposition of aggregated tau in the brain is a hallmark of tauopathies and AD. In sporadic AD (sAD), the tau neurofibrillary tangles (NFTs) pathology follows a very stereotypical pattern: NFTs start to form in the locus coeruleus and the entorhinal cortex, then appear in the hippocampal formation from where they "spread" through limbic areas to the forebrain and other cortical areas [83, 84]. Neuronal loss and cognitive decline in patients with AD correlates with the progression of NFT pathology. One main working hypothesis for the mechanism of tau progression in sAD is based on the trans-synaptic propagation of tau proteins between neurons [85]: misfolded tau travels from a neuron to downstream connected neurons, where it "seeds" the misfolding and aggregation of tau, which then leads to NFT formation in the recipient neuron, and continues to propagate further to other synaptically connected neurons; this hypothesis nicely explains the occurrence of tau aggregation in sAD in brain regions that are connected by major neuronal projections. However, the cellular mechanism of tau protein propagation between neurons is still unclear, and different possibilities are discussed. Tau could propagate either by general release and uptake in by neighboring cells, or across synapses by pre-synaptic release followed by uptake at the post-synapse. The release at the synapse has been shown for tau associated with synaptic vesicles [86], tau associated with exosomes [87, 88] or ectosomes [89], or by unusual protein secretion depending on lipids in the synaptic membrane [90]. Furthermore, tau propagation appears to be enhanced by the presence of amyloid-beta [91] and by neuronal activity in mice *in vivo* [92]. The species of tau released from neurons are discussed as well, and propagation has been reported for non-misfolded full-length tau [93], for misfolded tau [94–97], and for soluble oligomers of tau [98, 99]. An unexplored possibility is that matured liquid- or gel-like tau condensates, which seem to be stable outside of cells at least *in vitro* [30] and in in neuronal cultures, could be released and internalized by neurons.

For the uptake of tau, multiple mechanisms have been suggested to play a role depending on the state of tau: whereas neurons *in vitro* internalize monomeric soluble tau by Clathrin-mediated endocytosis [100], the uptake of pre-aggregated tau seems to involve heparan sulfates on the cell surface and to be facilitated by pinocytosis [101] or bulk endocytosis [99, 100] of tau. Largely unexplored is the process by which the uptake of tau (not associated with synaptic vesicles) could happen at the pre-synapse.

In context of tau liquid condensates [30] and/or tau associated with stress granules [81], one can imagine different mechanisms for the propagation of tau aggregation pathology in the brain, which do not rely on the physical travel of tau proteins between neurons. One could speculate that the progressive accumulation and aggregation of tau may be a consequence of neuron-intrinsic changes manifesting in certain neurons during the disease and favoring an aberrant condensation and subsequent aggregation of tau in these neurons. However, the intrinsic neuronal changes involved in aberrant LLPS or stress granule association in this model are speculative as well, and the signal that causes the changes may as well be tau protein that was received by neuron-to-neuron protein propagation.

Condensed Tau and Microtubules

The most prominent and best studies role of tau is the stabilization of microtubules in the axon. In fact, MAPT was discovered during co-purification of tubulin from the brain, as a co-factor enhancing tubulin polymerization into microtubules [102]. In the context of tau protein liquid phase separation, it is obviously important to examine if tau condensation processes are involved in the binding, bundling, and nucleation of microtubules.

Tubulin Co-condensation with Tau Nucleates Microtubules

Tau phosphorylation (by certain kinases at certain sites) regulates the microtubule binding of tau, is relevant for the regulation of microtubule dynamics [103], and is also discussed for the regulation of transport along on microtubules [104]. Accordingly, disease associated hyperphosphorylation [105] or mutations [75] decreases the microtubule binding of tau, thereby causing transport deficits and neurotoxicity. Tau can also nucleate microtubules *in vitro*, and only recently it was shown that the nucleation involves liquid phases of tau [29]. Non-polymerized tubulin co-partitions into preformed tau liquid droplets, where it reaches the critical concentration for microtubule nucleation, and minutes after the addition of GTP, microtubules start growing out bidirectionally from of the tau condensates, whereby the growing microtubules remain "coated" by a liquid phase of tau. This mechanism of *a priori* nucleation of microtubules could be important for local non-centrosomal microtubule growth and organization in the axon at sites of need, for example after injury, or during axon growth and branching. Furthermore, the liquid phase of tau seemed to reversibly bundle microtubules, which could be an important mechanism for microtubule stability in cells. Liquid-like Tau phases on microtubules.

Liquid Tau Phases on Microtubules

A more close view on the association of liquid tau phases with preformed microtubules *in vitro* showed that actually very little tau (~0.5 nM) is needed to form small local tau condensates on microtubules [106]. A similar inhomogeneous "coating" of microtubules by tau can also be observe in the brain of mice [107]. Interestingly, the local dense condensates seem to form preferentially at areas of high microtubule curvature, and one can imagine a mechanism for repair or stabilization of microtubules after forced bending or during axon branching. Furthermore, in the same study, the tau condensates appeared to selectively decrease the migration of some but not other motor proteins along microtubules, suggesting a mechanism for how tau may regulate the microtubule transport of cargo.

Methods Used to Study Tau Protein Condensation

To characterize the transition of tau between its different condensation states – soluble monomeric, liquid dense condensates, gel-like dense condensates, small soluble oligomers, and larger aggregates – one is confronted with various molecular sizes, interactions and dynamics. For example, highly soluble intrinsically unfolded tau monomers have minor amounts of transient structure and a large amount of interaction partners, whereas tau PHFs are larger by at least 2–3 magnitudes, have a highly ordered rigid amyloid-like structure (in their fibrils core), and presumably a limited amount of interaction partners. Tau liquid condensates can, to some extent, be seen as an intermediate between these two states: tau molecules remain somewhat mobile but with a reduced diffusion coefficient compared to free monomeric tau, the conformational sampling of tau in the condensed phase is more restricted than in its monomeric freely moving form, and tau molecules establish many transient interactions with themselves and with other constituents of the liquid phase.

To describe the characteristics of all the states that tau can adopt – monomers, liquids, oligomers, aggregates -, many different protein biochemical and biophysical methods have to be employed. The following techniques were used to describe the evolution of tau condensates, their liquid state, and their maturation into gel-like phases and aggregates: Light microscopy can be used to identify droplet-like tau condensates in solution and to describe their fusion/fission and size distribution; it is limited though to droplets with diameters larger than the diffraction limit [29, 30]. Optical tweezers can be used to measure the forces occurring during tau droplet fusion [29]. Bulk measurements of dynamic light scattering and turbidity can detect the formation of tau condensates in a solution [27, 28]. Nuclear

magnetic resonance and circular dichroism measurements were used to determine changes in the interactions and molecular structure of tau in the free soluble versus the condensed phase [28]. Electron microscopy and AFM could produce images of tau liquid condensates and aggregates, and *in vitro* aggregation assays could proof the formation of tau aggregates [27, 28, 30] from recombinant tau and in cells [30]. In future, the use of fluorescence life-time imaging and novel time-resolved light-scattering and x-ray techniques promises to give more insides into the dynamic structural changes during the maturation of tau condensates into aggregates, both *in vitro* and in cells. For the examination of tau condensation processes and their consequences in the brain, two-photon imaging in combination with calcium imaging and electrophysiology in different animal models, for example in *C. elegans* but also in rodent models, appear to be promising approaches to study the role of tau LLPS *in vivo*.

Conclusion and Outlook

The wide-spread biological relevance of protein condensation into dense phases with liquid- or gel-like biophysical properties has recently been discovered. In the case of tau, the roles of LLPS (and the resulting liquid dense condensates) for "normal" and pathobiological tau functions are still quite unknown. However, the concept of tau liquid phases opens up another level of complexity in tau biology, in which interactions, reaction kinetics, and molecular behavior differ from processes involving mono-molecular tau. All the different physiochemical states in that tau can exist in – monomers/dimers, small oligomers, larger aggregates, and now liquid phases – enable tau to establish different sets of interactions at individual time-scales, depending on their molecular size and their intrinsic conformational sampling rate. This may enable us to explain observations that are not yet understood. The liquid phase of tau offers us additional possibilities to explain the mis-regulations of tau in diseases like AD and tauopathies, and it should encourage us to rethink some of the long-standing paradigms in the context of LLPS. For example, enhanced LLPS in neuronal stress situations (elevated phosphorylation) or over-stabilized droplets of FTD-mutant tau could result in tau aggregation and thereby explain the enigmatic transition from highly soluble to ordered aggregated states of tau in the brain (Fig. 25.3). In another example, the aberrant regulation of tau LLPS on microtubules could lead to failures in motor protein processivity and, hence, mitochondria or other cargo transport observed in neurodegenerative diseases. Additionally, altered co-phase separation of other (yet unknown) biomolecules into tau liquid phases may result either in local microtubule destabilization, or in too high (or low) local concentrations of certain essential molecules; this, in turn, can deregulate molecular pathways by locally changing the reaction equilibrium in the axoplasm. Furthermore, tau LLPS characteristics likely depend on local distinct conditions in the cytoplasm, and thus may differ in the different cellular compartments (soma, axon, dendrites, synapses). While tau LLPS may be needed for functional microtubule bundling and nucleation in the axon, the condensation of tau in the soma could result in aggregation or other misfunctions of tau. For example, we recently discovered that phosphorylated tau, when in the soma and perinuclear space as in AD neurons, can interact with nucleoporins in the pore of the nuclear pore complex – another physiological liquid/gel phase in the cell – and that this interaction causes an impairment of nucleocytoplasmic protein transport [14]; this interaction may involve co-condensation of tau with FG-nucleoporins.

To conclude, it is now on us and others to experimentally test the hypotheses around functional and dysfunctional tau LLPS, and to evaluate the role of tau condensation in the healthy and the diseased brain.

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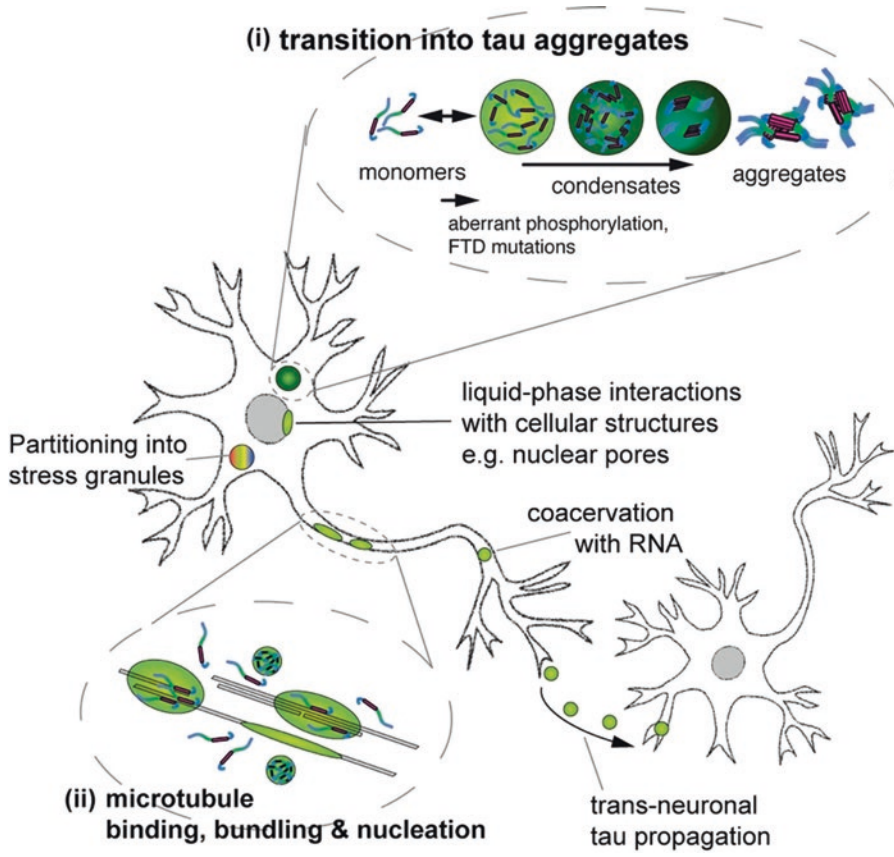

Fig. 25.3 Possible roles of neuronal tau condensation. Tau liquid-liquid phase separation could have multiple roles for the neuronal cell biology. There is evidence that, in the pathological setting of AD and tauopathies (i), tau phase separation is enhanced by hyperphosphorylation or FTD mutations and can initiate tau aggregation [30]. In the healthy neuron, tau liquid condensates can form on microtubules and facilitate efficient bundling and nucleation at sites needed (ii) [29]. Partitioning of tau into stress granules, which assemble through LLPS of RNA-binding proteins with RNA, could change the stress granule function and/or trigger tau aggregation [81]. The efficiency of coacervation of tau with RNA depends on the type of RNA [27]; tau-RNA condensates may, for example, be involved in RNA transport, transcription, and stability. More generally, co-condensation or partitioning of tau into other liquid-like condensates could lay the foundation for "unusual" interactions of tau, such as the interaction of phospho-tau with the hydrogel formed by FG-domain nucleoporins in the pore of the nuclear pore complexes [14]. Lastly, tau condensates could also be involved in the trans-neuronal propagation of tau between neurons in the brain. In this context, the travel of seeding-competent tau condensates could, for example, play a role for the spread of pathological tau aggregation in neurodegenerative diseases such as AD

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The Pathophysiology of Tau and Stress Granules in Disease

26

Anna Cruz, Mamta Verma, and Benjamin Wolozin

Introduction

Stress and the stress response are fundamentally central features of any disease. Stress and the stress response are also clearly central features in the pathophysiology of tauopathy and the biology of AD. Chapters 16, 23, 28 and 29 in this book address how stress modifies the phosphorylation of tau, the role of stress in the biology of the endoplasmic reticulum and the translational stress response, and the effects of behavioral stress and glucocorticoids on tau. The stress response is undoubtedly necessary to cope with the harm arising from chronic exposure to β-amyloid (Aβ), oligomeric tau and age or disease related reductions in cerebral blood flow. However, this chapter will address the paradoxical concept that particular elements of the stress response mediated by RNA binding proteins actually accelerate tau oligomerization and thereby accelerate disease progression in tauopathy. Conversely, we will also show how RNA binding proteins are therefore key targets for therapeutic intervention in tauopathies, including Alzheimer's disease.

Dysfunction of RNA Binding Proteins in Neurodegenerative Disorders

Reviewing the basic biology of RBPs, stress granules and the translational stress response is necessary in order to understand how and why tau would interact with RBPs. An increasing number of genetic studies show that RNA binding proteins (RBPs) are central to the pathophysiology of multiple neurological disorders. Mutations in genes that encode RBPs cause motor neuron diseases such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), multisystem proteinopathy (MSP), and frontotemporal lobar degeneration (FTLD) [40]. ALS is the most common motor neuron disorder that leads to progressive loss of upper and lower motor neurons, muscle weakness, atrophy and ultimately death [78].

TAR DNA-binding proteins 43 (TDP-43) is the major constituent of pathological

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ubiquitinated inclusions present in ALS and frontotemporal dementias defined by progranulin haploinsufficiency (FTD-TDP-43 or FTD-U) [69]. Mutations in TDP-43 also cause rare forms of familial ALS, which demonstrates that dysfunction of TDP-43 is sufficient to cause disease [83]. TDP-43 is not the only RBP to exhibit mutations linked to ALS, though. Multiple RBPs exhibit mutations that are genetically linked to ALS: heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1), fused in sarcoma/translocates in liposarcoma (FUS/TLS), Ewing's sarcoma breakpoint region 1 (EWSR1), TATA-box binding protein associated factor 15 (TAF15), Matrin3 (MATR3), and TIA1 cytotoxic associated granule binding protein (TIA1) [40, 53] (Table 26.1). In addition, polyglutamine (polyQ) expansions (27–33 Qs) in Ataxin-2 (ATXN-2) are an important genetic risk for ALS [19]. RBPs are generally defined by the presence of a homologous RNA binding domain, the RNA recognition motif (RRM); many RBPs also share homologous low complexity Gly-rich domains (LCDs) [31, 53]. FUS, EWSR1, and TAF15, which comprises the FET family, also share common zinc finger domains [31]. The RRMs allow the binding of these proteins to DNA and RNA in a sequence-specific manner, while the LCD is a prion-like domain that plays a critical role in the formation and the dynamic biophysical state of stress granules (SGs) [30]. These RBPs are multifunctional RNA processing proteins that predominantly reside in the nucleus and are generally expressed in many different types of cells and tissues [40].

RNA Binding Proteins Mediate Disease Through Stress Granules

The identification of disease-linked mutations in the genes that encode these RBPs, particularly TDP-43 and FUS, introduced a paradigm shift in the study of ribostasis and proteostasis in disease. Under physiological conditions, TDP-43 and FUS localize in the nucleus. But, in the presence of a cellular stress, they redistribute from the nucleus to the cytoplasm where they associate with SGs (Fig. 26.1). These granules serve as a cytoprotective mechanism against stress as it temporarily inhibits the translation of non-essential mRNA and promote the translation of transcripts necessary for cell survival [41, 49]. However, mutations in TDP-43 and FUS increase the propensity to aggregate, leading to the accumulation of persistent cytoplasmic SGs, and the formation of pathological inclusions of these proteins in the human brain [9, 16, 48]. Support for the hypothesis that the pathological inclusions in brain derive from SGs comes from observed colocalization with SG markers such as eIF3, eIF4G, TIA1 and PABP [48, 16].

This chapter will focus on SGs because the relationship to disease is clear, SGs provide a linear pathway between chronic stress and disease, and the disease-associated RBPs examined to date have been shown to co-localize with SGs. However, RBPs form many types of RNA granules to mediate many different functions, including splicing, transcription, ribosome genesis, RNA transport, RNA degradation, RNA translation, viral defense and many other functions. The pathophysiological principles linking SGs to disease presented below might also apply to these other types of RBPs, particularly for familial RBPs that exhibit mutations rendering the RBPs more aggregation prone. However, differing types of RNA granules likely differ in their propensity to precipitate the irreversible protein aggregation that causes disease because of differences in the types of proteins associated with each RNA granule type and/or differences in post-translational modifications, as discussed below. SGs currently appear to represent the RNA granule exhibiting the greatest propensity to elicit the irreversible protein aggregation associated with neurodegenerative diseases.

Many of the mutations in FUS are thought to increase cytoplasmic localization by disrupting the nuclear localization signal, which prevents the transportation of these proteins to the nucleus [16, 71, 94]. The disease associated mutations in TDP-43 occur predominantly in the LCD domain, increase the tendency to aggregate, and formation of cytoplasmic SGs [48, 38]. In most cases,

Table 26.1 Physiological and pathophysiological functions of RBPs and non-RBPs implicated in neurodegenerative diseases

	Proteins	Functions	Contributions to pathophysiology of neurodegenerative diseases
Nucleating RBPs in stress granules	**T-cell intracellular antigen 1 *(TIA1)***	Primarily a nuclear protein that is involved in RNA metabolism and in the assembly of cytoplasmic SGs in response to cellular stress.	LCD mutations in TIA1 are associated with ALS and myopathies. The mutations accelerate TIA1 aggregation, and promote formation of TDP-43 pathology.
	Fragile X mental retardation protein *(FMRP)*	An mRNA-binding protein present in the neuronal cell body, proximal dendrites, and axons. It mediates translation and mRNA trafficking, including dendritic localization.	Moderate expansion of CGG nucleotide repeats in FMR1, the gene coding for FMRP, are linked to FXTAS. Major expansions silence cause FXS and silence expression.
	Tristetrapolin *(TTP)*	Member of a family of proteins containing tandem CCCH zinc fingers. It is responsible for regulating mRNA dynamics such as stabilizing mRNA, regulating mRNA decay, mediating posttranscription, and regulating TNF-α transcript.	TTP positive inclusions in the cytoplasm increase with disease state, but observed to co-localize with phospho-tau only in late stage diseased brains of transgenic mice expressing human P301L mutant 4R tau isoform.
	Ras GTPase activating binding protein *(G3BP1 & 2)*	A cytoplasmic RNA binding protein that functions in cell proliferation and RAS signaling pathway, and regulates protein nuclear localization through its NTF2 –like domain.	G3BP cytoplasmic granules increase in number and size with disease severity in the brain of transgenic mice expressing human P301L mutant 4R tau isoform.
Secondary RBPs in stress granules	**TAR DNA-binding protein *(TDP-43)***	Primarily a nuclear protein that binds to DNA and RNA and is involved in RNA metabolism, microRNA biogenesis, apoptosis, and cell division.	TDP-43 is a major constituent of pathological, ubiquitinated inclusions present in ALS, AD and some FTDs.
	Fused in Sarcoma *(FUS)*	A member of FET family and primarily binds to DNA, RNA, and splicing factors, and regulates DNA damage-repair, transcription, and splicing.	FUS mutations are linked to ALS and result in the cytoplasmic aggregates of FUS in ALS, FTD, and polyglutamine diseases.
	Ataxin-2 *(ATXN2)*	A cytoplasmic protein that is involved in multiple RNA processes including RNA metabolism, splicing, and degradation. It also plays a role in endocytosis, mTOR signaling, and in mitochondrial functioning.	ATXN2 with more than 34 contiguous CAG repeats are associated with SCA2, while repeat lengths between 27-33 increase the risk of ALS.
	Survival of motor neuron *(SMN)*	Functions in the assembly of snRNPs and in the formation of 3'-end of histone mRNAs. It mediates the localization of mRNA and RBPs, and serves as a molecular chaperone for RNA complexes.	Disease-associated mutations in SMN1 gene locus result in the reduction of SMN1 protein levels causing the molecular defects associated with SMA.
	Heterogenous nuclear ribonucleo-protein *(hnRNPA2/B1)*	An RBP associated with pre-mRNA in the nucleus and is responsible for pre-mRNA metabolism and transport. It primarily binds to UAG[G/A] motifs in 3' UTR.	Mutations in the LCD of hnRNPA2/B1 accelerate aggregation, promote hnRNPA2/B1 sequestration into cytoplasmic SGs, and lead to aggregates in multisystem proteinopathy and ALS.
	Ewing Sarcoma RBP1 *(EWSR1)*	A member of the FET family that is primarily localized in the nucleus for most cell types. It is involve in RNA transcription, processing, and DNA repair.	Mutations in EWSR1 are associated with ALS. Motor neurons from sporadic ALS patient samples exhibit a cytoplasmic distribution of EWSR1 that can be diffuse or localized to punctate granular structures.
	TATA-box binding protein associated factor 15 *(TAF15)*	A member of the FET family and also involve in RNA processing. Interacts with RNA polymerase II and associated with TFIID complexes to take part in gene transcription.	In FTLD-FUS or ALS, TAF15 co-localizes with FUS, where it occurs in cytoplasmic inclusions in neurons and glia, as well as in dystrophic neurites of neurons.
Non-RBPs	**Caprin-1**	A cytoplasmic phosphoprotein that is ubiquitously expressed and is necessary for the progression of cells in the G1-S phase of the cell cycle . It is also present in mRNPs of postsynaptic granules in neuronal dendrites.	Caprin-1 overexpression induces eIF2α phosphorylation which stalls protein translation and drives the formation of cytoplasmic SGs.
	Histone deacetylases 6 *(HDAC6)*	A histone deacetylase that primarily resides in the cytoplasm where it binds with non-histone proteins. HDAC6 is important for cytoskeletal regulation and the stress response.	In AD, HDAC6 co-localizes with tau, which augments tau toxicity. While, in PD, HDAC6 promotes sequestration of α-syn toxic oligomers, which is cytoprotective to dopaminergic neurons.
	Sirtuin 6 *(SirT6)*	A nuclear sirtuin that plays essential roles in cellular stress response, energy homeostasis, inflammation, DNA repair, genomic stability, and aging.	SirT6 depletion in cells leads to increases in tau stability, tau phosphorylation by GSK3β and apoptosis. Brains from AD cases exhibit reduced SirT6 protein and mRNA.
	Nucleoporin 98 *(Nup98)*	Functions in the assembly of NPC. Nup98 contains the Phe-Gly repeat domain and is responsible for nuclear import and export, mitotic progression, and regulation of gene expression.	Nup98 triggers tau aggregation *in vitro*, interacts with phospho-tau in neurons, and co-localizes with NFTs *in vivo*.
	Transportin 1 *(Karyopherin-β2; KPNB2)*	A nuclear transport receptor that interacts with nuclear localization signals to transport proteins through the nuclear pore into the nucleus. For FUS, it has been shown to mediates nuclear transport through binding to the PY motif.	NLS-associated mutations in FUS reduces binding to KPNB2 and impairs nuclear import; this deficit results in the redistribution of FUS in the cytosol, recruitment SGs and formation of cytoplasmic aggregates of FUS.

ALS: Amyotrophic Lateral Sclerosis; fALS: familial ALS; AD: Alzheimer's disease; FXTAS: Fragile X-associated Tremor/Ataxia syndrome; FXS: fragile X syndrome; TNF-α: Tumor Necrosis Factor Alpha; NTF2-like domain: N-terminal Nuclear Transport Factor 2-like domain; FTD: Frontotemporal Dementia; SCA2: Spinocerebellar Ataxia Type 2; NPCs: Nuclear Pore Complex; NLS: Nuclear Localization Signal; mRNP: Ribonuceloprotein; RBPs: RNA binding proteins; snRNPs: Small Nuclear Ribonucleoproteins; SMA: Spinal Muscular Atrophy.

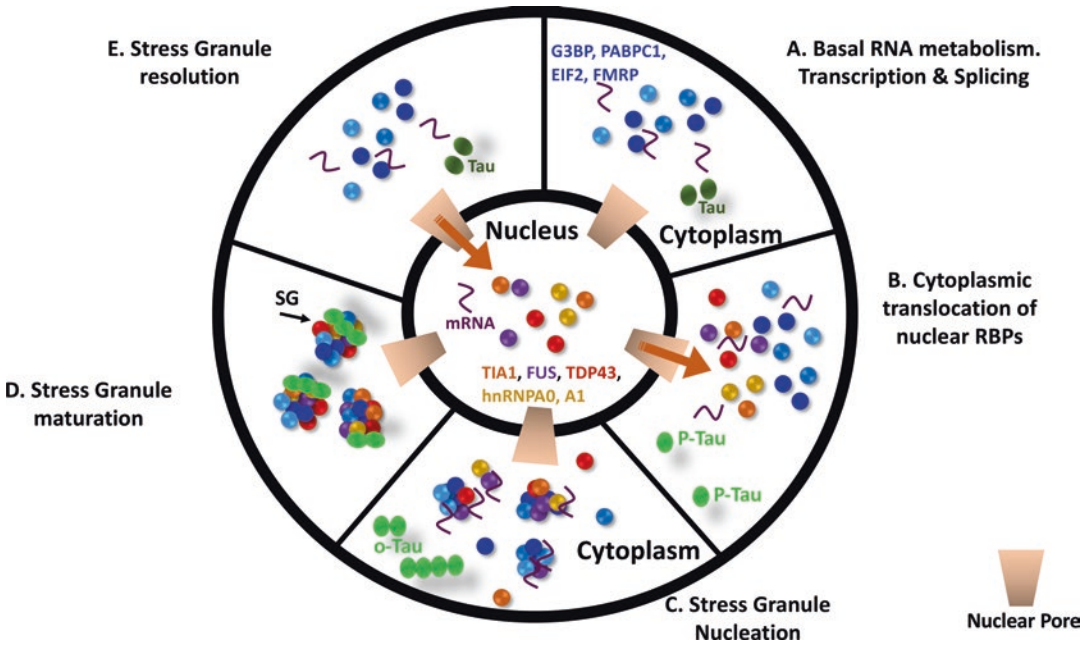

Fig. 26.1 The stress granule cycle. (**a**) Under basal conditions most RNA binding proteins are nuclear, while some are predominantly cytoplasmic. (**b**) With stress, nuclear RNA binding proteins exit the nucleus through the nuclear pore (arrow) and enter the cytoplasm. (**c**) The SGs begin to nucleate. Proteins such as TIA1, TIAR, TTP, G3BP and FMRP are primary nucleators of SGs. (**d**) The SGs mature over time, consolidating and beginning to incorporate multiple secondary proteins that include RNA binding proteins as well as proteins with functions independent of RNA (e.g., autophagy, apoptosis). (**e**) Upon resolution of the stress, the SGs dissolve and nuclear RNA binding proteins return to the nucleus

the increased tendency to aggregate shifts the biophysical state of these granules, that is thought to extend their persistence, resulting in the formation of persistent pathological SGs which can then consolidate to form the classic pathologic structures that are associated with disease [55]. Finally, it is important to note that cleavage of TDP-43 can produce fragments with a strong tendency to aggregate through a mechanism that is not strongly linked to the translational stress response [71, 94].

The Biology of Stress Granules and the Translational Stress Response

SGs are cytoplasmic complexes that form in concert with inhibition of RNA translation; stimulate of SG include a wide variety of stresses that include nutritional stress, heat or osmotic shock, DNA damage or proteostatic dysfunction [14, 25, 41, 42, 56, 57, 64]. SGs are classically made up of mRNAs, RNA-binding proteins, small 40S ribosome subunits, translation initiation factors, and broadly non-RNA binding proteins [12, 64, 76]. SG formation occurs in stages, with core nucleating RBPs initiating SG formation, followed by secondary association of a wide variety of proteins. The complexity of SGs varies with the type of stress, the type of cell and the duration of the stress [54].

The initial changes in RNA metabolism induced by stress result in polysome stalling and nucleation of SGs by a set of core nucleating RBPs, which include TIA1, G3BP1 and 2, FMRP, TTP and TIAR; proteomic experiments point to a comprehensive list of core RBPs (Fig. 26.1) [5, 54]. Nucleation of these RBPs is controlled by PTMs, which are described below, and by location. TIA1 for instance, translocates from the nucleus to the cytoplasm during stress [41].

These core nucleating components associate with the mRNA transcripts and protein components of the stalled initiation complexes including eIF3, eIF4F (consist of eIF4E, eIF4A and eIF4G), eIF4B, small ribosomal subunits and Poly A Binding protein 1 (Table 26.1) [41, 42]. A wide variety of secondary proteins associate with SGs. These proteins include many different RBPs, including those associated with ALS, such as TDP-43, FUS and hnRNPA0 [41, 42]. However, secondary proteins that associate with SGs also include scaffolding proteins, such as caprin, signaling proteins such as HDAC6 and SirT6, nuclear pore proteins such as nup98 [17], disaggregases such as karyopherin-b2 (Transportin-1, Kapnb2) (Table 26.1) [16, 27, 28, 32], and proteins linked to cell death pathways, such as TRAF and FAST (Table 26.1) [11, 23, 44].

Phase Transition and the Role of Protein Aggregation in the Biology of SGs

The formation and consolidation of SGs (and other RNA granules) appears to depend on the biophysical processes of liquid-liquid phase separation (LLPS) and protein aggregation. The process of LLPS is described in detail in Chaps. 24 and 25 (Kosik & Han, Wegmann), but a brief overview will be given in this chapter because of its fundamental importance for understanding the biology of SGs. The roles of LLPS and protein aggregation are the essential features that make SGs so important for the pathophysiology of neurodegenerative diseases generally, and tauopathies specifically. Under transient stress conditions SG components assemble and disassemble quickly, forming the highly dynamic structures that are governed by the biophysics of phase separation [5, 46]. The dynamic nature of these phase separated proteins enables transitions between multiple protein conformations. A fundamental weakness of this biology gives rise to neurodegenerative diseases. With extended time, such as might occur with chronic stress, some SG proteins transit into highly stable amyloid like states, similar to that observed in the protein aggregates that form in neurodegenerative diseases [65, 74]; mutations that are associated with familial disease frequently increase the rate of amyloidogenic transition [65, 74]. Secondary nucleation also allows the association of SGs with other proteins, such as tau, that exhibit a high propensity to aggregate into stable amyloids [88].

Three different biophysical considerations explain the biology of LLPS. (1) The physical chemistry of liquids creates the conditions for LLPS. Chemicals that are in liquid form and exhibit strong physical differences, such as oil and water, will phase separate to minimize the free energy of the mixture, reduce unfavorable interactions and promote weak bonding. The aqueous nature of proteins thus provides the conditions that allow for phase separation. The phase separation is promoted by weak bonding of low complexity protein sequences that consist primarily of alanine, glycine, glutamine and proline, with some extra complexity arising from interspersed arginine and asparagine [34, 75]. (Note that the phase separation of proteins does not produce the extreme concentrations (e.g., 55 M) occurring when oil and water phase separate because the weak interactions present in proteins are only moderately favored over interactions between proteins and water in the aqueous solution) (Fig. 26.1a). The low affinity binding of multiple short regions of low complexity domains produces the dynamic phase separation that characterizes RNA granules [91, 92]. (2) The low complexity regions that promote LLPS occur in intrinsically disordered regions (referred to as IDRs in the literature). The lack of order enables the "sticky" sequences in these regions to move in a dynamic manner forming the multiple weak associations that drive the LLPS. (3) The final consideration is polymer chemistry. RNA greatly facilitates protein based LLPS by forming a scaffold that helps to stabilize the phase separating proteins, keeping them generally in the same region [20]. Thus, RBPs bound to RNA phase separate at a lower concentration than is required in absence of RNA [20]. The tendency of RBPs to cluster around RNA combines with the presence of intrinsically disordered regions that can

self-associate in a low affinity manner to render LLPS a prominent feature of RBP biology, leading to formation of many types of RNA granules, including SGs.

Tau and Stress Granules

Tau Is Sorted to the Somatodendritic Domain in Stress

The information presented above provide a clear mechanistic pathway through which RBPs, SGs and RNA metabolism contribute to pathological aggregation and the pathophysiology of motoneuron diseases. The section below will explain how this mechanism involves tau, and the profound manner in which RBPs, SGs and the translational stress response contribute to the pathophysiology of tauopathy.

Tau is normally most abundant in the axons of neurons, where its primary function is to stabilize microtubules [4]. In AD, as well as in stress, tau becomes phosphorylated by proline directed kinases, such as glycogen synthase kinase β (GSK3β), cyclin dependent kinase 5 (CDK5) and microtubules affinity-regulating kinases (MARKs); this type of phosphorylation will be referred to as "hyperphosphorylation" [89]; this hyperphosphorylated tau accumulates in the somatodendritic arbor where it eventually forms neurofibrillary tangles. Originally hyperphosphorylated tau was through to translocate from the axon to the cytoplasm, but the translocation model didn't fit the pathology, which does not show extensive axonal tau phosphorylation. More recent data indicate that accumulation occurs as newly synthesized tau becomes phosphorylated and is prevented from entering the axon [33, 84, 96]. In the presence stress, tau is phosphorylated and localizes in the soma and dendrite where it can interact with RBPs associated with SGs [33, 84]. The accumulating phospho-tau arises from phosphorylation of *de novo* synthesized tau rather than translocation of phospho-tau from the axon [96]. The physiological logic for which the neuron would change the distribution of tau represents a fundamental question for tau research, and is one that has never been explained. We propose that hyperphosphorylated tau accumulates in the somatodendritic arbor during stress to adapt protein synthesis to address the stress [88].

Tau Regulates Stress Granules

The role of tau in the translational stress response is evident from studies of RBPs in neurons during stress. The relationship of tau to SGs is readily apparent when examining TIA1, an RBP that is one of the core nucleating SG proteins. In cell lines TIA1 is completely nuclear under basal conditions, and translocates into the cytoplasm under stress. Translocation of TIA1 has been demonstrated in response to many different stresses as well as viral infections (for a general review of TIA1, see Anderson et al. [1]), but also includes stresses that are very relevant to disease, such as arsenite, glucocorticoids, Aβ and tau oligomers [37, 82, 87, 88]. In each case, the resulting TIA1 SGs co-localize with hyperphosphorylated tau. However, comparison of SGs associated with TIA1 and G3BP1 demonstrate that SGs are not uniform species [54]. The neuronal SY5Y cell line exhibits a strong stress response after glucocorticoid treatment, exhibiting both TIA1 and G3BP1 positive SGs [82]. Interestingly, hyperphosphorylated tau inclusions strongly colocalize with TIA1-positive SGs, but show little colocalization with G3BP1-positive SGs [82]. The relevance of this point becomes clearer when considering tau pathology *in vivo* (discussed below), where one sees induction of both TIA1 and G3BP1 pathology with disease, but only TIA1 colocalizes with hyperphosphorylated tau [87]. In neurons under basal conditions TIA1 is also abundant in the nucleus, but also has some presence in the cytoplasm [88]. However, in tau knockout neurons, TIA1 is completely nuclear [88]. In addition, under conditions of stress (e.g., Aβ toxicity), TIA1 exhibits reduced translocation to the cytoplasm in tau KO neurons (Wolozin, personal communication). Conversely, over-expressing tau increases SG formation and the associated repression of protein synthesis [60, 87, 88].

Independent approaches support the intimate link between tau, SGs and translational control. Tau is known to exist in dendrites near dendritic

boutons [36]. These small tau granules appear to be linked to translational control/protein synthesis because chemicals that modulate protein synthesis affect the tau granule distribution. Cycloheximide, which inhibits protein synthesis and inhibits SG formation, also prevents clustering of tau into granules in the dendritic arbor. Conversely, puromycin, which also inhibits protein synthesis but stimulates SG formation, enhances clustering of tau into granules in the dendritic arbor [88]. Other SG inhibitors, such as the protein kinase R inhibitor C16 or the PERK inhibitor GSK2606414 also prevent coalescence of tau with SGs [88]. Immunoprecipitating TIA1 identifies tau in the protein interactome network, as well as many other RBPs for which binding to TIA1 requires tau, and immunoprecipitating tau identifies multiple co-associating RBPs [26, 35, 55, 88, 90]. These data demonstrate that the biology of tau is tightly connected with that of SGs and translational control.

Tau exhibits a tendency to phase separate in the presence of RNA *in vitro* much like RBPs, as discussed in the Chaps. 24 and 25 by Kosik and by Wegmann [18, 97]. The propensity of tau to phase separate might facilitate its interaction with RBPs and the formation of SGs, although this point has yet to be empirically demonstrated. Hyperphosphorylation of tau increases its propensity to form droplets *in vitro*, which suggests that tau hyperphosphorylation might function to promote the formation of phase separated complexes of tau, RBPs and RNA [18]. However, tau also has a strong tendency to fibrillize, and studies of human tau in neurons show that hyperphosphorylation renders tau prone to irreversible aggregation [18]. Thus, the consolidation of tau into droplets and SGs might increase the tendency of tau to aggregate, thereby enhancing a pathway that leads to neurodegeneration.

Tau Colocalizes with RNA Binding Proteins in Disease

These cell culture studies complement cogent pathological data. The connection between tau and RBPs is evident in pathological tissues from human cases of AD, FTD-tau as well as animal models of tauopathy. Molecular pathology studies show colocalization of pathological tau (hyperphosphorylated or misfolded) with multiple RBPs [2, 3, 55, 82, 87, 88]. The degree of co-localization detected in human tissues, though, likely under-represents reality because detection of RBPs *in situ* decreases dramatically with fixation time [55]. RBPs are abundantly detectable at <2 h of fixation and remain readily detectable at <24 h of fixation, but become difficult to detect at >48 h of fixation [55]; this sensitivity to fixation duration impacts greatly on staining of human tissues because most human cases have been fixed for much more than 48 h.

The pattern of SG pathology and co-localization with tau differs dramatically based on the RNA binding protein examined. TIA1, co-localizes strongly with pathological tau in human tissues [87]. In contrast, rasGAP-binding protein (G3BP) only shows weak colocalization with phosphorylated tau, despite exhibiting increased accumulation in neurons with increasing disease severity [87]. In animal tissues, where shorter fixation times are possible, tau is observed to co-localize with multiple other RBPs, including DDX6, eIF2α, hnRNPA0, and PABP [55, 82]. Interestingly, the pattern of reactivity appears to differ with the type of pathology. Co-localization of tau with RBPs is strongest with smaller inclusions; mature neurofibrillary tangles exhibit accumulation of RBPs adjacent to the pathological tau tangles, suggesting the hypothesis that the RPBs become excluded as tau consolidates to form the mature tangle [55].

The putative dysfunction of RBPs and RNA metabolism in tauopathy can be tested by examining RNA splicing. If RBPs become sequestered as protein aggregates in persistent pathological SGs, then one might expect to observe effects on RNA metabolism when examined through the lens of RNAseq. Indeed, multiple transcriptome studies show dramatic changes in RNA transcriptomes in tauopathies. Studies from several laboratories, including our own, show that splicing is dramatically altered in animal models of tauopathy as well as in cases of AD [2, 3, 6, 27, 28, 63, 81]. The changes in splicing are far greater than the comparatively modest changes in transcript levels. Since the splicesome is made up of RBPs, the large

changes in splicing that occur with disease are consistent with a model in which RBPs become sequestered away from splicesomes in the nucleus leading to dysfunctional splicing.

Tau Oligomers Mediate Interactions with RNA Binding Proteins

Animal models provide insight into the mechanisms underlying the interaction of tau with RBPs, SGs and the translational stress response. Our laboratory recently crossed PS19 P301S tau mice with TIA1−/− mice, and demonstrated that reducing TIA1 *in vivo* provides striking rescue of the degenerative phenotype associated with the PS19 P301S tau mice [2, 3]. Reducing TIA1 expression by 50% greatly decreased the number and size of cytoplasmic pathological TIA1 granules (which colocalize with SG markers). The TIA1 reduction also yielded striking rescue of behavior, neuronal and synaptic degeneration, cortical thickness, as well as a 26% increase in survival despite the continued five-fold over-expression of tau [2, 3]. TIA1 reduction also decreased the amount of hyperphosphorylated tau evident at 3 months of age [2, 3]. This acute reduction in tau pathology is consistent with cell culture studies showing that TIA1 knockdown also provides neuroprotection and reduces tau pathology [88].

Insights into the mechanism of tau/TIA1 interactions arise from our studies examining the mice at later ages, as well as from a subsequent study comparing the propagation of oligomeric and fibrillar tau. Aging of the P301S tau::TIA1+/+ and +/− mice showed dramatic changes in the aggregation of tau. The P301S tau::TIA1+/− exhibited striking (90%) reduction in the accumulation of oligomeric tau at 9 months, and an equally striking increase (>10-fold) in fibrillar tau at 9 months of age. Analysis by immunoelectron microscopy demonstrated that TIA1 binds to phosphorylated tau oligomers but not tau fibrils; the ability of TIA1 to increase tau oligomerization (assessed by ELISA) confirmed this observation [88]. These data suggest the hypothesis that TIA1 interacts selectively with phosphorylated tau oligomers.

The selective interaction between tau oligomers and TIA1 is supported by independent studies of tau propagation. Tau oligomers and fibrils were isolated from 9-month P301S tau mice, and propagated in both WT and P301S tau mice; the results were similar for both but more striking in the mice over-expressing human tau [37]. Both oligomeric and fibrillar tau exhibited robust propagation, which is consistent with Chaps. 30 and 31 and multiple reports in the literature [15, 47, 79]. The experimental design also allowed side by side comparison of toxicity, with the results showing that oligomeric tau produced abundant tau pathology and abundant neurodegeneration while the fibrillar tau produced abundant tau pathology but no degeneration evident after 3 months of propagation [37]. This confirms prior studies suggesting that oligomeric tau is much more toxic than fibrillar tau. [80, 86]. These studies suggest that oligomeric or misfolded forms of tau are toxic, drive cognitive decline, and act through a mechanism that occurs before or independently of the development of NFTs [73, 80, 95].

The propagation studies also demonstrated the strong link between oligomeric tau and TIA1-positive SGs. The oligomeric tau propagated tau pathology that co-localized with cytoplasmic TIA1-positive SGs, as shown by co-localization with TIA1, PABP and eIF3η; this was true in both the ipsilateral and contralateral cortex providing clear demonstration of tau propagation [37]. In contrast, the fibrillar tau propagated nicely, but showed no colocalization with TIA1-positive granules. An additional mechanistic link between TIA1 and tau was evident from similar studies performed in P301S tau::TIA1+/−. These mice showed abundant propagation of fibrillar tau, but very little (if any) propagation of oligomeric tau, which provides support for the hypothesis that TIA1 selectively interacts with oligomeric tau.

A Model for the Interactions of Tau in Stress and with RNA Binding Proteins

The accumulating data presented above suggest a model in which phosphorylated tau accumulates

in the somatodendritic arbor where it oligomerizes and then interacts with TIA1 and possibly other RNA binding proteins and/or ribosomal proteins. Binding of tau to these proteins appears to promotes the translational stress response, which reduces synthesis of specialized proteins (such as those related to synaptic function) and increases synthesis of proteins needed for the stress response. The proline directed phosphorylation that is characteristic of the stress response also inhibits binding of tau to microtubules, perhaps allowing for more tau oligomerization and interaction with the translational machinery. Chronic stress, though, leads to the accumulation of oligomeric tau which is toxic, although the mechanism of toxicity is not currently known.

Therapeutic Approaches Based on Modulating SGs and the Translational Stress Response

One of the most important aspects of studying the relationship between tau, RBPs, SGs and the translational stress response is the possibility of innovative disease therapies. The biology of SGs involves multiple biochemical pathways, some of which not been considered previously in the context of neurodegenerative diseases.

The classic SG/translational stress response is regulated by phosphorylation of eukaryotic initiation factor 2 (eIF2α-P); this pathway has been studied by a number of different laboratories. In presence of stress, stress-related kinases, including PERK, PKR, HRI and GCN2, phosphorylate eIF2α trigger the assembly of SGs that inhibit global cellular protein synthesis [93]. Chronic diseases produces a sustained stress response, persistent SGs and continued global translational repression [8, 45, 77, 85]. These observations suggest the hypothesis that the translational stress response is too active, and inhibiting the stress response might be beneficial in neurodegenerative disease. One of the first studies demonstrating the value of inhibiting the translational stress response was performed in a mouse prion model, where Mallucci and colleagues demonstrated that reducing eIF2α phosphorylation (by expressing the phosphatase adapter protein, GADD34) relieves the translational repression caused by eIF2α-P and delays neurodegeneration [66]. Studies in *drosophila* and in rat primary cortical neurons expressing TDP-43 show that inhibiting the phosphorylation of eIF2α alleviates the toxicity induced by TDP-43 [43]. Similar approaches have now also been shown to apply to inhibition of toxicity associated with tau, as well as with Aβ [51, 52, 59, 88]. These studies point to inhibition of eIF2α phosphorylation as a potential therapeutic intervention to neurodegenerative diseases. However, the clinical value of each of these approaches is limited by toxic liabilities. For example, PERK inhibition leads to pancreatic toxicity, although partial inhibition might be clinically tolerated [29], and inhibition of PKR enables reemergence of retroviruses [68].

Other teams are developing innovative small molecule therapeutics to inhibit the accumulation of persistent pathological SGs. Our group used neuronal PC12 cells inducibly over-expressing TDP-43 to screen a library of brain penetrant small molecules [10]. This screen lead to the identification of 16 hits that reduced the accumulation of TDP-43 inclusions [10]. Some of these compounds were able to improve survival of neurons in a C. elegans model of over-expressing TDP-43, suggesting the potential for *in vivo* efficacy [10]. Cell lines expressing TDP-43 have also been used to screen a variety of other putative therapeutics, with promising results although these approaches have yet to be tested *in vivo* [13, 67].

An alternative approach for disease modifying therapy has been direct therapeutic targeting of RBPs for therapy. Studies in this area have focused on delaying neurodegeneration in models of tauopathy, based on tau over-expression, and models of ALS based on TDP-43 over-expression [2, 3, 7]. The discussion of tau, described above, demonstrates how reducing TIA1 in the P301S tau mouse model rescues memory loss, reduces neurodegeneration and improves survival [2, 3]. Protection is also be achieved by TIA1 knockdown with shRNA directed against TIA1 [88]. TIA1 reduction also

protects against tauopathy in a model of tau propagation, which indicates that reducing TIA1 provides generalized protection against tau pathology produced within neurons as well as propagated among neurons [37]. These results suggest that reducing TIA1 might provide broad-based neuroprotection in AD and other tauopathies.

The therapeutic potential of reducing RNA binding proteins has also been examined in a model of ALS based on over-expressing TDP-43 [7]. These studies focused on ATXN2, which participates in RNA metabolism, contributing to RNA splicing, and degradation [39, 72]. ATXN2 contains a domain with a small number of CAG trinucleotide repeats (producing glutamines) whose expansion is associated with disease. Disease linked mutations in ATXN2 that expand the CAG domain to 34 or more repeats cause the neurodegenerative disorder spinocerebellar ataxia type 2 (SCA2) [24]. However, disease linked mutations that expand the CAG domain to 27–33 repeats increase the risk of ALS, with associated TDP-43 pathology [19]. The link between ATXN2 and TDP-43 was strengthened with the observation that reducing ATXN2 significantly extends survival in an animal model of ALS based on TDP-43 overexpression [7]. Knockout and antisense-mediated knockdown of ATXN2 in TDP-43 transgenic mice decreased SGs containing TDP-43, reduced the accumulation of phosphorylated TDP-43 spinal cord inclusions, improved the motor performance and increased the median lifespan by 35% [7]. These findings indicate that ATXN2 plays a crucial role in the development of pathological SGs and augmentation of TDP-43 toxicity.

Targeting other regulators of RNA metabolism has also been shown to ameliorate disease. McGurk et al. [58] found that downregulating Tankyrase 1 and 2, a poly (ADB-ribose) polymerase, reduced the formation of cytoplasmic TDP-43 foci without affecting the SG assembly. PAR binds to the PAR-binding motif in the N-terminal region of TDP-43 and is necessary for its sequestration in SGs in mammalian cells and neurons upon exposure to stress [58]. Inhibiting Tankyrase significantly increases nuclear TDP-43 and decreases cytoplasmic TDP-43 without affecting the total TDP-43 expression levels and increases survival percentage of flies expressing TDP-43 in the brain. Inhibiting Tankyrase prevents the stress-induced formation of cytoplasmic TDP-43 foci without altering the dynamics and assembly of SGs. TDP-43 also becomes phosphorylated upon prolonged exposure to stress, but the phosphorylation appears to stimulate aggregation of TDP-43 through a pathway that does not co-localize with SG markers [58]. Since phospho-TDP-43 is associated with disease pathology, this TDP-43 phosphorylation pathway might identify a disease-relevant pathway [70]. Since tankyrase downregulation increases nuclear TDP-43 and decreases cytoplasmic TDP-43, inhibiting tankyrase might reduce the amount of cytoplasmic TDP-43 that is available to be phosphorylated and provide a potential pharmacological intervention for diseases associated with pathological TDP-43 [58].

The most striking success in delay of neurodegenerative disease based on targeting RNA metabolism comes from the field of spinal muscular atrophy (SMA). This disease is caused by a missense mutation that causes exon skipping that produces an inactive form of the gene survival of motor neuron 1 (SMN1) [50]. Teams of investigators from Ionis Pharmaceuticals and Biogen developed antisense oligomers capable of correcting the exon skipping, which increases levels of SMN1. Multiple clinical trials now demonstrate that application of these antisense oligomers to children with SMA produces striking rescue from disease, and prolonged delay of disease progression [21, 22, 61, 62]. The striking ability of these antisense oligomers to delay disease progression and actually improve clinical conditions in patients with SMA now serves as a bench post for future therapies.

Conclusion

The work covered in this chapter presents a cogent paradigm for understanding the pathophysiology of tauopathy. Accumulating data suggest that tau becomes hyperphosphorylated and oligomerize as part of an endogenous mechanism to promote the translational stress response. Tauopathies, though,

are chronic diseases. The prolonged stress leads to a persistent stress response, which provides time for oligomeric tau to accumulate, cause toxicity and neurodegeneration. We show that tau-mediated neurodegeneration occurs through a mechanism that is mediated by RNA binding proteins and the translational stress response. Discovery of the role of RNA metabolism in tauopathy opens a wide variety of novel therapeutic approaches. The abundant studies targeting TDP-43-mediated disease demonstrate the large variety of approaches designed to therapeutically modulate RNA metabolism, and indicate strong potential for success in disease modification in models of ALS based on abrogating TDP-43 mediated disease. These studies complement the documented success of targeting RNA metabolism modify disease progression in animal models of tauopathy as well as β-amyloidosis. The success in treating SMA point to a new era for therapy of neurodegenerative diseases, and suggest that the right type of therapy can actually go beyond delay of disease progression to actually halt disease progression and improve clinical outcomes for some neurodegenerative diseases. Taken together, these approaches point to a future in which novel therapeutic approaches might be able to significantly delay disease progression in tauopathy.

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Tau Oligomers

27

Sumihiro Maeda and Akihiko Takashima

Prediction of Non-fibrillar Aggregates of Tau Protein

Neurofibrillary tangles (NFTs), a pathological hallmark of Alzheimer's disease (AD), are composed of filamentous polymers of tau protein [1]. NFTs are insoluble and resistant to proteases. Thus, even after neurons are lost, NFTs remain as "tombstones" of the NFT-bearing neurons and are called ghost tangles. In AD, the number of neurons lost and the number of NFT/ghost tangles should be the same if all of the neurons were lost after NFT formation. But they aren't. More neurons are lost than ghost tangles remain [2, 3]. Although the number of NFTs, neurons lost, and the severity of the disease all correlate well with each other [3], several findings point to a missing element. NFT formation and neuronal loss have been reported to be distinct events. Suppression of human tau (hTau) expression in an hTau transgenic mouse model did not block tau filament formation but reduced neuronal loss [4], and NFT-bearing neurons were functionally intact in hTau transgenic mice [5]. In an hTau *Drosophila* model, hTau over-expression induced neuronal loss without NFT formation [6]. Therefore, over-expression of tau, at least, induces neuronal toxicity in an animal model, but the toxic species of tau was not the tau filament itself, but something produced in the process of tau aggregation. In that sense, granular tau oligomers, the intermediate form of tau filament, meet this criterion for the toxic tau species.

Tau Oligomer as an Intermediate Species of Tau Filament

Tau is highly hydrophilic and does not aggregate by itself. The core of the tau filament, the microtubule binding domain (MBD), is positively charged, and that charge prevents intermolecular interactions of tau. Under physiological conditions, the positively charged residues of tau interact with negatively charged residues of tubulin [1]. Under pathological conditions, tau is highly phosphorylated, and the negative charge of phosphor residues is thought to neutralize the positive change of the MBD, induce detachment of tau from tubulin, and allow tau-tau interactions [1].

Polyanionic compounds, such as RNA or heparin, induce aggregation of recombinant tau probably by neutralizing the positive charges of tau [7–9]. Lipids, such as arachidonic acid, also induce tau aggregation above the critical micelle concentration because the surfaces of lipid micelles are negatively charged [10]. In an

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in vitro tau aggregation assay, non-filamentous tau aggregates called granular tau oligomers were found [11]. The non-filamentous tau aggregates could be separated from tau monomers and filaments by sucrose gradient centrifugation (Fig. 27.1). Tau filaments do not form in vitro without aggregation inducers. However, simply concentrating tau oligomers induces tau filament formation without additional heparin, and under atomic force microscopy (AFM), filaments formed in vitro or purified and AD brain degraded to granular tau structures [11] (Fig. 27.2). This suggested that the tau oligomer is an intermediate species of the tau filament. In addition, tau oligomers were found in animal models and human brains [12–14] (Fig. 27.3). The amino acid sequences critical for tau filament formation, PHF6 and PHF6* [15], are also reported to be key for tau oligomerization by western blots (WBs) and nuclear magnetic resonance analysis [16, 17]. In human brains, the number of tau oligomers was increased in the frontal cortex of Braak stage I patients, when NFTs have yet to form. Thus, tau oligomers form before NFTs [12–14].

Various Types of Non-filamentous Tau Aggregates

Definition and preparation of tau oligomers vary among researchers. For example, Maeda et al. induced tau aggregation by mixing recombinant tau with heparin and defined tau oligomers as granular structures under AFM [11]. The Davies group reported non-filamentous tau species that were extracted by a conformation-dependent antibody, MC1 [18]. The non-filamentous tau species formed filaments after concentration,

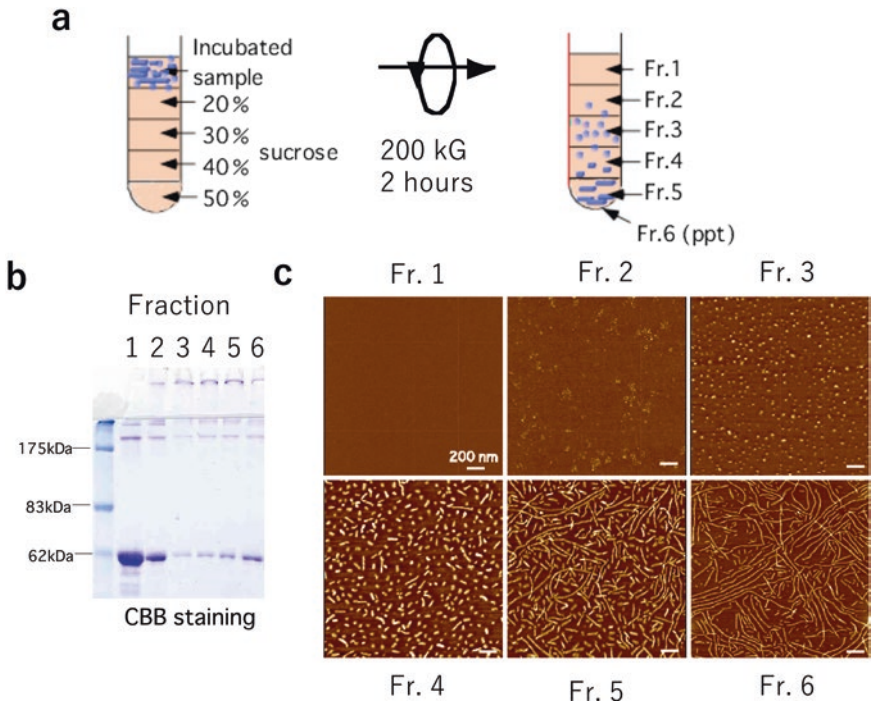

Fig. 27.1 The purification of granular tau oligomers using sucrose step gradient centrifugation. (**a**) Tau proteins were aggregated in vitro and layered onto 20–50% sucrose step gradients and centrifuged at 200,000 × G for 2 h to separate non-aggregated tau, granular tau oligomers, and tau filaments. (**b**, **c**) Staining with Coomassie brilliant blue (CBB) (**b**) and AFM images of all fractions (**c**) are shown. Granular tau oligomers could be recovered in fraction 3 as indicated (**b**, **c**). In fraction 1, tau monomers and multimers were collected (**b**), but no aggregated structure were observed under AFM (**c**). The amount of tau in fraction 3 was less than that in fraction 1 (**b**), but granular structures were detected under AFM (**c**). Longer filaments were collected in fractions 4–6 (**c**) [11]

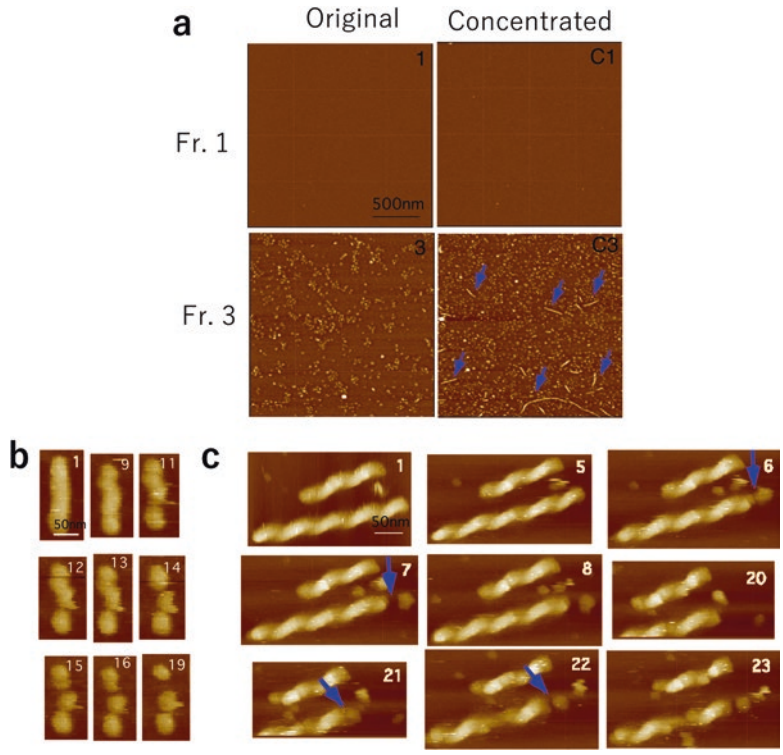

Fig. 27.2 The formation and degradation of tau filaments. (**a**) Granular tau oligomers in fraction 3 and non-aggregated tau in fraction 1 were concentrated without adding heparin. Filaments were observed by AFM in concentrated fraction 3 (C3) but not in the concentrated fraction 1 (C1). Blue arrows indicate the generated filaments in C3. (**b**, **c**) Structures were exposed to mechanical damage by AFM tips. Continuous AFM observation revealed that mechanical damage degraded tau filaments formed in vitro (**b**) or purified from an AD brain (**c**) to granular tau structures. The numbers at the top right indicate the order of the images. It took about 9 min to take an image [11]. Blue arrows indicate the breaking points of the tau filament

indicating that the oligomers extracted here are identical with the oligomers described above. Berger et al. reported a correlation of tau multimers and behavioral scores in an hTau transgenic mouse model [19]. Multimers were defined by the molecular mass by WB (140- and 170-kDa bands). The multimer can be a component of higher order aggregates, such as globular oligomers or tau filaments, but it degrades to a multimeric form under WB denaturing conditions. Thus, one component of tau oligomers may be the 140-/170-kDa multimers. Multimers might also be complexes of tau and other molecules, but another group reported that similar multimers may form only from recombinant tau [16]. Mandelkow's group induced tau oligomers using recombinant protein produced in insect cells (i.e., Sf9 cells) because highly phosphorylated tau proteins can be extracted, whereas recombinant tau protein produced in *E. coli* had no post-translational modifications [20]. They confirmed that phosphorylation enhanced tau aggregation [20, 21]. The Kayed group generated tau oligomers by cross-seeding with amyloid beta (Aβ) oligomers [22]. Those oligomers induced cell death when added exogenously to cells [22, 23]. Anti-tau oligomer antibodies were generated using the cross-seeded tau oligomers, and the antibodies blocked tau-induced neurodegeneration in hTau transgenic mouse models that lack Aβ aggregates and could not form Aβ seeds [24, 25]. Thus, tau aggregates, like tau oligomers seeded by Aβ aggregates, can be induced in the absence of Aβ aggregates. Binder's group [26]

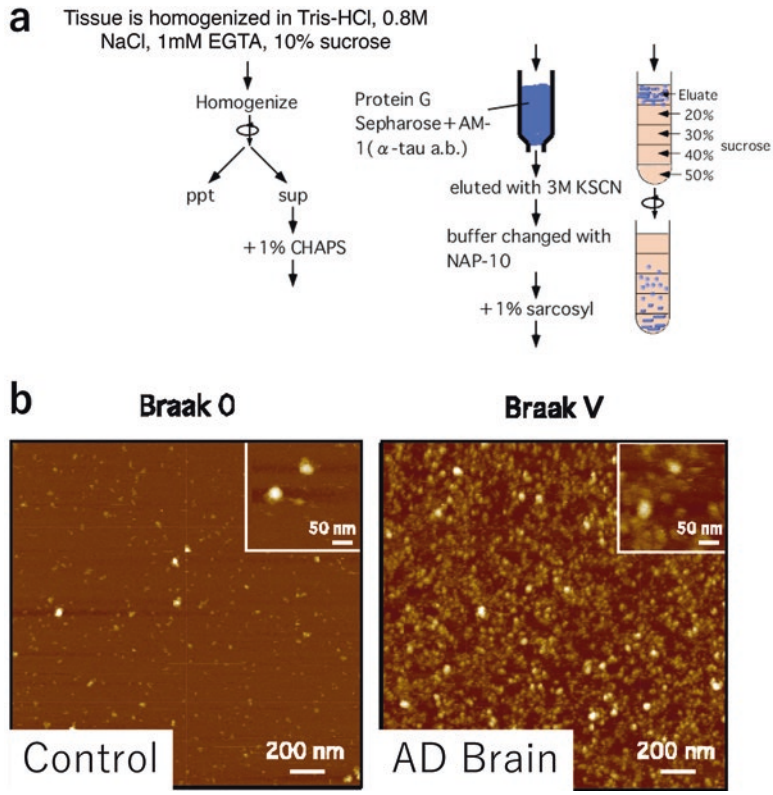

Fig. 27.3 Purification of granular tau oligomers from human brains. (**a**) Granular tau oligomers in human brains were purified in a combination of immune-affinity column chromatography with a pan-tau antibody and sucrose step gradient centrifugation [12]. (**b**) AD brains (Braak stage V) contained more granular tau oligomers than control brains (Braak stage 0) [12]. Inserts show the magnified images of granular tau oligomers purified from AD or control brains

stabilized tau aggregates with a chemical cross linker and found that, by WB, the tau dimer is a component of tau aggregates and that cross-linked tau dimers form short filaments and oligomeric globular structures but not long filaments.

Tau Oligomer Detection Methods EM and Oligomer Antibodies

Granular tau oligomers can be detected by AFM and electron microscopy (EM) [11, 22, 26], but not by other methods. To expand their ability to study tau, researchers generated antibodies specific for tau oligomers.

The Davies group generated two conformation-dependent antibodies, Alz50 and MC1 [27], that react with tau filaments purified from AD brains. Alz50 recognizes discontinuous sequences at the N-terminus and MBD. During abnormal conformation changes, inter- or intramolecular attachment of the N-terminus to the MBD may generate the Alz50 epitope [28]. MC1 is a second-generation conformation-dependent antibody that was raised against tau aggregates purified with an Alz50 immunoaffinity column. MC1 reacts with aggregated tau species more specifically than Alz50 [27, 29] and detects both tau filaments and oligomers [2]. With a combination of sucrose step gradients and the MC1 antibody, oligomeric species can be detected even in mouse tissue lysates [14]. However, the requirement for sucrose step gradient centrifugation limits the ability to do mechanistic studies of tau oligomer-dependent pathogenesis.

Researchers next sought to distinguish tau oligomers from tau filaments without gradient centrifugation. They generated more specific antibodies against tau oligomers than Alz50 and MC1 antibodies. Binder's group found the 180-kDa tau species in AD brain lysates [26]. To examine the pathogenesis of this tau species, they cross-linked tau dimers to obtain stable lower-order tau aggregates. When incubated with arachidonic acid, cross-linked tau dimers formed granular tau oligomer but not long fibrils. The

researchers immunized tau-knockout mice with the cross-linked tau dimers and obtained the Tau Oligomeric Component-1 (TOC1) antibody [26]. TOC1 preferentially labels granular tau oligomers and the end of tau filaments, supporting the idea that tau oligomers are an intermediate species of tau filament and the attachment of tau oligomer to tau filament will elongate the filament [11]. Unlike another tau-conformation dependent antibody, Alz50 that recognizes discontinuous tau sequences, amino acids 2–10 and 312–342, TOC1 recognizes amino acids 209–224 of tau.

TNT1 is another conformation-dependent antibody generated by the Binder group [30]. Its epitope is the tau phosphatase-activating domain (PAD) in the N-terminal domain. The N-terminus attaches to the MBD under physiological conditions. It is detached by aggregation or phosphorylation and impairs axonal transport via phosphatase activation, GSK-3β activation, and kinesin light chain phosphorylation [30, 31]. As with all other tau oligomer antibodies, reactivity depends on conformation. Notably, the PAD sequence lacks the endogenous mouse tau sequence, and thus, TNT1 cannot detect mouse tau even though mouse tau aggregates like human tau [32]. Both TOC1 and TNT1 are pan-tau antibodies in WB because their epitopes are freely accessible even under denatured conditions [33].

The Kayed group raised antibodies against the cross-seeding oligomeric species of tau and obtained the T22 (rabbit polyclonal) and TOMA (mouse monoclonal) antibodies [25, 34]. For unknown reasons, the epitopes of these antibodies are preserved even in WB, whereas other conformation-dependent antibodies function as pan-tau antibodies in WB because all sequences of tau protein are accessible to the antibodies. Normal tau immunization induced neurologic deficits in normal mice [35]. However, immunization with these antibodies blocked tau-induced neuronal dysfunction in hTau transgenic mice as mentioned above, suggesting that granular tau oligomers, not tau monomers, should be explored as therapeutic targets for tauopathies [24, 25].

Tau Oligomerization Enhancers, Blockers, and the Toxic Mechanism

Not all FTDP-17 mutations increase tau filament formation [36, 37]. However, all FTDP-17 tau mutations examined so far for tau oligomerization enhanced tau oligomerization. Notably, the P301L mutation decreases the size and increases the number of tau oligomers [14].

Tau aggregates (mainly filaments) have been suggested to spread from one brain region to another trans-synaptically [38]. The spreading may be mediated by the tau aggregates themselves, which are called prion-like tau species, but not by the dysfunction of projecting neurons [38]. Although it is not clear that the soluble fraction has prion-like activity [39, 40], tau oligomers may mediate the propagation [41]. They may function as a template for newly formed tau oligomers that can be amplified by adding non-aggregated tau [22]. Thus, the tau oligomer itself may increase tau oligomer numbers.

Heat shock proteins (HSPs) are involved in tau oligomerization [42]. HSPs, including Hsp90 and 27 that block tau aggregation, are inversely correlated with tau oligomer levels in human brains [16]. Thus, HSPs are an intriguing target for therapies to prevent tau pathogenesis [43, 44]. Other small molecules also block tau oligomerization. A screen of a library of natural compound derivatives for tau-binding compounds revealed several positives. For example, 1,2-dihydroxybenzene blocked tau oligomerization, and DL-isoproterenol reduced tau aggregation and neuronal cell loss in human tau transgenic mice [45]. These findings also support the idea that tau oligomer is a culprit of tau-mediated pathogenesis.

Tau aggregates accumulate inside of neurons. Thus, to assess their toxicity, tau oligomers must be introduced into neurons. However, tau oligomers are thought to be incorporated into cells [46] and to induce synaptotoxicity and Ca dysregulation [20, 47]. Surprisingly, the synaptotoxicity may be separate from cell viability, even though neuronal cell loss correlates well with the NFT formation as mentioned above. Ca dysregulation

by tau oligomers may be mediated by M1 and M3 muscarinic receptors [48], even though aggregated tau species showed less toxicity than monomeric non-aggregated tau in that system.

The mechanism of tau toxicity is unknown, but a hint might be provided by its conformation. Tau is reported to have a paper-clip conformation: the N-terminus is bent over to MBD in native state [49]. Tau aggregation disrupt that conformation and exposes the N-terminus. The exposure allows the PAD domain to induce the impairment of axonal trafficking [30].

Conclusion

Tau aggregation is toxic to cells. Thus, many researchers have visualized tau aggregation in their experimental models to search for a common feature between AD patients and the models. The exact mechanism of tau aggregation and toxicity and the targets of tau aggregates are still open issues. The discrepancy of tau filaments from neuronal cell loss implicates tau oligomers as the culprit of tau-mediated pathogenesis. Researchers have characterized tau oligomers by various methods. However, to directly examine their toxicity mechanisms, we will need methods to enhance or block tau oligomerization inside neurons. Also, to develop drugs that target tau oligomers, we will need tau oligomer probes that can be used for the target engagement in human live imaging. Antibodies specific for tau oligomers were used to show that tau oligomers have a structure distinct from other tau species. Thus, it might be possible to develop tau oligomer–specific probes for positron emission tomography. Those probes would be a powerful tool in combination with similar probes for tau filaments [50].

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28 Experimental Models of Tauopathy – From Mechanisms to Therapies

Julika J. Götz and Jürgen Götz

Introduction – The Concept of 'Tauopathy'

One of the routinely used 'labels' for neurodegenerative diseases that provides a clue about the underlying pathomechanisms is the term 'proteinopathy'. Proteinopathies come in many variations, including 'tauopathy', a term coined by Drs Bernardino Ghetti and Michel Goedert to categorize a group of human neurological disorders that have tau inclusions in neurons or glia as their common, defining denominator. Previously, when Alzheimer's disease (AD) research was dominated by studies into the processing and toxicity of amyloid-β (Aβ, in part owing to the fact that disease-causing mutations had been identified in the amyloid precursor protein-encoding gene *APP*, but not in the tau-encoding gene *MAPT*) [1], it was helpful to stress the point that tau pathology is a defining feature of more than two dozen so-called primary tauopathies, whereas in the secondary tauopathy AD, tau pathology exists on par with Aβ pathology [2]. It was at this time that Götz et al. generated the first human wild-type tau transgenic mouse model that reproduced aspects of the human pathology, including the somatodendritic accumulation of tau and its hyperphosphorylation at selected epitopes [3].

The focus of this book chapter is on animal models for tauopathies that have been established by manipulating the germline (i.e. viral approaches or the intracerebral injection of brain extracts will not be discussed). Although AD is a tauopathy which pathologically involves not only tau but also Aβ and even TDP-43 deposition [4], we will, in this chapter, restrict our discussion to tau models, their validity, the insight they have provided into pathomechanisms, as well as the therapeutic interventions that have been tested and validated in these models. We will then finish the chapter with an outlook into what the future may hold as far as animal models for tauopathy (or brain diseases more generally) are concerned.

Approaches to Model Tauopathy in Animals

The mouse (*Mus musculus*) is the most frequently used species for modeling tauopathy because of its ease of breeding and genetic manipulation, as well as the relatively low cost of its husbandry. As discussed recently, there are a few principal approaches in generating animal models, and these strategies have been applied to the study of tau under both physiological and pathological conditions [5].

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For the generation of classical transgenic models, the gene of interest and a particular promoter are combined and delivered into the zygote by pronuclear injection [6]. The injected zygotes are then transplanted into pseudopregnant females to obtain pups that are screened for the expression of the transgene and then used to establish a new animal strain [5]. During the creation of the first human wild-type tau transgenic mouse strain [3], the longest human tau isoform (2N4R) in its wild-type form was expressed by cloning the tau-encoding cDNA into a human Thy1 (hThy1) expression vector. At the time, the authors also tested the human rhombotin 1, the rat neuron-specific enolase and the murine neurofilament light chain promoter; however, neither of these expression vectors yielded significantly high expression levels [3]. Subsequently, stronger promoters were chosen for transgene expression. These included the murine Thy1.2 (mThy1.2) promoter, which has been successfully used to overexpress different frontotemporal dementia (FTD) mutant forms of tau, achieving tau filament formation [7, 8], the murine prion protein (PrP) promoter [9, 10], or the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) promoter [11, 12]. In addition to promoter choice, these strains differ in terms of the type of tau isoform they express, and whether or not they carry pathogenic mutations of FTD. An excellent recent review by Dr. Simon Dujardin and colleagues provides a comprehensive, although as they write, 'non-exhaustive' overview of 50 tau transgenic mouse strains (some in combination with other genetic modifications), a small number of rat and *C. elegans* strains, and around 25 *Drosophila* strains [13]. When one assesses the additional information provided in this review, the heterogeneity of the models becomes evident: there are differences in the tau isoform being expressed, the presence or type of FTD mutation(s), the promoter used to drive transgene expression, the distribution pattern of tau, the degree of overexpression compared to endogenous tau levels, and the type and time-course of tau pathology, including its phosphorylation pattern. The way these models were generated did not allow for a controlled integration of the transgene, which often forms multiple tandem repeats, and interestingly, for most of the strains the analysis is incomplete and more often than not, motor or cognitive deficits have not been assessed [13].

The first mouse strain with significant tau filament formation in neurons was reported in 2000; this mouse expressed the prevalent P301L mutation of FTD tau in neurons [14]. Whereas the majority of vectors that have been used target tau expression to neurons, a few have also achieved pathology in glia [15–17]. Surprisingly, glial tau pathology has remained an underexplored research area, despite the fact that in tauopathies such as corticobasal degeneration (CBD) or progressive supranuclear palsy (PSP), glial pathology often outnumbers neuronal pathology. A transactivator-based strategy was chosen to generate P301L transgenic mice that are characterized by massive neuronal cell loss and early-onset tau pathology [18]. Other regulatable systems expressed tau fragments which were either prone to or resistant to aggregation [19]. Whereas tau transgenic mice are generally maintained on a C57Bl/6 wild-type background, breeding a P301L tau transgene onto a senescence-accelerated mouse-prone 8 (SAMP8) background over ten generations achieved an accelerated tau phenotype, indicating that age (the major risk factor for AD) accentuated tau pathology [20]. Finally, the entire tau-encoding MAPT locus has been introduced into the mouse genome, yielding a more pronounced pathology when endogenous tau was removed [21].

For models based on homology-directed recombination (HDR) mechanisms, the targeting vector containing the sequence to be introduced into the host genome is flanked by homology arms corresponding to the recipient genomic site of interest and also contains a selection marker. In the first step, the targeting vector is introduced into embryonic stem cells that are initially selected for an HDR event rather than random integration. These are then microinjected into blastocysts, followed by transfer into pseudopregnant females to establish a new line [5]. To obtain models based on gene editing (such as transcription activator-like effector nucleases

(TALEN) or CRISPR), a donor template containing homology arms is injected into the pronucleus of zygotes together with the method-specific components (either TALENs or the Cas9 enzyme and guiding RNA molecules, respectively) to introduce breaks in the genomic DNA at specific sites.

Embryonic stem cell-based approaches have also been used to generate tau knock-out mice. The first strain was established by Harada et al. in 1994 but, surprisingly, did not display any pathological phenotype [22]. In a compensatory mechanism, the tau homolog MAP 1A was upregulated and the axon caliber was altered to one typically found in dendrites. Subsequently, in 2001, two additional strains became available. The 'Dawson' strain was established by Dawson and colleagues, who used the same strategy as Harada and colleagues by replacing the first coding exon of tau with a neomycin selection cassette, thereby abrogating tau expression [23]. The second strain was a 'knock-in' generated by Tucker and colleagues, who inserted a GFP cassette in frame, resulting in a fusion protein that contained the first 31 amino acids of tau [24]. Neither of these two strains revealed any obvious impairment (at the time, the Tucker strain was only used as a 'tool' to study neurotrophins, although it has since been used for behavioural studies: [25]). Interestingly, in the Dawson strain, MAP 1A levels were increased twofold at birth, whereas they returned back to normal levels as the mice became older, suggesting that MAP 1A may compensate for the loss of tau during early brain development, but not in the mature brain [23]. Potential compensatory functions attributable to closely related MAPs can be excluded in *C. elegans*, where protein with Tau-like repeats (PTL-1) is the sole homolog of TAU/MAP 2/MAP 4 [26, 27], meaning that shared physiological functions of the different family members can be addressed. Knock-out studies in *C. elegans* revealed that PTL-1 has an essential role in maintaining neuronal health with age and in regulating whole organism lifespan [28]. Several follow-up studies using the above tau knock-out mouse strains mostly failed to report significant memory and motor impairments; however, one group reported parkinsonism [29] and another reported subtle dopamine-independent motor deficits with age [30] (for a detailed discussion see [31]).

With the aim of reproducing tau pathology without relying on an overexpression approach, a P301L tau knock-in mouse was generated in 2012 [32]. However, no overt tau pathology developed, suggesting that endogenous levels of even pathogenic tau were not sufficient to achieve tau pathology, including tau filament formation, within the relatively short lifespan of a mouse – this has only been achieved through the generation of overexpression models. In 2016, we used the TALEN gene editing tool to generate knock-in mice in which a photoconvertible mEOS2 tag was introduced in-frame into the carboxy-terminus of the *MAPT* gene [33]. The mice expressed all three major brain isoforms of tau, with the isoform ratio being similar to that reported for wild-type mice. Interestingly however, we found that the level of tau expression was approximately 15-fold lower than that of wild-type animals. By establishing primary cultures from these mice, the distribution of tau in different subcellular compartments could be analyzed using live-cell imaging, photoconversion and FRAP (fluorescence recovery after photobleaching) assays. It was found that Tau-mEOS2 followed a proximo-distal gradient in axons and a subcellular distribution similar to that of endogenous Tau in neurons obtained from wild-type mice. These features were abolished, when either human wild-type or P301L mutant tau were overexpressed. These results highlight the potential confound of overexpression systems. They simultaneously highlight the value of gene-edited models in gaining a deeper understanding of the mobility and dynamics of tau in response to both physiological and pathological stimuli.

To address the issue of a potential overexpression artifact in the context of AD and FTD pathogenesis, attempts have been made to humanize the relevant murine genes and then introduce pathogenic mutations found in familial cases of AD and FTD. This approach has not remained without challenges. Most progress has been made towards modeling Aβ pathology. When the sequence in the murine A*PP* locus was humanized

by replacing three amino acids, predictably, these mice did not develop Aβ plaques [34]. However, the simultaneous knock-in of *APP* harboring both the *APP*^{swe} and the Beyreuther/Iberian (I716F) mutations has led to Aβ deposition by 6 months of age, with the introduction of an additional *APP* mutation, the Arctic (E693G) mutation, leading to the formation of Aβ deposits as early as 2 months of age in homozygous mice [34]. These strains presented age-dependent memory impairments corresponding to the number of mutations that had been introduced [34], together with an Aβ pathology that was more pronounced in female mice [35]. Obviously, this strategy combines mutations and generates a hybrid protein that does not exist in nature and may lead to an altered interactome. Humanizing tau is even more challenging due to the large size of the *MAPT* locus and the coding sequence, and the difference in isoform composition in adult mice and humans [36]. There is also the question of which sequences should be humanized. The challenge that these issues present is reflected by the absence of published outcomes despite attempts by several laboratories.

As reviewed previously [37], other species, such as the sea lamprey (*Petromyzon marinus*) and zebrafish (*Danio rerio*), have also been explored as a means to dissect specific aspects of tau pathology. Large animal species in which neurodegeneration has been successfully modeled include the pig and sheep [38, 39], with features of AD pathology being modeled in transgenic pigs [40].

Comparative Analysis of Tauopathy Models

With advances in research technologies, there is often a change in focus and therefore also in the expectations of what an animal model needs to deliver. As discussed above, an initial goal has been to reproduce aspects of the human pathology, at least at a histological and biochemical level. Considering that AD and FTD are neurodegenerative diseases, an authentic model would need to present not only with a tau (and Aβ) pathology, but also with the accompanying synaptic and neuronal degeneration. These aspects have been achieved in a subset of models and generally required the overexpression of FTD mutant tau [5, 13]. A major challenge lies in comparing the different models and assessing their validity, due to either incomplete analysis or the lack of standardized analytic techniques.

A further challenge that arises is modeling the role of tau pathology in impairing various domains of cognition and motor function. Such impairments occur due to the accumulation of pathological tau in particular brain areas, which is determined by several factors including the integration site of the transgene (which determines the expression pattern) and the copy number (which is somewhat linked to expression levels). The pattern of expression and pathology inevitably differs from that encountered in for example AD brains, where the pathology follows a stereotypical pattern with disease progression as formulated in the Braak staging scheme [41], which is believed to be due to a stereotypical process of tau spreading [42, 43], in part mediated by exosomes [44–46], and an area discussed elsewhere in this book in more detail **Chaps. 21**, **34** and **35**).

Finally, a recent review by Ahmed et al. of mouse models of FTD (which goes beyond tau and also includes C9ORF72, progranulin, TDP-43 and VCP) [47] discusses them in the context of the functional impairments observed in human tauopathy. These include behavioral, socioemotional, motor and memory functions, as well as language, eating and metabolism. Several of these impairments have indeed been modeled in mice, adding to the validity of such models for the study of tauopathy. For example, it has been reported that impaired ultrasonic vocalizations in aged mutant tau mice correlate with tau pathology in the mid-brain and brainstem nuclei controlling vocalization and respiration [48]. This may partially resemble the language disorders observed in FTD patients, although the finding awaits confirmation in other lines. Another FTD tau mutant mouse model displayed age-dependent signs of impulsivity, decreased social exploration, and executive dysfunction. The deficit in

executive function was initially limited to decreased spatial working memory, but with aging extended to impaired instrumental short-term memory. Importantly, tau pathology was prominent in brain regions underlying these behaviours [49]. One of the propositions of the review by Ahmed and colleagues [47] was to (re) examine existing FTD mouse models based on the functional impairment in human patients. They further suggested that defining FTD mouse models, in isolation or in combination, based on functional deficits driven by clinical observations may improve drug development and testing.

Insight into Pathomechanisms

It is impossible to provide an exhaustive overview of the pathomechanistic insight that has been gained in animal models and pay tribute to the wealth of data that have been obtained. Here, we would like to focus on two findings made in our laboratory. Both involved studies in which cell culture systems were complemented by animal models.

The first study focused on why tau, which is mainly perceived as an axonal protein, accumulates in the somatodendritic domain. Early in development, tau is distributed throughout the neuron; with maturation, however, it becomes enriched in the axon [50]. In AD and related tauopathies, tau accumulates in both the soma and the dendrites in a hyperphosphorylated form [51]. It is generally assumed that hyperphosphorylated tau in the axon detaches from the microtubules and passes through the axon initial segment, which serves as a diffusion barrier for physiologically phosphorylated tau, before accumulating in the cell body and dendrites, a process that is partly mediated by Aβ [52, 53]. We asked whether Aβ employs a mechanism other than relocalization of tau to account for the massive accumulation of this protein in the somatodendritic compartment. Because tau is known to interact with the Src kinase Fyn [25, 54], we investigated this crosstalk in more detail, initially by co-transfecting tagged tau and Fyn expression vectors into HEK293 cells. This revealed that tau levels were massively induced by Fyn [55]. In addition, tau was found to be strongly phosphorylated. This boosting was not seen when the dendritic tau homolog MAP 2 or GFP was co-expressed with Fyn, indicating the specificity of this effect [55]. Importantly, the boosting effect of Fyn on tau was suppressed with cycloheximide and not with actinomycin D, suggesting (and subsequently confirmed) that Fyn induces tau translation rather than transcription. We further showed that Fyn activates the ERK/S6 pathway in an mTOR-independent manner. We then incubated primary neurons with Aβ and showed that the oligomeric form of the peptide induced the *de novo* protein synthesis of tau via a Fyn/ERK/S6 pathway. We further revealed that Aβ employed this pathway to achieve *de novo* protein synthesis specifically in the somatodendritic compartment. Activation of the same pathway was demonstrated in a range of cellular systems, and *in vivo* in brains from Aβ-depositing APP23 mice [56], Aβ-injected wild-type mice, and Fyn-overexpressing FynCA mice [57] with tau accumulation [55]. Our findings even extended to human patients, where tau levels in the cerebrospinal fluid of AD patients, but not patients with FTD, were shown to rise steeply, suggesting that Aβ is a driver of the tau increases that occur in AD [58, 59]. We believe that tau is not only a substrate of the Aβ/Fyn/EEK/S6 cascade, but may also have a role in the translational machinery itself. Indeed, tau has been shown to interact with RNA-binding proteins such as TIA1, which has been reported to facilitate the formation of stress granules in tauopathy [60]. These stress granules are believed to sequester specific mRNAs, altering the synthesis of particular sets of proteins and therefore potentially having a role in tau pathology [60, 61]. Together, these findings indicate that there are distinct differences in the pathomechanisms of primary and secondary tauopathies.

The second example has to do with the fact that tau is best perceived as a scaffolding protein. This means that, with its pathological accumulation in the somatodendritic domain, it may bind to additional proteins. We showed this to be the case, when we investigated the parkinsonism and

axonal transport impairment phenotype in K369I tau transgenic K3 mice [62, 63]. We found that the pathological tau which accumulated in the soma trapped the kinesin adaptor JIP1, thereby preventing it from loading distinct cargoes, including mitochondria, onto the kinesin 5 motor, resulting in their impaired axonal transport [62, 63]. Interestingly, in very recent work, we identified a similar trapping effect as an underlying pathomechanism when we tried to understand why pathological tau (this time wild-type and P301L human tau) impairs mitophagy [64, 65]. Here, we complemented a HEK293 cell system with a *C. elegans* model of tauopathy to reveal that pathological tau traps parkin in the soma, thereby preventing it from localizing onto mitochondria [65]. These findings add a new dimension to our understanding of tau toxicity, illustrating the existence of a vicious cycle whereby tau not only contributes to mitochondrial dysfunction, but also inhibits the clearance of the resultant damaged mitochondria [66, 67].

From Mechanisms to Therapeutic Interventions

Strategies that directly target tau and have reached clinical development involve blocking its aggregation and vaccination, whereas indirect strategies involve stabilizing microtubules, as well as manipulating kinases and phosphatases that govern the post-translational modification of tau [68]. Other important approaches that have been tested in animal studies include reducing tau oligomerization, facilitating autophagy and protein clearance, supporting mitochondrial function and mitigating oxidative stress, as well as reducing tau levels, hyperphosphorylation and pathological tau propagation, either directly or through vaccination [68]. As far as the latter is concerned, guidance has been provided by immunization strategies targeting Aβ, although tau is a more challenging target because it is mainly deposited intracellularly and because we still do not fully understood which post-translational modifications and tau species need to be targeted for therapeutic intervention. Considering the subtle phenotype of tau knock-out mice [69], the view has emerged that, rather than targeting distinct post-translational modifications, it may suffice to reduce overall tau levels. In this context, pan-tau-specific antibodies represent a potential treatment strategy.

Many phospho-epitopes have already been tested in mice. The active immunization approaches used a peptide containing important tau phospho-epitopes (pS396/pS404 (known as the PHF1 epitope), pS202/pT205 (AT8), pT212/pS214 (AT100), pT231/pS235 (AT180) and pS422), proving efficacious in preventing or reducing pathology in tau transgenic models, in the absence of overt side effects [68]. Complementing these active approaches, passive strategies have also been pursued using the PHF1 antibody and the conformation-dependent anti-tau antibody MC1, resulting in behavioral and cognitive improvements in mouse models [68]. Neuronal uptake of therapeutic anti-tau antibodies is believed to be crucial for vaccine efficacy, although such uptake has not been reported in all preclinical studies. Because tau is also present in the extracellular space, however, antibodies that have not been shown to be taken up into neurons are predicted to block both the uptake and the seeding activity of extracellular tau aggregates, and have been demonstrated to ameliorate the cognitive deficits in tau transgenic mice [70]. A recent study has shown that different antibodies targeting extracellular tau might have different modes of action, and some antibodies have been shown to facilitate the clearance of extracellularly added tau aggregates by microglia-like BV2 cells, although others do not [71]. Overall, several tau-targeted active and passive vaccines are currently in phase I and II clinical trials in AD and PSP, with PSP being an attractive testing ground because of the absence of Aβ pathology as a comorbidity factor [68].

More recently, we have begun to investigate ultrasound as a new treatment strategy for AD and tauopathies more generally. Ultrasound is a mechanical pressure (sound) wave with a frequency above 20 kHz, the highest frequency that can be detected by humans [72]. Ultrasound is used in a wide range of diagnostic imaging

applications, primarily in the fields of obstetrics and cardiology, but also for examining the abdomen and musculoskeletal system. In this context, ultrasound waves are transmitted from the transducer into the patient [72]. The wave is partially reflected at tissue interfaces and the 'echoes' are detected by the same transducer. Bone tissue highly attenuates the propagation of ultrasound, so current neurological ultrasound imaging must be performed at 'acoustic windows' in the skull, where the bone is thin enough for adequate signal transmission. To date, this approach has only proven suitable for imaging structures in limited areas within the brain, such as blood vessels that are basal to the skull. Therapeutic ultrasound uses similar instrumentation at a higher power, and can be broadly categorized as either thermal or non-thermal. In the latter case, ultrasound is combined with intravenously injected microbubbles to achieve transient opening of the blood-brain barrier (BBB). Microbubbles are biologically inert and have either a lipid or polymer shell and a stabilized gas core. They can be systemically administered and subsequently exposed to non-invasively delivered focused ultrasound pulses [72, 73]. Microbubbles within the target volume become 'acoustically activated' by what is known as acoustic cavitation. In this process, the microbubbles (with a diameter of up to 10 μm, matching that of small capillaries) expand and contract over several cycles. These dilations and contractions displace the vessel wall [74, 75]. More specifically, the mechanical interactions between ultrasound, microbubbles and the vasculature transiently open tight junctions and facilitate size-dependent transport across the BBB [76]. These microbubbles are clinically approved and routinely used as contrast agents for diagnostic ultrasound.

We and others have shown that microbubble-mediated BBB opening in the absence of any therapeutic agent reduces the Aβ pathology in APP mutant APP23 mice [77–80]. Following 5–8 weekly treatments of 12 month-old APP23 mice with ultrasound in a scanning mode, Aβ species ranging from monomers to oligomers to high molecular weight species could be reduced effectively, plaques were partially cleared, and memory functions were restored in three complementary behavioral tests [78]. Our results also revealed that the underlying clearance mechanism involved the internalization of Aβ by brain-resident dormant microglial cells that became activated by unidentified blood-borne factors which entered the brain as a consequence of the transient opening of the BBB. Importantly, we found that tau pathology (being mostly intraneuronal) could also be partially ameliorated with this approach, as shown in the pR5 transgenic mouse model of tauopathy which accumulates hyperphosphorylated tau in neurons [81]. In this particular study, we performed four treatments and also tested the synergistic effects of scanning ultrasound using a single chain variant (ScFv) antibody fragment targeting the 2 N isoform of tau [81]. However, the mechanism by which ultrasound treatment clears tau remains to be determined. Long-term safety in wild-type mice has also been demonstrated, based on a wide range of behavioral, electrophysiological and imaging modalities [82, 83], together with related studies in sheep [84]. Considering the safety and efficacy profile of ultrasound, which is highly tunable, these studies suggest that therapeutic ultrasound may offer a potential treatment strategy for tauopathies, either on its own or in combination with a therapeutic agent [85].

Is There a Future for Animal Models of Tauopathies?

There is an ongoing need for animal models of tauopathies. We believe that there is value in the existing models, which can be further probed to reflect clinical features, to dissect additional pathomechanisms and to validate therapeutic interventions. To increase their utility, a standardized approach of analysis is highly recommended. It can also be anticipated that gene editing tools will be increasingly employed to generate improved animal models and to study key molecules such as tau and the enzymes with which it interacts, such as Fyn, Pyk2 and STEP,

at a subcellular level, using tools such as super-resolution microscopy [86]. Mice are likely to remain a major species for modeling and analyzing tauopathy. However, although used less frequently than mice, rats present with several advantages for the study of human diseases, including their relatively large brain size, which facilitates surgery. Rats are also much easier to handle than mice and are less easily stressed by human contact. In addition, several of the confounds in the behavioral paradigms found in the mouse studies do not apply to rats [87]. It is anticipated that the increased availability of gene-editing tools is likely to pave the way for a multitude of new genetic models based on rats [87]. There is also a continued need for invertebrate models such as flies and worms. These have short generation times, are cheap in their handling, do not require animal ethics approval and are ideal for fast genetic screening. In an ideal world, pathological findings would be demonstrated in multiple models, either in two or more strains of the same species (such as two complementary tau transgenic mouse models) or in a cellular system complemented by an invertebrate and a vertebrate model. There is also value in large animal models such as minipigs, sheep or non-human primates; however, their availability, the time required to establish an aged colony, the hurdles posed by ethics approval, and the cost and effort required for their analysis presents practical impediments for their widespread use. Cell culture systems, even when derived from human induced pluripotent stem cells [88], cannot model brain or immune interactions or the BBB, and aging can only be modeled to some extent. Whether organoids provide a viable solution remains to be determined [89]. There is no doubt that this technology opens up numerous possibilities for scientific discovery in developmental biology, as well as in translational research, but whether organoids can truly live up to this challenge is, for some, still an open question [90]. Transplanting human cell-derived organoids into the brains of animals such as mice would be a compromise and may allow the brain to function as a Petri dish for human tissue [91].

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29

Cerebrospinal Fluid and Plasma Tau as a Biomarker for Brain Tauopathy

Mikio Shoji

Introduction

Presence of tau in cerebrospinal fluid (CSF) was first discovered by Vandermeeren in 1993 [1]. They developed specific monoclonal antibodies against human tau, and phosphorylated tau (ptau) [2, 3], and set up specific ELISA consisted of AT120 antibody and rabbit anti-human tau antiserum. Quantitation of total 190 CSF samples from Alzheimer's disease, controls and other neurological disease showed assay detection limit of CSF tau was less than 5 pg/ml and increased tau levels in AD compared to those of controls. However, marked overlap value between AD and other neurological diseases was observed in this initial report. In 1995, 5 assay results of CSF tau were published at once. Vigo-Pelfrey group measured CSF tau from 181 patients using ELISA using 16G7 and 16B5 antibody to tau [4]. We also developed different ELISA system using anti-Ht-2 and F-F11 antibodies and confirmed increased CSF tau in AD patients [5]. Data using present common ELISA system for tau and ptau provided Innogenetics N.V. in Belgium (now Fujirebio Europe N.V.) published from this year. Hock reported that CSF tau levels are increased in AD, preclinical stage of AD, familial AD cases, and correlated with severity of dementia. In this assay, AT120 was adopted for captured antibody and combination with HT7 and BT2 was used for reporter antibodies [6]. Blennow first reported the presence of phosphorylated tau at threonine 181 (p181tau) and threonine 231 (ptau231) by ELISA using AT180 for ptau231 and AT270 for ptau181 as captured antibodies and combination of HT7 and AT120 for reporter antibodies. Although tau and ptau levels were significantly increased compared with controls, overlap of assay values were observed also between AD and other neurological diseases [7]. Presently, this p181tau assay system is altered to use HT7 for capture and AT270 for detection antibodies [8]. Arai showed significant increase of CSF tau and presence 50~65 kd tau bands in CSF samples by western blot suggesting that CSF tau might reflect the progressive accumulation of altered tau due to progressive death of neurons in the AD brain [9]. This is short history of developing of CSF tau and ptau assay. Afterward these basic findings, huge number of studies of CSF tau and ptau open up doors to the definite biomarkers for diagnosis and prediction of AD, and the clarification of tauopathy mechanisms, establishment for global standardization, and finally valuable tools for development of essential therapy for AD.

Major papers about CSF tau studies are reviewed and summarized here, and the vison of future is commented.

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Definite Biomarker for AD

In the same 1995, another way of AD biomarker had initiated. Motter first reported that CSF Aß42 levels were decreased in spite of increased levels of CSF tau in AD patient [10]. CSF samples from 37 AD, 32 neurological disease and 20 nondemented controls were evaluated by ELISA using 266/277-2 antibodies for Aß42 and 16B5/16G7 antibodies for tau. In 1998, Kanai published the first longitudinal and multicenter study of CSF Aß40, Aß42 and tau. The study consisted of 93 AD, 33 non-AD dementia, 56 other neurological disease CSF samples were evaluated by Innogenetics tau assay and common Aß ELISAs of BAN-50 for capture and BA-27/BC-05 for detecting Aß40/Aß42, respectively. A significant elevation of tau and correlation between the tau levels and the clinical progression were observed in AD. A significant decrease of the Aß42 levels and a significant increase of Aß40/42 ratio were observed in AD suggesting that CSF tau increases with clinical progression of dementia and alteration of Aß42 and Aß40/42 ratio starts at early stages in AD. Efficient diagnostic sensitivity (71%) and specificity (83%) were revealed by using combination of tau and Aß40/42 ratio values. Improvement in sensitivity up to 91% was obtained in longitudinal evaluation [11]. Additional reports by different assay systems supported these findings [12, 13].

Different assay methods of ptau also reported. Increased level of ptau at serine 199 using ELISA with HT-7 and Anti-PS199 was published by Ito in a large scale multicenter study consisted of 570 CSF samples, in 2001 [14]. However, in global standardization study in comparative CSF study among ptau231, ptau181, and ptau199 showed that ptau181 assay reached specificity levels greater than 75% when sensitivity was set at 85% or greater [15].

For clinical application in differential diagnosis of AD, another large-scale multicenter study by Shoji analyzing total 1,031 samples from 366 AD and 168 non-Alzheimer dementia, 316 non-demented neurological disease and 181 normal controls showed the cut-off value of CSF tau was 375 pg/ml, 59% sensitivity and 90% specificity for diagnosis AD compared to other groups. Simultaneously, elevation of CSF tau level was observed in other chronic and acute brain damage disease suggesting required attention for clinical practice [16]. Andreasen also showed sensitivity, specificity and stability of CSF tau in AD in a community-based patient samples showed the cut-off value of CSF tau was 302 pg/ml, 93% sensitivity and 86% specificity for diagnosis AD compared with controls and suggested some neurological conditions (e.g., stroke) increases CSF tau [17]. Finally, these findings were validated by prospective comparisons between antemortem CSF tau and Aß and autopsy-confirmed dementia diagnosis [18].

Systemic Review and Meta-Analysis

From these basic findings era, CSF biomarker study expanded to clarify the diagnostic efficacy of CSF tau and ptau in huge number of cohort studies including AD dementia, mild cognitive impairment (MCI) due to AD, cognitively unimpaired (CU) state in AD and other neurological diseases. According to recent systemic review by Olsson, 231 articles comprising 15,699 AD and 13,018 controls were analyzed and showed CSF tau, ptau and Aß42 strongly differentiated AD form controls, and MCI due to AD from stable MCI. Total 164 cohorts with AD and 153 control cohorts representing 11,341 AD and 7,086 controls showed that average tau concentration ratio between AD and controls are 2.54. In CSF ptau, 98 studies comprising 7,498 AD from 96 cohorts and 5,126 controls from 91 cohorts showed the ptau concentration ratio is 1.88. Comparison between 12 cohorts with 307 MCI and 570 stable MCI showed that average concentration ratio of CSF tau is 1.76 and those analysis with 9 cohorts comprising 251 MCI due to AD and 501 stable MCI of CSF ptau is 1.72 [19].

Differential Diagnosis and Prediction for Onset of AD

During these 20 years, based on development of basic research and neuroimaging tools, clinical classification and diagnosis criteria of neurogenerative dementia have been refined and subdivided in detail. These developments facilitate rigorous evaluation of CSF biomarkers in newly identified neurogenerative diseases. Toledo examined pathology confirmed neurodegenerative dementia patients and showed 26.8% of AD combines another pathology and 14~17% underestimation of biomarker accuracy. CSF tau and ptau are increased in AD and AD-Dementia with Lewy bodies (DLB) [20]. Both tau and ptau values themselves did not discriminate behavior variant frontotemporal lobar degeneration (bvFTLD) and frontotemporal dementia (FTD) without combination of Aß42 values [21, 22]. The systemic review and meta-analysis of idiopathic normal-pressure hydrocephalus indicated significantly reduced levels of tau, ptau and Aß42 compared to healthy normal state [23]. In Creutzfeldt-Jakob disease (CJD), highly elevated CSF levels of tau and 14-3-3 protein are established biomarkers. Rumeileh showed tau yielded 80.6% sensitivity and 75.3% specificity in distinguishing AD from CJD. However, ptau alone showed no eligible significance. Cut-off value was proposed to be >1200 mg in tau and >16.4 in tau/ptau ratio [24]. Ewers studied diagnostic value of CSF Aß42, tau and ptau in 675 CSF samples from controls, AD dementia, subjective memory impairment, vascular dementia, LBD, and FTD, depression, and other neurological disease. As the results, Aß42 showed the best diagnostic accuracy among them. At a sensitivity of 85%, the specificity to differentiate AD dementia against other diagnosis ranged from 42% for DLB, 77% for FTD. However, significant overlap with other non-AD dementia, possibly reflected the underlying mixed pathology [25].

Recently, we have independently reexamined CSF tau and ptau in a total of 213 CSF samples from various neurological diseases and CU subjects [26]. Tau levels were 259.3 ± 162.8 pg/ml in CU, and were significantly higher at 738.4 ± 290.6 pg/ml in AD dementia/MCI ($p < 0.0001$), 1,337 ± 1554 pg/ml in encephalopathy (ENC) ($p = 0.036$), and 415.7 ± 158.2 pg/ml in multiple system atrophy (MSA) ($p = 0.0164$) than in CU. One patient with CJD had a CSF tau level of 1,554 pg/ml (Fig. 29.1a). P181tau levels were 41.69 pg/ml in CU and significantly increased to 88.62 ± 29.69 pg/ml in AD dementia/MCI ($p < 0.0001$). No significant changes were observed in other diseases. The CSF tau/p181 tau ratio was 6.0 ± 1.8 in CU, and increased to 8.2 ± 1.2 in AD dementia/MCI ($p = 0.0038$), 38.8 ± 55.6 in ENC ($p = 0.013$), and 10.2 ± 3.1 in MSA ($P < 0.0001$). In CJD, this ratio was 25.9. Specific changes due to AD processes were recognized in P181tau levels. This study corresponded of previous 1,031 subjects multicenter study showing overlap values in the tauopathy and other neurological disease groups, with moderate measurement sensitivity and specificity as a biomarker using mean ± 2 SD as a cutoff value [16]. Total tau was increased in some diseases because of different pathological processes, including tauopathy due to AD, acute brain injury by ENC and CJD, and axonal degeneration in MSA. No significant changes were detected in total tau, p181tau, or their ratio in CBD, PSP, or FTD. The present results on the CSF tau/p181tau ratio suggest that it was 6:1 and that the phosphorylated-tau tangle pathology increased p181tau and secondarily induced brain injury due to increased total tau levels (8:1), even in AD.

ADNI and DIAN Study Demonstrated Signature of AD

Then, epochal 2 global studies initiated; One is Alzheimer's Disease Neuroimaging Initiative (ADNI) from 2003, which demonstrated natural course of cognitive function, neuroimaging and CSF biomarkers in cognitively unimpaired (CU), MCI and dementia stage of pure sporadic AD. Another is further convincing study of signature of biomarkers in dominantly inherited AD, Dominantly Inherited Alzheimer's Disease (DIAN) from 2008.

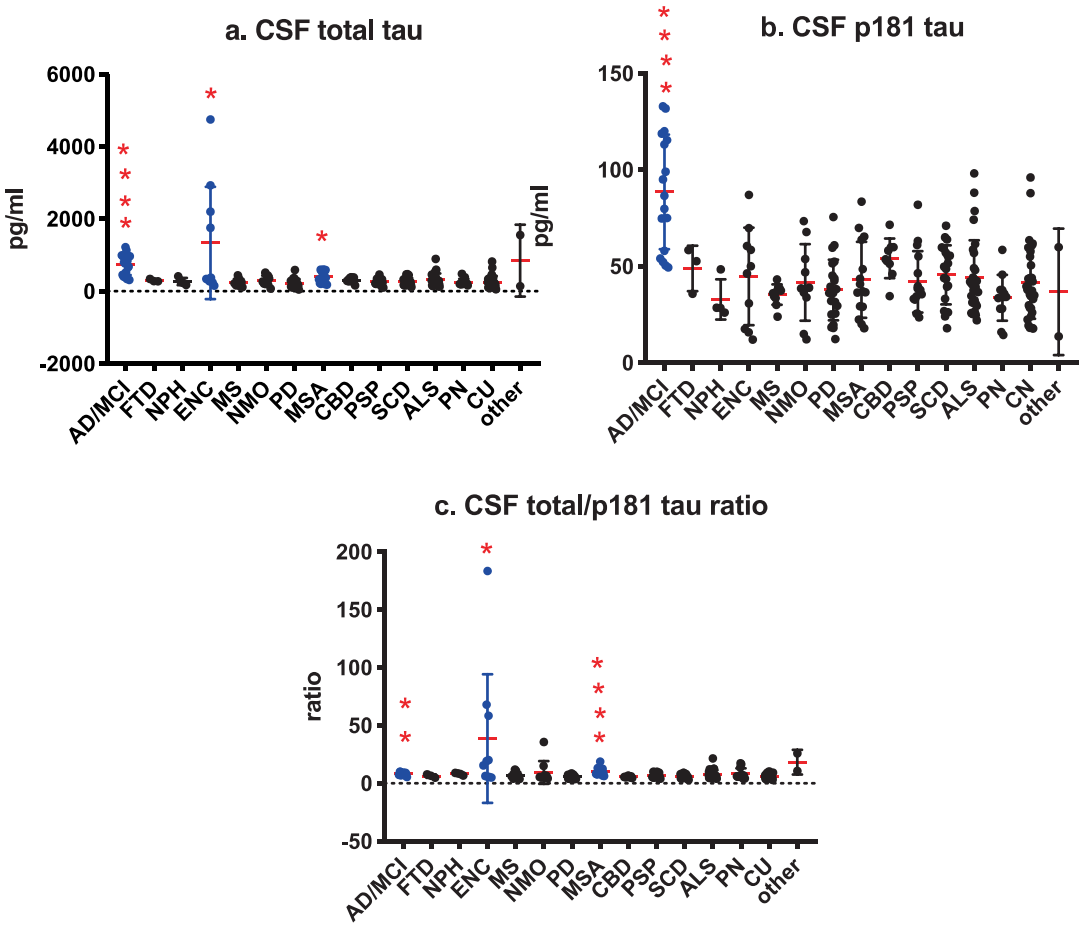

Fig. 29.1 Total tau, phosphorylated-tau in CSF from 231 neurological diseases
∗: $p < 0.05$; ∗∗: $p < 0.005$; ∗∗∗: $p < 0.0001$
Abbreviations: *ADD* Alzheimer dementia, *MCI* mild cognitive impairment, *FTD* frontotemporal dementia, *NPH* normal pressure hydrocephalus, *ENC* meningoencephalitis, *MS* multiple sclerosis, *NMO* neuromyelitis optica, *PD* Parkinson's disease, *MSA* multiple system atrophy, *CBD* corticobasal degeneration, *PSP* progressive supranuclear palsy, *SCD* spinocerebellar degeneration, *ALS* amyotrophic lateral sclerosis, *PN* polyneuropathy, *CU* cognitively unimpaired control subjects. Units of CSF total tau and phosphorylated-tau were pg/mL

Shaw showed that CSF Aß42 was the most sensitive biomarker for AD in ADNI cohort and autopsy-confirmed subjects. Cut-offs, sensitivity and specificity discriminating between AD and CU subjects were 93 pg/ml, 69.6% and 92.3% in tau, 23 pg/ml, 67.9%, and 73.1% in ptau 181, 192 pg/ml, 96.4% and 76.9% in Aß42, and 0.39, 85.7% and 84.6% in tau/Aß42 [27]. Multicenter quality control study of ADNI samples using INNO-BIA AlzBIo3, xMAP technology showed intra center assay CV% was 5.3% in Aß42, 6.7% in tau and 10.8% in ptau181. Those of inter-center CV% was 17.9% in Aß42, 13.1% in tau and 14.6% in ptau181 [28]. Follow-up during 48 months in ADNI cohort showed that low Aß42 values were associated longitudinal increase in ptau181, and high baseline ptau181 values were conversely not associated win changes of Aß42 levels [29]. Recent report from ADNI consisted of 56 CU, 73 MCI and 17 AD over 1~7 years follow-up divided by Aß+ and Aß− groups depend on Aß42 cut-offs 192 pg/ml, showed significantly increased baseline levels of CSF tau and ptau in Aß + CU, Aß + MCI and Aß + AD

dementia. Longitudinally, tau levels increased in both Aß + CU and Aß + MCI, but, decreased in Aß + AD dementia. Longitudinally, ptau levels increased in Aß + CU and significantly decline in Aß + AD dementia. Both follow-up study showed increase of tau and ptau mainly increase MCI stage, and conversely decline in dementia stage [30].

DIAN study is the unique study of dominantly inherited AD (DIAD). DIAD is caused by mutations of *APP, APP* duplication and *PSEN-1/-2* mutations. Although the onset age is variable depend on each gene mutation type, penetrance is 100% and the onset age and prognosis is essentially identical with carrier's parent with DIAD. These findings indicate that survey of mutation carrier can reveal definite preclinical alteration and natural course of biomarkers and cognition before onset. In 2012, Bateman clearly showed the order and magnitude of pathologic processes in AD. CSF Aß42 levels appeared to decline 25 years before the symptom onset. Aß deposition in the brain detected by PiB amyloid PET, increased CSF tau and brain atrophy initiated before 15 years of onset. Cerebral hypometabolism and episodic memory disturbance appeared before 10 years. Finally, global cognitive impairment was detected 5 years before onset. Then, 3 years after onset, patients met diagnostic criteria for dementia [31, 32]. Thus, DIAN study conclusively established big data of all alterations of biomarkers and cognitive functions which gradually progress during 25 years and confirmed these orders speculated by ADNI study. Both results by ADNI and DIAN study provide us extremely useful tool to open interventions for prevention of AD.

Prospective Study for Prediction of MCI and Dementia Due to AD

Natural course of CSF biomarkers of incipient AD from CU to MCI was also studied. Hansson examined 137 MCI during 5.2 years follow up and showed 42% developed AD dementia and 15% develop other form of dementia. CSF biomarkers at baseline yielded sensitivity 95% and specificity 83% in combination with tau and Aß42, and those of 95% and 87% in combination ptau181 and Aß42 for detection AD dementia converter [33]. DESCRIPIA prospective study from 20 memory clinic across Europe during 2003–2005 showed CSF AD profile (low Aß42/high tau value) was common in 52% of subjective cognitive impairment (SCI), 68% of non-amnestic MCI (naMCI), 79% of amnestic MCI (aMCI) and 31% CU and associated with cognitive decline in naMCI and aMCI [34]. In subjects with MCI and abnormal CSF Aß42 profile, CSF tau and ptau and hippocampal atrophy can predict further cognitive decline [35]. Prospective 9-year study of 44 CU showed that 6 of 12 with low baseline CSF Aß42 developed AD, but other 6 subjects with low Aß42 and 32 with normal Aß42 did not develop AD. CSF tau and ptau did not predict development AD/DLB over 9 years [36].

Adult Children Study of 169 middle-aged CU during 6 years prospectively also revealed that longitudinal reduction in Aß42 were observed in some individuals as early middle age and low Aß42 levels were associated with the development of cortical amyloid deposition, especially in mid middle age during 55–64 years. CSF tau and ptau as neuronal injury markers dramatically increased in some individuals in mid and late middle aged during 55–74 years [37]. CSF Aß42 was correlated only with PiB binding, but, CSF tau, ptau and hippocampal volume were correlated with the longitudinal alteration in global cognition [38, 39]. Toledo recently reported a global large multicenter study of CSF samples from 1,233 healthy cohort subjects, 40–84 years, from 15 cohorts from 12 different centers by Luminex® assay of Aß42, tau and ptau in Gothenburg Laboratory. At 40 years of age, 76% of subjects were classified normal Aß42, tau and ptau and their frequency decreased to 32% at 85 years. Normal Aß42 and increased tau/ptau group frequency increased slowly from 1% at 44 years to 16% at 85 years. Low Aß42 with high tau/ptau frequency increased from 1% at 53 years to 28% at 85 years. Abnormal low Aß42 were already frequent in middle-life and APOE genotype strongly affects the Aß42, tau and ptau in

Swedish BioFINDER (n = 277) and ADNI (n = 646) [40].

Standardization and Newly Developed Assay Technology

To assay AD biomarkers simultaneously and automatically, the flow cytometric-based Luminex xMAP® technology involves coupling of specific monoclonal antibody sets to the surface of microbeads uniquely identified with a combination of fluorescence dyes in a single sample assuming no cross-reactivity of particular antibodies. The standard assay system named The INNO-BIA AlzBio3 is commonly used to measure Aß42, tau and ptau181 consisted of corresponding capturing antibodies (tau: AT120, ptau181: AT270, Aß42:4D7A3) and biotinylated detection antibodies (HT7 and 3D6). Intra-and interassay CVs were less than 10% for all analytes [41]. Multiplexed quantification correlated with results by usual ELISA assay and improved sample management and quality control of assay [42]. Recently, Roche Diagnostics developed Elecsys assays that utilize the automated cobas 601 analyzer exhibited further precision accuracy, reliability, reproducibility with between-laboratory CV of approximately 4% [43, 44]. Large concordant study between cut-offs for Elecsys assay and amyloid PET using Swedish BioFINDER (n = 277), ADNI (n = 646) and clinical progression in MCI (n = 619) showed tau/Aß42 and ptau/Aß42 ratios were highly concordant with PET classification in BioFINDER (overall agreement: 90%) and ADNI classification (overall agreement: 89–90%) and predicted greater 2-year clinical decline in MCI [44]. The Alzheimer's Association quality control program participated by 40 laboratories in 2011 showed total CVs among centers were 16–28% for ELISA,13–36% for xMAP, and 16–36% for Meso Scale Discovery [45]. Extended quality control program participated 84 laboratories reported that CVs between laboratories were around 20–30%; within-run CVs, less than 5–10%; and longitudinal with-in laboratory CVs were 5–19%. For tau and ptau, between-kit lot effects were less than between-laboratory effects [46]. Temperature at stored, non-frozen time, contamination such as detergent and blood, centrifugation and tube materials have a significant effect on assay variability [47].

Origin of CSF Tau and pTau

During these 25 years, no one believed tau is normally secreted into CSF. Everyone also tried to clarify the missing link between Aß amyloidosis and tauopathy in AD pathological processes. Sato illuminated these issues using kinetic study of stable isotope labelling tau and mass spectrometry in the human central nervous system and iPSC-derived neurons. Brain full length tau is C-terminally truncated at residues 210–230 and released from human neurons in 3 days and ~14 days into CSF. Average half-life of CSF tau is 23 days and its production rates are 26.3 ± 9.2 pg/ml/day. Increase in CSF tau in AD is due to an increase in synthesis and release and positively correlated with amyloidosis. There was no correlation between tau fraction turnover rate and tau PET imaging. Increased tau production and soluble tau secretion are initiated by amyloid toxicity in very early MCI. Then, increased aggregated tau and decreasing elevated CSF tau appear and induce trans-synaptic spreading of aggregated tau, causing cortical cognitive function deficits in early to mild AD dementia stage [48]. He reported that Aß plaque facilitates the rapid amplification of aggregated tau seeds into large tau aggregates in dystrophic neurites of senile plaques, induces formation and spread of neurofibrillary tangles and neuropil threads as secondary seeding events [49].

Association Between CSF Biomarkers and Newly Developing Tau Neuroimaging

Tau PET ligand [^{18}F] flortaucipir have been developed to detect in vivo tau accumulation in AD. Brain tau accumulation initiated from temporal lobe at early MCI stage, progressively

extended to parietofrontal lobes and closely associated with cortical cognitive functions and severity of dementia. Comparison between postmortem brain pathology and regional in vivo uptake of [^{18}F] flortaucipir showed close correlation with density of tau-positive neurites, intrasomal neurofibrillary tangles and total tau burden. No correlations between [^{18}F] flortaucipir and Aß pathology were found [50]. CSF tau and ptau increase from preclinical AD, despite normal [^{18}F] flortaucipir retention, suggesting that appearance of positive tau PET findings initiates later stage of MCI than those of CSF tau and ptau [51, 52].

Plasma Phosphorylated Tau as Possible Biomarker for AD

Recent studies have clarified that the plasma Aß42/40 ratio is inversely correlated with cortical amyloid burden in AD, which can be converted to MCI, and that the plasma Aß42/40 ratio is a useful screening marker for brain Aß amyloidosis in normal individuals [53, 54]. In a similar way, quantitation of plasma tau and ptau as a screening biomarkers for brain tauopathy are developing. Mattsson studied of plasma tau levels using total of 1,284 participants from ADNI and BioFINDER cohorts. Plasma tau partially reflects AD pathology, but the overlap between normal aging and AD is large, especially in patients without dementia [55]. Tatebe tried to quantitate plasma ptau181 using modified Simoa™ Tau 2.0 kit on Simoa HD-1 analyzer (Quantrex). Plasma ptau181 levels were significantly increased in AD and Down syndrome patients compared to controls [56]. Mielke measured plasma ptau 182 using the Meso Scale Discovery platform with antibody AT270 for capture ptau181 and antibody SULFO-TAG-LRL for detection tau from 172 CU, 57 MCI, 40 AD dementia with concurrent Aß and tau PET. Plasma tau and ptau181 levels were higher in AD dementia than those in CU. Plasma ptau181 was more strongly associated with Aß and tau PET [57]. The values of ptau181 measured Simoa or Meso Scale Discovery are very small but totally different. In former report, plasma ptau181 levels are 0.171~0.045 pg/ml. In the later report, those are 6.4~11.6 pg/ml. There are 100 times differences between recent reports. Basic studies such as plasma Aß kinetics from brain to CSF, plasma and mass spectrographic identification studies have not yet performed. Presence of big tau, a close homologues with brain tau presented in human body organs and peripheral nerve systems, has not been clarified yet [58, 59]. Based on these issues, further developing basic study and large scale confirmation studies are expected.

Recommendation in the Diagnostic Evaluation of MCI and Dementia

In 2017, evidence-based guidelines in the diagnostic evaluation of MCI and dementia due to AD were proposed based on systematic reviews using Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) methods by working group comprised 28 international members [60, 61]. The former report recommends the use of CSF markers in predicting the functional or cognitive decline or conversion to AD dementia within 3 years and counseling both before and after the biomarker evaluation. The later report recommends the use of CSF AD biomarkers as a supplement to clinical evaluation, to identify or exclude AD as the cause of dementia, for prognostic evaluation, and for guiding management of patients, particularly in atypical and uncertain cases. As summarized here, huge numbers of basic and clinical dedications during these 25 years for CSF biomarkers revealed the total figures occurred in person due to AD. However, essential aim of CSF biomarkers for contribution of developing disease modifying therapy and intervention in AD pathological processes are still ongoing. In 2018, National Institute on Aging -Alzheimer's Association (NIA-AA) has proposed research framework using ATN classification system of biomarkers toward a biological definition of AD. In this criteria, AD is considered as a continuum, and cognitive staging is

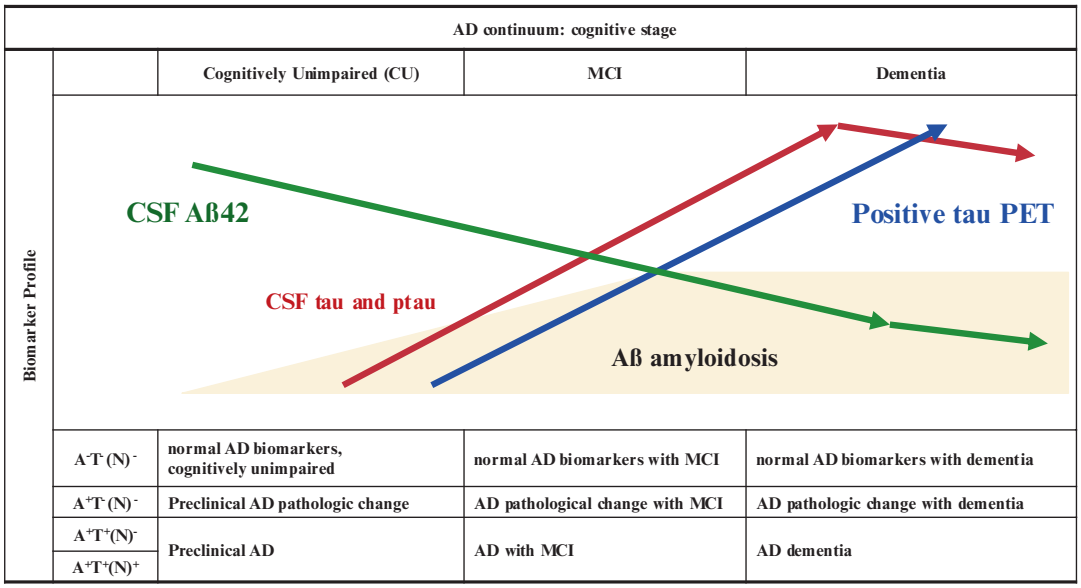

Fig. 29.2 NIA-AA Research Framework and natural course of CSF biomarkers

classified into cognitively unimpaired (CU), MCI and dementia. Biomarker profile is classified in to 3 groups; A: Aggregated Aß or associated pathologic state including CSF Aß42, or Aß42/Aß42 ratio, amyloid PET; T: Aggregated tau (neurofibrillary tangles) or associated pathologic state including CSF ptau and tau PET; (N): neurodegeneration or neuronal injury including Anatomical MRI, FDG PET and CSF tau (Fig. 29.2). Based on this novel NIA-AA research framework criteria, AD process in human brain will be biologically defined further and essential therapy of next generation will be evaluated hopefully [62].

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Dementia Therapy Targeting Tau

30

Luc Buee

This final chapter addresses some of the various researches on a therapeutic potential around the tau protein and its gene, MAPT. Some have fallen into oblivion and others have appeared. This research reflects the different streams of ideas about tau protein and neurofibrillary degeneration.

Currently, MAPT is considered to encode a pleiotropic protein but this was not always the case. Tau is primarily a microtubule-associated protein regulated by its phosphorylation state but has functions in chromatin structure, signal transduction, and nucleic acid protection [1]. Alternative splicing and tau expression are also new aspects of its biology. Finally, its new functions, its other post-translational modifications that phosphorylation, its half-life, its secretion and its degradation recently aroused new interests, as you could discover it in the various chapters of this work.

This chapter is not exhaustive and it covers only a few representative examples. Other recent reviews on tau therapy may also be of interest [2]. Keep in mind that tau is a recent therapeutic target. The hypothesis of the amyloid cascade made of tau a secondary actor. However, there have been some pre-clinical and clinical therapeutic approaches.

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In the following sections, we investigate tau silencing, tau alternative splicing, post-translational tau modifications, tau metabolism, microtubular tau function, tau aggregation, tau immunotherapy and brain homeostasis.

Tau Silencing

To stop tau toxicity, one of the most obvious approaches is probably to modulate the expression of tau protein. Indeed, there is some evidence that decreasing tau expression is beneficial in reducing the electrophysiological and/or behavioral disturbances found in models of Alzheimer's disease [3–5]. This positive side should not obscure the fact that tau protein is essential for synaptic plasticity, signal transduction and nucleic acid protection [6–8].

To silence tau, the antisense oligonucleotide (ASO) and siRNA approaches are the most common ones. MicroRNAs may also be of interest since they have been involved in many tau (dys-) functions [9, 10]. ASO are single-strand oligonucleotides (8–50 nucleotides) designed to complement pre-mRNA/mRNA with unique specificity. Such approach is already in clinical trials (ClinicalTrials identifier NCT03186989) following promising results in rodents and monkeys [11].

Similar approaches are also investigated to modulate tau alternative splicing.

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Tau Alternative Splicing

Alternative splicing of tau has quickly emerged as a therapeutic target. Indeed, some mutations on the MAPT gene in frontotemporal lobar degeneration (FTLD) are responsible for misplicing. Thus, the intron mutations around exon 10 favor its default insertion within tau transcripts leading to overexpression of isoforms with four microtubule binding domains. There are also some pathologies such as type 1 myotonic dystrophy where CTG expansions in the 3' UTR of the DMPK gene are responsible for misplicing or deregulation of alternative splicing. The resulting CUG expansions of the transcripts would lead to the sequestration of certain splicing factors like muscleblind (MBNL). This loss of MBNL function would result in a decrease or absence of insertion of exons 2, 3 and 10 in the tau transcripts and lead to neurofibrillary degeneration (for reviews, [12, 13]).

Correcting the alternative splicing of tau thus seems to be an excellent strategy for some tauopathies like FTLDs and myotonic dystrophies. On the one hand, it will be a question of modulating the insertion of the exon 10 of the other to correct the loss of function of MBNL. The modulation of splicing of exon 10 in FTLDs was not only tested by the use of ASO [5] [14] for its exclusion but also by spliceosome-mediated RNA trans-splicing for its inclusion [15]. In any case, it is possible to modulate the alternative splicing of tau. Concerning myotonic dystrophies, the most advanced results concern MBNL [16] A gene therapy approach with a truncated form of MBNL would correct the alternative splicing of tau in the presence of CUG expansions (Patent WO2015158740A1).

Acetylation and Phosphorylation

In the 1980s, tau was considered the constituent of neurofibrillary degeneration but the notion of phosphorylation was not clear. In the mid-1990s, the notion of pathological phosphorylation is established because even if this phosphorylation is physiological and disappears in healthy subjects due to post-mortem dephosphorylation [17, 18], there is also a "pathological" phosphorylation [19–23]. In addition, some works have suggested that hyperphosphorylated tau proteins may serve as nucleation agents for tau aggregation [24, 25]. Thus, inhibition of tau phosphorylation is clearly considered as a therapeutic approach. Other post translational modifications such as acetylation may also be of interest. For instance, acetylation on Lys residues 274 and 281, as found in AD, can impair AMPAR trafficking and LTP [26].

Acetylation is the second most important post-translational modification after phosphorylation. Tau protein contains 44 lysine residues that can be acetylated. Acetylated tau protein was observed linked to neurofibrillary degeneration in the brains of patients with AD. In addition, tau acetylation would reduce its own degradation when it is phosphorylated, suggesting a link between hyperphosphorylation, accumulation and aggregation of Tau in tauopathies [27]. Acetylated tau protein no longer polymerizes tubulin into microtubules and aggregates more rapidly *in vitro* [28]. Nevertheless, the real role of acetylation on tau pathology remains poorly defined. Other studies have shown the opposite effect where Tau protein nonacetylated aggregates more rapidly *in vitro* than acetylated Tau protein [29, 30]. Salsalate and salicylate have been shown to reduce tau acetylation, tau amounts and decrease cognitive deficits in a mouse model of tauopathy [31].

In fact, modulating phosphorylation has been a major approach: inhibitors of different kinases such as glycogen synthase kinases (GSK) and cyclin-dependent kinases and MAPK (erk, JNK, p38 …) are used.

Lithium chloride and GSK3ß inhibitors have been widely studied [32]. Lithium has been very promising in preclinical models [33]. It has been used for many years in mood disorders [32]. Unfortunately, clinical trial with Lithium did not reach its endpoints [34]. Similarly, GS3ß inhibitors were used in preclinical models of tauopathy [35–37]. In PSP, the GSK3ß inhibitor, Tideglusib, did not allow for any clinical improvement [38].

More recently, the PERK activation is also considered as a therapeutic target in tauopathies [39] with CCT020312, a drug developed for cancer [40] but there is no conclusive data yet.

Finally, kinases involved in AMPK and mTOR pathways have also been considered as therapeutic targets [41–43].

Regarding tau phosphorylation, there are 85 phosphorylation sites. Among them, five are Tyr residues. Thus, we can also cite tyr kinase inhibitors even if these molecules do not target tau phosphorylation per se but mostly signal transduction pathways such as src/fyn and abl pathways. Regarding the Fyn inhibition, the most advanced molecule is saracatinib, which is in clinical trial [44]. Regarding Abl tyr kinase, its inhibition by nilotinib, a drug cancer, may turn on autophagy [45] and allow for the degradation of misfolded proteins including tau proteins [46].

Another way to modulate phosphorylation is dephosphorylation by phosphatases [47]. The activation of an enzyme is always tricky and the broad spectrum of phosphatase activities is very challenging to find a therapeutic approach. Sodium selenate has been shown to activate PP2A and restore synaptic plasticity [37, 48]. Finally, indirect approaches have also been suggested such as Pin1 modulators [49]. Pin1 is a peptidyl cis/trans isomerase and its isomerization catalysis may allow for regulating the (de)phosphorylation of specific pThr/pSer-Pro motifs [50–53, 47]. Other isomerases such FKBPs may also modulate tau phosphorylation and degradation [41, 54, 55].

Finally, N-acetyl glucosamine linked to Ser/Thr residues (O-GlcNAc) is also a post-translational modification that regulates tau phosphorylation. O-GlcNac is a reciprocal to phosphorylation. Thus, therapeutic approaches to inhibit deglycosylation on Ser/Thr residues have been also used. For instance, Thiamet G is a O-GlcNacase inhibitor and has shown promising results in preclinical models [56, 57]. Some similar compounds such as MK8719 and ASN120920 are currently in clinical trials.

Tau Metabolism

As indicated earlier, some kinase modulators may also play on tau metabolism by facilitating tau proteolysis such as nilotinib, rapamycin and CCT020312 [39, 43, 46]. Other compounds such as chaperone modulators also modulate tau metabolism. For instance, trehalose, an enhancer of autophagy, has been shown to lead to tau degradation in preclinical model [58]. Another approach is proteasome modulation by PROTACs. Protac (Proteolysis Targeting Chimeric Molecule) acts as a bridge, bringing together E3 ubiquitin ligase with a protein target, resulting in its ubiquitination and degradation [55]. Tau could be degraded through a Keap1-dependent peptide PROTAC [59]. Finally, sGC-1061 is a NO mimetic which activates the cGMP signaling [60]. It restores cognition and lowers Aß and tau amounts in mouse models [61]. More recently, tau clearance via the lysosome has also been explored. Activation of this pathway by inhibiting the enzyme farnesyltransferase blocks the attachment of a neuronal protein, Rhes, to the cell membrane. Farnesyltransferase inhibitors are already in use in human patients for treating cancer. The authors treated a mouse model of tauopathy with one such drug, lonafarnib, and were able to prevent the formation of tau inclusions, decrease microgliosis and brain atrophy, and attenuate behavioral abnormalities [62].

In addition, it has been suggested that neurons may suffer from toxicity of persistent stress granules. These latter are cytoplasmic RNA-protein macro-complexes which appear as a normal cell response to stress. They inhibit translation of transcripts not required for the stress response, sequestering these non-essential transcripts. However, in neurodegenerative disorders such as ALS and tauopathies, some RNA binding proteins such as TIA1 may allow for the persistence of stress granules facilitating both formation of tau oligomers, tau toxicity and tau spreading [63]. Thus, the modulation of such binding proteins may provide insights into possible therapeutic approaches to reduce the spread of neurodegeneration in tauopathy [64].

Finally, some drugs have pleiotropic effects and may act on both amyloid and tau degradation [65, 66]. Some of those such as AZP2006 are in clinical trials (http://www.alzprotect.com/en/rd/azp2006-clinical-trials/).

All of these approaches target proteolytic pathways and modulate tau metabolism in a non-specific way.

Microtubule Stabilizers

Tau is a microtubule-associated protein and its binding to microtubules is regulated by post-translational modifications such as acetylation and phosphorylation as described earlier. Some compounds are also able to stabilize microtubules. For instance, taxol and its derivates have been widely in cancer to stop proliferation of cancer cells by interfering with tubulin [67]. However, these compounds do not cross the blood-brain barrier. Other drugs such as Epothilone D [68] and TPI-287 (abeotaxane) [69, 70] have been developed and shown positive outputs in experimental models. Nevertheless, Epothilone D, a small molecule microtubule stabilizer, has been tested in phase 1 and discontinued. TPI-287 has finished phase 1 clinical trials in AD and SSP in 2018. As indicated earlier, tau is more than a microtubule-associated protein [1] and thus, it may be more complicated than a simple microtubule stabilization.

Anti-Aggregating Agents

Tau aggregation is considered as deleterious even if it is likely more complicated. In fact, there is first post-translational modifications which may lead to gain of toxic functions. Such tau species may be aggregated as inert species. However, with time, such aggregates grow and also become toxic for the cell. Such postulate is based in different observations in other proteinopathies and experimental models of tauopathies [71, 72]. However, different ß-breakers and anti-aggregating agents were tested in preclinical models [73]. Among them, methylene blue and its derivate Rember™ have been in clinical trials but shown some bias due to the blue color lacking in placebo [74]. TRx 0237 (LMTX™) is the new generation of anti-aggregating agent [75]. It is currently in phase 2/3 trial for AD.

Immunotherapy

With the success of immunotherapy in cancer and multiple sclerosis, this approach has been widely tested in other pathologies. With the initial work of Dale Schenk on amyloid vaccine, tau vaccine has initiated by the group of Einar Sigurdsson in 2007 [76] and followed by tau immunotherapy [77]. The main concern about tau immunotherapy is the antigen location. Tau is a cytosolic protein and it is not clear how antibodies can "clear" intraneuronal aggregates even if some the endosome-lysosome [78] or the cytosolic TRIM21 Fc receptor [79] pathways have been suggested. With the prion-like propagation hypothesis, tau becomes an extracellular target and thus may be easy to eliminate [80]. However, there is no consensus on the seeding and spreading tau species which may be truncated, monomers, oligomers or high molecular weight species [81–83]. More research is needed to specify which tau species have to be targeted. Thus, we should be all conscient that current trials jeopardize tau pathology as target since tau research is not advanced enough. However, numerous pre-clinical and clinical trials are ongoing.

ACI35 and AADvac-1 are the most advanced clinical trials in vaccination. ACI35 is a liposome-based antigen. It allows for the targeting of pS396 and pS404 and has shown promising preclinical effect [84]. AADvac-1 is microtubule-binding peptide around the PGGG repeat sequence. Such peptide belongs to the core of PHF and may act as seeds for aggregation [85].

Different passive immunotherapy approaches are also ongoing and positive preclinical studies have allowed for phase 1 and phase 2 clinical trials in AD and PSP. For instance, phosphorylation at Ser422 is considered as pathological [21] and its targeting has shown improvement in behavior and decrease in tau aggregation [86]. An antibody anti-phosphoSer422, has also shown promising data in preclinical studies even if some lysosomal defects were reported [78]. Unfortunately, clinical trial with passive immunotherapy RG7345, has been stopped in phase 1. Other antibodies are currently in clinical trials with different isotypes (see Table 30.1). Human isotype IgG4 may be preferred in a number of studies since it has low antibody-dependent cellular cytotoxicity and low binding to Fc receptors [87] which may allow for a better targeting of extracellular tau. For

Table 30.1 Examples of current clinical trials for tauopathies

Mode of action	Drug	Target	Status
Tau RNA	BIIB080	Tau mRNA	Phase 1
Tau PTMs	Tideglusib/Lithium	GSK3ß inhibition	Phase 1 discont.
	Nicotinamide	Desacetylase inhibitor	Phase 2
	Saracatinib *AZD0530*	Tyr kinase inhibition	Phase 2
	Nilotinib		
	VEL015 Selenate	PP2A activation	Phase 2
	ASN120290	O-GlcNacase inhibitor	Phase 1
Tau metabolism	AZP2006	N.A.	Phase 1
Tau microtubule	Epothilone D	Stabilization microtubule	Discontinued
	TPI-287	Stabilization microtubule	Phase 1
Anti-aggregating agents	Rember	ß-breakers	Discontinued
	LMTX	ß-breakers	Phase 3
Active immunotherapy	AADvac1	$[KDNIKHVPGGGS]_4$	Phase 2
	ACI-35	VYKpSPVVSGDTpSPRHL	Phase 1
Passive immunotherapy	BIIB076	Full tau	Phase 1
	BIIB092	EVMEDHAGTY	Phase 2
	C2N-8E12	DQGGYT	Phase 2
	JNJ-63733657	Tau mid-region	Phase 1
	LY33035690	N-terminal conformational	Phase 2
	NPT088 (not real immunoglobulin)	Fused Ig-general amyloid interaction motif	Phase 1
	RG7345	pS422	Phase 1 discontinued
	RO7105705	pS409	Phase 2
	UCB0107	SPSSAKSRLQTA	Phase 1
Others	CERE-110	NGF	Phase 2

GSK3β glycogen synthase kinase 3ß, *PP2A* protein phosphatase 2A, *NGF* Nerve Growth Factor

instance, both BIIB092 and UCB0107 are IgG4, which have been tested in phase 1 clinical trials and shown convincing preclinical studies [88–91]. They are both in phase 2 clinical trials. Some antibodies are also fused immunoglobulin with general amyloid interaction motif and target Aß, tau, synuclein amyloids [92].

Other immunotherapies are going and summarized in Table 30.1.

Brain/Body as a Whole

Finally, many modulators of brain homeostasis have also been tested in experimental models of tauopathy and in clinical trials. They are not directly linked to tau pathology but are clearly of interest. Physical exercise has shown a beneficial effect in tau transgenic mice [93]. Energy metabolism is also very important for brain homeostasis: key actors are linked to tau pathology such as adenosine and its A2A receptor which can be modulated by a non-selective antagonist caffeine) [94, 95] and AMPK which is hyperactivated in neurofibrillary tangles [42, 96].

NGF and growth factors may also represent a nice therapeutic strategy linking tau pathology and loss of cholinergic neurons [97, 98]. Modulation of cholesterol metabolism by acting on its esterification as well as insulin may also be of interest [99–101].

Finally, recent findings suggest that peripheral signaling such as microbiote and different infections may also participate to tau pathology and thus represent new therapeutic targets [102–104].

In conclusion, there are many (maybe too many) therapeutic targets for tau pathology. Unfortunately, tau pathology is ill-defined and thus, it is difficult to identify those which are the most promising ones.

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