Isolated from a heat stable fraction of tubulin-bound proteins in 1975, Tau protein was associated a decade later with the paired helical filaments (PHFs) found in neurofibrillary tangles (NFTs) of Alzheimer’s disease (AD) brain. As the main constituent of PHFs, Tau has thus become a pharmacological target of interest. Although phosphorylation is involved in the regulation of Tau microtubule binding activity allowing for neuronal plasticity, Tau in AD brain is found in an abnormally hyperphosphorylated form which combines high level with abnormal phosphorylation (i.e., phosphorylation sites that are not detected in normal adult brains). Tau is mainly found in the axonal compartment of adult mature neurons under physiological conditions, separated from the somatodendritic compartment by a diffusion barrier in the axon initial segment. In contrast, Tau missorting is an early marker of Tau dysfunction in AD together with hyperphosphorylation and aggregation. Furthermore, decreasing Tau levels has a positive effect on AD cellular and in vivo models suggesting that at least part of Tau effect in neurodegeneration comes from a gain of toxic function and/or accumulation. Hence, the current model of Tau pathogenesis in AD proposes that (hyper)phosphorylated Tau detaches from microtubules, accumulates in diverse neuronal compartments, and aggregates into intraneuronal PHFs.

Together Tau protein and β-amyloid (Aβ) peptide are AD molecular biomarkers that both form amyloid assemblies. Aβ peptide as the main component of the extracellular amyloid plaques—which is provided by the enzymatic cleavage of the amyloid precursor protein (APP) by the presenilins—has been in the forefront over the past decades as a promising target for the development of AD therapeutics according to the amyloid cascade hypothesis. The etiological role of APP in familial AD (that represents 1% of total cases) related to autosomal dominant mutations of either APP, presenilin-1, or presenilin-2 genes, together with the extraneuronal localization of amyloid deposits, has first motivated attempts to target Aβ peptide in a therapeutic strategy aiming at reducing amyloid plaques, however with poor outcome up to now. Hence, new strategies directed against Tau protein have emerged. The role of Tau in AD has been highlighted by the finding that mutations of the MAPT gene encoding for Tau have been linked to the etiology of other dementia called tauopathies indicating that Tau itself is able to trigger neurodegeneration in the absence of Aβ. Furthermore, the neurofibrillary degeneration is, unlike Aβ deposits, in good correlation with AD progression through the brain and severity of cognitive decline. The number and extent of NFT lesions are used in postmortem definite diagnosis of AD. In addition, phospho-epitope staining allows for defining AD stages and progression of neurofibrillary degeneration through the brain, referred to as Braak staging. A spreading of neurofibrillary degeneration in AD brain in a specific temporal and regional pattern is observed, but molecular mechanisms of transmission between neurons and the involved pathogenic specie(s) remain to be defined.

It has been suggested that abnormal phosphorylation could be accompanied by conformational change(s) that would turn the native into an aggregation-prone conformation as first suggested by AD-specific, conformation-dependent antibodies. This is even more intriguing for the disorder structure of Tau protein—classified as an intrinsically disordered
protein (IDP)—that seemingly escapes the dogma of structure-function relationships. However, the molecular details still have to be described, a difficult task given the complexity of Tau post-translational modifications. Tau oligomers seem to be the most toxic species and have also been involved in the spreading of Tau pathology. Hence, transformation of Tau protein involves progressive conformational changes from a monomeric form into soluble, prefibrillar oligomers that constitute the nuclei of the fibrillation process and finally into bona fide PHFs containing β-structures. Furthermore, truncated forms of Tau, other post-translational modifications such as lysine ubiquitination, acetylation or methylation, or other proteins found in PHF extracted from AD brain account as potential factors involved in the acquisition of the aggregation capacity.

This volume of *Methods in Molecular Biology* dedicated to Tau protein covers basic and advanced methods and protocols from in vitro assays to in vivo models to address the molecular and functional aspects of Tau physiopathology, and deals with many related technical issues. Hereafter are described protocols for the conformational studies of native Tau protein and investigation of its physiological function in microtubule binding and tubulin polymerization; in vitro methods of formation of Tau oligomers and PHF-like fibrils, the study of fibrillization kinetics and a screening assay of Tau aggregation inhibitors; protocols for the characterization and in vitro introduction of post-translational modifications on Tau protein for further functional studies; analytical tools for the detection of Tau proteins, their modifications and cellular interactions, and MAPT gene mutations in various biological samples; and cellular and in vivo models for the investigations of Tau physiopathology.

**Part I**

Although it has been studied for several decades, Tau protein has still not finished revealing its secrets. Several studies have suggested that distinct conformations could drive Tau function and toxicity, but a description of diverse Tau species is still required at the molecular level. The flexibility and structural dynamics of Tau and other IDPs enlarge the concept of “structure-function relationships” requiring the development of new structural methodologies to decipher the fascinating and intriguing world of IDPs. Detection of transient folding and secondary structure elements in the monomeric form of Tau requires a combination of biophysical methodologies including, amongst others, circular dichroism and small-angle X-ray scattering described in Chapter 1 and Raman Spectroscopy in Chapter 2. Detection of subtle conformational changes of Tau protein upon phosphorylation or other post-translational modifications is challenging for most experimental methods. Atomic molecular dynamics simulations described in Chapter 3, combined with experimental NMR data, provide structural and dynamic information on folding of a short phospho-peptide encompassing the AT8 epitope (pS202/pT205). The study of Tau:tubulin or Tau:microtubule interactions is of major interest in the context of Tau physiopathology when one wants to decipher the role of post-translational modifications or cofactors on microtubule binding and tubulin polymerization, or the intricate molecular mechanism by which Tau promotes tubulin assembly. Microtubules constitute a dynamic protein machinery while Tau protein keeps a high degree of flexibility even at the microtubule surface. Together these behaviors hamper the full structural description of the Tau:tubulin and Tau:microtubule complexes at the molecular level. Furthermore, negatively charged patches at the microtubule surface could promote Tau self-association when studying interactions of Tau with tubulin or microtubules leading to misinterpretation of dissociation constants and kinetic parameters.
Chapter 4 describes tubulin preparation from lamb or pig brains for in vitro polymerization assays. Various experimental conditions are proposed to study Tau:tubulin and Tau: microtubule interactions in vitro and in living cells using turbidimetry, co-sedimentation, and FRET assays. Pitfalls and cautions related to data interpretation are emphasized.

**Part II**

In vitro aggregation assays both aim to decipher the fibrillization process at the molecular level and to screen small-molecule inhibitors interfering with fibril formation in a perspective of AD cure. Part II focuses on in vitro methods and protocols for the study of Tau fibrillar structure, fibrillization process, and screening of small-molecule inhibitors of Tau aggregation. In addition, protocols using Tau oligomers as seeds in in vitro aggregation assays and in in vivo model of Tau spreading are included. Due to its high solubility, external aggregation inducers are required to stimulate Tau fibrillization in vitro that otherwise would not be of any practical use, i.e., in a few day timescale. Molecules enabling Tau aggregation are polyanions such as heparin, polyglutamate or RNA, anionic micelles and vesicles made up of fatty acids, as well as small-molecule anionic compounds or negatively charged gold nanorods. Inducers stimulate Tau fibrillization into PHF-like fibrils with morphologies resembling fibrils extracted from AD brains, hence modeling an authentic pathological process. A combination of biophysical techniques are used for the specific detection of amyloid-type fibril formation, conformational changes upon aggregation, quantification of fibril amounts and monitoring of fibrillization kinetics, as well as the description of fibril morphology. Chapter 5 describes the co-crystallization procedure of a short Tau peptide encompassing the minimal motif of fibrillization—the PHF6 peptide—with small-molecule compounds, collection of X-ray diffraction data from microcrystals and data processing. This model has provided structures of a pharmacophore for rational design of small-molecule compounds targeting Tau amyloid fibrils. Chapter 6 presents protocols for in vitro fibrillization of full-length Tau protein from the monomeric form using either Geranine G, ODS, or anionic microspheres as external aggregation inducers combined with three complementary methods of fibril detection, transmission electron microscopy (TEM) imaging, filter-trap immunoassay, and Thioflavin dye fluorescence. The advantages and disadvantages of each method are emphasized. Chapter 7 describes three alternative methods for the detection of Tau fibrils formed with heparin and the study of fibrillization kinetics. Vibrational spectroscopy such as Fourier transform infrared (FTIR) and ultraviolet resonance Raman (UVRR) spectroscopy can discriminate between different classes of secondary structure, therefore allowing for the detection of specific β-structure formation upon fibrillization. These techniques are combined with atomic force microscopy (AFM) for fibril imaging. Chapter 8 presents a screening method of Tau aggregation inhibitors (TAI) combining a cell-free Tau-Tau binding immunoassay and a cell-based assay involving a stable cell line expressing an aggregation-prone Tau fragment and inducible for the expression of full-length Tau. This method allows investigation of Tau aggregation inhibition with small molecules together with evaluation of their properties of cell penetration and toxicity. Chapter 9 provides a method for the in vitro preparation of Tau oligomers with Aβ42 or α-synuclein seeds, methods for isolation of Tau oligomers from biological sources, and their detection using Tau oligomer-specific antibodies in ELISA, dot blot, Western blot, and immunohistochemistry assays. This method provides important tools for the early detection of Tau pathogenic species in vivo and the study of Tau pathology propagation.
Part III

The disordered nature of Tau induces a high level and diversity of post-translational modifications. Therefore, the characterization of such a complex array of post-translational modifications is very challenging, and deciphering their role in modulation of Tau functions has been rendered even more complicated. In particular, phosphorylation has focused attention due to its close link with pathogenesis. The number of potential phosphorylatable Ser/Thr residues (80 sites) as well as the number of involved kinases has complicated the description of pathological patterns of phosphorylation and the sequence of events. While phosphorylation has been extensively studied, other post-translational modifications have only been recently described and their characterization is thus less comprehensive. Part III deals with methods for the characterization of Tau post-translational modifications generated either in vivo or in vitro and methods of production of Tau proteins with specific pattern of post-translational modifications as tools for the study of their role in Tau functions. Chapter 10 presents identification and quantification of lysine-directed modifications in Tau protein by liquid-chromatography-tandem mass spectrometry. A method for the characterization of Tau phosphorylation and acetylation patterns by high-resolution NMR spectroscopy is described in Chapter 11. Protocols in this chapter include the recombinant production of the activated ERK2 kinase and a functional fragment of the Creb-binding protein in E. coli for the in vitro modification of isotopically labeled Tau protein that fulfills the amount required for NMR analyses. Additionally, a protocol for the preparation of a rat brain extract is provided for the production of phosphorylated Tau samples. As an alternative to the enzymatic strategy that affords heterogeneous patterns of modification, a protocol of Expressed Protein Ligation (EPL) is described in Chapter 12 for the introduction of selective and quantitative phosphorylation or O-β-N-acetylglucosaminylation (O-GlcNAcylation) in the C-terminal region of Tau protein. This method is complemented by a protocol for traceless purification of the ligation product. As in vitro enzymatic O-GlcNAcylation of Tau is challenging, Chapter 13 describes an alternative method for high-level incorporation of O-GlcNAc moieties in Tau protein with co-expression of Tau and O-GlcNAc transferase (OGT) in E. coli and enrichment of the O-GlcNAc-modified Tau fraction, as well as selective detection of O-GlcNAc-Tau in brain lysates with the S400-O-GlcNAc antibody.

Part IV

Part IV describes basic and advanced analytical tools to detect Tau proteins, Tau post-translational modifications, and Tau interacting partners from various biological sources. Additional protocols are presented to identify mutations of the MAPT gene encoding for Tau protein. Although mainly found in neurons Tau protein is also detected in the extracellular space. An increase of Tau and phospho-Tau levels is detected in the cerebrospinal fluid (CSF) of AD patients. Synaptic release and postsynaptic uptake of Tau species are responsible, at least in part, for interneuronal spreading of Tau pathology. Hence, release and diffusion of Tau in the interstitial fluid (ISF) account for the extracellular Tau content. In addition, intraneuronal aggregates of hyperphosphorylated, misfolded PHF-Tau become extraneuronal when neurons die (referred to as “ghost” tangles). Detecting intraneuronal and extracellular Tau species is thus a key issue not only in understanding Tau physiology.
and pathology but also for (early) diagnosis of neurodegenerative diseases. Challenging characterization of the neuropathological lesions such as neurofibrillary tangles required development of methods for the isolation and identification of their proteinaceous constituents. With a high content of various post-translational modifications coupled to the expression of six isoforms, proteomic analyses of Tau proteins and PHF-Tau become very difficult. Chapter 14 describes a two-dimensional electrophoresis-based protocol for the analysis of Tau isovariants extracted from human or mouse brain and cells. Chapter 15 highlights pitfalls and offers tips to circumvent detection of nonspecific signals when analyzing mouse brain proteins by Western blotting with anti-Tau monoclonal antibodies. Chapter 16 deals with quantitative flow cytometry analysis of Tau in synaptosomes—isolated nerve terminals enriched in synaptic proteins obtained during nerve tissue homogenization—as a model system for the study of Tau release and synaptic Tau pathology. This method includes a protocol for the preparation of crude (P2) synaptosomal fraction from cryopreserved postmortem tissue of AD brain and P2 immunolabeling. Flow cytometry allows purification of synaptosomes from P2 fraction based on a particle size selection achieving a high degree of purity and single-cell quantitative analysis of (phospho)Tau. Chapter 17 presents a protocol of in vivo microdialysis in freely moving mice to detect extracellular Tau proteins in brain interstitial fluid. Changes of Tau levels in ISF related to drug administration can be monitored using this protocol. While Tau was first identified as a tubulin-binding protein, it has been recently described as a scaffold protein with several protein partners. Chapter 18 describes the proximity-ligation assay (PLA) method to detect Tau interacting partners into neurons. This procedure is illustrated by the characterization of interactions between Tau and the bridging integrator 1 (BIN1). BIN1 is one of the most important genetic risk factors in late-onset AD identified by genome-wide association study while BIN1 protein was shown to modulate neurofibrillary tangle pathology. Finally, Chapter 19 describes a method for PCR amplification, bidirectional Sanger sequencing of the MAPT gene from genomic DNA of patients, and further sequence analyses in order to detect single nucleotide MAPT mutations combined with exon trapping. As MAPT mutations that affect splicing of exon 10 are commonly associated with tauopathies, exon trapping enables to functionally connect DNA variants to dysregulation of exon 10 splicing of MAPT.

Part V

Part V concerns various cellular and in vivo models for investigation of Tau physiopathology. Chapter 20 provides a detailed protocol to grow primary neurons, as well as a cell fixation and staining protocol for immunofluorescence microscopy of endogenous Tau. Chapter 21 proposes a nonviral transfection protocol of primary neurons ensuring low levels of Tau comparable to endogenous levels and thus providing a good model to investigate Tau sorting and transport into neurons. Chapters 22 and 23 provide two protocols of cellular assays based on fluorescence biosensors for the detection of Tau aggregation within the cell. Chapter 22 describes the Tau-BiFC cell-based model that uses Bimolecular Fluorescence Complementation as a convenient tool to investigate intracellular tau aggregation. An example of modulation of Tau-Tau interactions in cell by Forskolin, a kinase activator, is given. Chapter 23 describes a rapid and sensitive FRET-based flow cytometry biosensor assay to quantitatively measure Tau seeding activity. Chapter 24 investigates Tau fibrillar aggregates in the mouse retina as a part of the central nervous system accessible for
noninvasive in vivo imaging using confocal scanning laser ophthalmoscopy in combination with fluorophore staining of fibrillar Tau in the retinal ganglion cell layer. This method can be exploited to evaluate the efficiency of potential drugs in preclinical studies with a follow-up of mice over several months of treatment. Chapter 25 presents an in vivo hyperthermic stress model to study the effects of increased oxidative stress on Tau functions in mouse brain. Chapter 26 describes the Drosophila model to study the modifiers of Tau toxicity. This method includes a protocol for the expression of Tau in the Drosophila eye which is used as a readout in a genetic screen of Tau toxicity modifiers. Chapter 27 deals with a protocol of in situ hybridization of neurotrophin mRNA with labeled riboprobes in the THY-Tau22 mouse model exhibiting an AD-like Tau pathology. This method is combined with immunohistochemical and immunofluorescence analysis of brain slices allowing for double labeling of neurotrophin mRNA and Tau phospho-epitopes. Chapter 28 describes Pin1 knockout mouse model and its use for the study of Tau pathology. This chapter includes several behavior tests as well as a protocol to harvest mouse brain and prepare paraffin-embedded sections for immunostaining and brain lysate for immunoblotting.

I am indebted to all authors who contributed, sharing their protocols and technical expertise, and who made this book possible. I would also like to thank Professor John Walker, the series editor, for inviting me to edit this volume of the Methods in Molecular Biology series. I am very grateful to him for this opportunity. Finally, I would like to warmly thank my colleagues for their encouragements and meaningful discussions.

Lille, France

Caroline Smet-Nocca
Tau Protein
Methods and Protocols
Smet-Nocca, C. (Ed.)
2017, XVIII, 432 p. 83 illus., 64 illus. in color., Hardcover
ISBN: 978-1-4939-6596-0
A product of Humana Press