Four-repeat tauopathies

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Abstract

Tau is a microtubule-associated protein with versatile functions in the dynamic assembly of the neuronal cytoskeleton. Four-repeat (4R-) tauopathies are a group of neurodegenerative diseases defined by cytoplasmic inclusions predominantly composed of tau protein isoforms with four microtubule-binding domains. Progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease or glial globular tauopathy belong to the group of 4R-tauopathies.

The present review provides an introduction in the current concept of 4R-tauopathies, including an overview of the neuropathological and clinical spectrum of these diseases. It describes the genetic and environmental etiological factors, as well as the contemporary knowledge about the pathophysiological mechanisms, including post-translational modifications, aggregation and fragmentation of tau, as well as the role of protein degradation mechanisms. Furthermore, current theories about disease propagation are discussed, involving different extracellular tau species and their cellular release and uptake mechanisms. Finally, molecular diagnostic tools for 4R-tauopathies, including tau-PET and fluid biomarkers, and investigational therapeutic strategies are presented.

In summary, we report on 4R-tauopathies as overarching disease concept based on a shared pathophysiological concept, and highlight the challenges and opportunities on the way towards a causal therapy.

Keywords: 4R-tauopathy, microtubule-associated protein tau; progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease, glial globular tauopathy, Alzheimer's disease

Abbreviations: AD, Alzheimer's disease; **AGD**, argyrophilic grain disease; **AGE**, advanced glycation end-products; **ALS**, autophagy-lysosome system; **ARTAG**, aging-related tau astrogliopathy; **bvFTD**, behavioral variant of FTD; **CBD**, corticobasal degeneration; **CBS**, corticobasal syndrome; **CDK5**, cyclin-dependent kinase 5; **CMA**, chaperone-mediated autophagy; **CSF**, cerebrospinal fluid; **CTE**, chronic traumatic encephalopathy; **CxI**, mitochondrial complex I; **DLX1**, distal-less homeobox 1; ***EIF2AK3***, eukaryotic translation initiation factor 2-alpha kinase 3 (encoding PERK); **eTau**, extracellular tau; **FDG**, fluorodeoxyglucose; **FTD**, frontotemporal dementia; **FTLD**, frontotemporal lobar degeneration; **GGT**, globular glial tauopathy; **GSK-3**, glycogen synthase kinase 3; **GWAS**, genome-wide association study; **HSPGs**, heparan sulfate proteoglycans; **iPSC**, induced pluripotent stem cell; **ISF**, interstitial fluid; **MAPT**, microtubule-associated protein tau; **MBD**, microtubule-binding domain; **MOBP**, myelin-associated oligodendrocyte basic protein; **MSA**, multiple system atrophy; **mTOR**, mammalian target of rapamycin; **NfL**, neurofilament light chain; **NFT**, neurofibrillary tangle; **O-GlcNAc**, O-linked *N*-acetylglucosamine; **OGA**, *O*-GlcNAcase; **PD**, Parkinson's disease; **PERK**, pancreatic endoplasmic reticulum kinase; **PET**, positron-emission tomography; **PHF**, paired helical filaments; **PiD**, Pick's disease; **PSP**, progressive supranuclear palsy; **pTau**, phosphorylated tau; **Rab**, Ras-related protein; **RS**, Richardson's syndrome; **SNPs**, single nucleotide polymorphisms; **STX6**, syntaxin 6; **TDP-43**, transactive response DNA-binding protein 43; **UPR**, unfolded protein response; **UPS**, ubiquitin-proteasome system.

1. Introduction

Tauopathies are a group of adult-onset neurodegenerative diseases including Alzheimer's disease (AD) and frontotemporal lobar degeneration (FTLD) spectrum diseases. They are defined by

intracellular aggregation and transcellular propagation of the microtubule-associated protein tau (MAPT). Physiologically, tau participates in the formation of microtubule fibrils from tubulin monomers (Cleveland *et al.*, 1977a, b; Weingarten *et al.*, 1975). In neurons, tau is primarily located in axons to regulate microtubular stability, dynamics, and transport processes (Lee *et al.*, 2001; Trojanowski *et al.*, 1989). Tau is encoded by the *MAPT* gene on chromosome 17q21. Six different tau isoforms are generated by alternative splicing (Lee *et al.*, 2001). Three isoforms, each deriving from alternative splicing of exon 10, encode the second of a maximum of four repeat regions within the microtubule-binding domain (MBD). This generates three isoforms each with three or four repeats, respectively, defining 3R- and 4R-tau isoforms.

AD, the most frequent tauopathy, is considered a secondary tauopathy since genetic and biochemical evidence coherently points to amyloid-beta being the primary driver of the disease (Selkoe and Hardy, 2016). In contrast, tau is the principal pathogenic protein in some FTLDs, which are considered primary tauopathies. In AD brains, both 3R- and 4R-tau isoforms aggregate. Primary tauopathies can be mixed 3R/4R-tauopathies, pure 3R-tauopathies, or pure 4R-tauopathies. 4R-tauopathies are sub-classified by their histopathological stigmata and clinical manifestations. They attract increasing research interest, leading to an impressive gain of knowledge about involved disease mechanisms, enabling the launch of well-founded clinical trials to test novel hypotheses in human patients. This review presents the recent scientific progress in our understanding of the neurobiology of 4R-tauopathies.

2. Neuropathology of 4R-tauopathies

Tauopathies are biochemically and morphologically heterogeneous diseases. Neuropathological phenotypes are distinguished by the involvement of different anatomical areas, cell types, and the

presence of distinct tau isoforms in pathological deposits.

2.1. *Classification of tauopathies*

As outlined above, tauopathies can be divided into **3R-, 4R-** or mixed **3R/4R-tauopathies**. In **primary tauopathies**, the tau pathology is predominant, whereas in **secondary tauopathies** additional driving forces or etiologies are involved (Fig. 1). On biochemical and neuropathological basis, sarkosyl-insoluble fractions of brain extracts show distinct ratios of 3R- and 4R-tau and two or three major phospho-tau bands (60 and 64; or 64 and 68; or 60, 64 and 68 kDa) (Lee *et al.*, 2001; Sergeant *et al.*, 2005; Spillantini and Goedert, 2013). The morphology of tau immunoreactivities and isoform-specific antibodies allow the differentiation and classification of primary tauopathies (Kovacs, 2015).

While most tauopathies are sporadic, rare mutations in *MAPT* cause autosomal dominant FTLD (**FTLD-*MAPT***) with variable 3R-, 4R- or 3R/4R-tau pathologies, overlapping with neuropathological features of the primary tauopathies (Forrest *et al.*, 2018; Ghetti *et al.*, 2015).

A prototypical **primary 3R-tauopathy** is **Pick's disease (PiD)** with spherical tau inclusions (Pick bodies) and 60 and 64 kDa bands on tau Western blots.

A **primary mixed 3R/4R-tauopathy** with 60, 64 and 68 kDa bands on tau Western blots is the neurofibrillary tangle (NFT) predominant senile dementia (**NFT-dementia** or "tangle-only" dementia), which is now included in the larger category of primary age-related tauopathies (PART) (Crary *et al.*, 2014).

Several conditions with diverse etiologies are associated with **secondary mixed 3R/4R-tauopathies**. Tau pathology in **chronic traumatic encephalopathy (CTE)**, a progressive neurodegenerative condition triggered by repetitive mild traumatic injury, comprises both 3R- and

4R-tau isoforms and includes NFTs and astrocytic fibrillary deposits (McKee *et al.*, 2015). In the **anti-IgLON5 (IgLON family member 5)-related encephalopathy**, tau pathology is associated with an autoimmune process, and predominantly affects subcortical structures with mostly neuronal 3R- and 4R-tau isoforms (Gelpi *et al.*, 2016). In **AD**, neuronal mixed 3R- and 4R-tau pathology is associated with amyloid-beta pathology (Selkoe and Hardy, 2016).

The **aging-related tau astrogliopathy (ARTAG)** represents different constellations of a **4R-tauopathy** with astrocytic tau pathologies (thorn-shaped astrocytes, TSAs and granular/fuzzy astrocytes, GFAs) in subpial, subependymal, and perivascular locations of white and gray matter (Kovacs *et al.*, 2016). Importantly, affected brain areas and morphologies of astroglial tau pathologies overlap between ARTAG and CTE and ARTAG and 4R-tauopathies (Kovacs *et al.*, 2017b). Since ARTAG can be seen associated with different conditions, it is yet to be defined whether specific constellations of ARTAG types can also be considered primary tauopathies.

Primary 4R-tauopathies with 64 and 68 kDa bands on tau Western blots comprise **progressive supranuclear palsy (PSP)**, **corticobasal degeneration (CBD)**, **argyrophilic grain disease (AGD)**, and **globular glial tauopathy (GGT)** (Kovacs, 2015). They will be described below.

2.2. Histopathology of 4R-tauopathies

4R-tauopathies show a spectrum of neuronal and glial tau pathologies. The severity of these characteristics and their distribution define the various entities (Fig. 1).

The diagnosis of typical **PSP** is based on the existing criteria (Hauw *et al.*, 1994), most importantly a high density of NFTs and neuropil threads in the basal ganglia and brainstem. Tufted tau-positive astrocytes or their processes can confirm the diagnosis (Hauw *et al.*, 1994). In addition, oligodendroglial coiled bodies are seen in variable amounts and distributions (Kovacs, 2015).

Further alterations in typical PSP comprise atrophy in the subthalamic nucleus, accumulation of pigments and spheroids in the globus pallidus and substantia nigra and grumose degeneration in the dentate nucleus (Kovacs, 2015). The neuropathological criteria await an update to include clinicopathological variants and contemporary tau immunohistochemistry.

Neuropathological hallmarks of **CBD** are 4R-tau-positive neuronal lesions, threads in the white and gray matter, coiled bodies and astrocytic plaques (Dickson *et al.*, 2002). The neuronal tau pathology differs from PSP and encompasses diffuse cytoplasmic granular immunoreactivity and small spherical inclusions. Further neuropathological features include the presence of achromatic ballooned neurons in affected cortical areas and neuronal loss in cortical and subcortical structures. CBD and PSP brains display subtle ultrastructural differences of tau filaments and certain distinctive biochemical features (Hoglinger, 2018; Taniguchi-Watanabe *et al.*, 2016). While neuronal tau pathology is more pronounced in the forebrain in CBD, PSP mainly affects hindbrain structures (Dickson, 1999). CBD and PSP are neuropathologically distinguished by astroglial tau pathology (astrocytic tufts in PSP, astrocytic plaques in CBD), the degree of involvement of the white matter (more in CBD), and the presence of subcortical NFTs (PSP).

AGD is characterized by an atrophy of the ambient gyrus and so-called tau-positive core lesions, of which the presence of argyrophilic (Gallyas-positive) and 4R-tau-immunoreactive grains in medial temporal lobe structures are essential for diagnosis (Tolnay and Clavaguera, 2004). Pretangles, oligodendroglial coiled bodies and astrocytic tau pathology are also characteristic findings. Astroglial tau pathology is compatible mostly with GFAs, as seen in gray matter ARTAG, but tufted astrocytes can also be detected. Thus, the astroglial tau pathology and anatomical vulnerability in AGD overlap with PSP (Yokota *et al.*, 2018). Moreover, argyrophilic

grains are frequently seen in PSP (18-80% frequency) and thought to be a constant feature of CBD (Gil *et al.*, 2018; Tatsumi *et al.*, 2014).

The three subtypes of **GGTs** are characterized by argyrophilic and 4R-tau-immunoreactive globular oligodendroglial and non-argyrophilic, 4R-tau-immunoreactive globular astroglial inclusions (Ahmed *et al.*, 2013). Neurons show diffuse cytoplasmic immunoreactivity or globular or tangle-like tau immunoreactivity. The morphology of oligodendroglial inclusions and the spectrum of immunostaining patterns for oligodendroglial and myelin markers show a considerable overlap with the alpha-synucleinopathy multiple system atrophy (MSA), The pattern of anatomical vulnerability, however, is completely different (Kovacs *et al.*, 2008; Rohan *et al.*, 2016).

2.3. Distribution of tau pathology in 4R-tauopathies

Pathological alterations seem to follow over time a stereotypic pattern of hierarchical involvement of specific brain structures in some tauopathies.

This is exemplified by the three stages of **AGD** according to Saito and colleagues (Saito *et al.*, 2004), later expanded to four (Ferrer *et al.*, 2008). Accordingly, involvement of the ambient gyrus and most anterior part of the hippocampus CA1 subregion is followed by the amygdala, the lateral tuberal hypothalamic nucleus, the dentate gyrus and presubiculum; finally, the CA2/3 subregions, further hypothalamic nuclei, anterior temporal, cingulate, insular and orbitofrontal cortices, accumbens nucleus and septal nuclei are affected, and rarely, further expansion to the neocortex and brainstem is noted (Ferrer *et al.*, 2008; Saito *et al.*, 2004).

Williams et al. suggested a grading system for **PSP** that reflects a progressive involvement of different anatomical areas (Williams *et al.*, 2007). It was shown that the pallidonigroluysian system

is affected early, followed by the basal ganglia, pontine nuclei, and dentate nucleus, and later the frontal and parietal lobes, and finally other neocortical areas and cerebellar structures (Williams *et al.*, 2007).

In **CBD**, neuronal and glial tau pathology initially predominate in frontoparietal and motor cortical areas and the striatum followed by other subcortical nuclei and finally the brainstem (Dickson, 1999; Kouri *et al.*, 2011).

In one **GGT** subtype the white matter pathology was shown to involve limbic areas early on (Kovacs *et al.*, 2008). A recent study has described sequential stages of distinct astroglial tau pathologies in **CBD** and **PSP** (four stages) and **ARTAG** types (Kovacs *et al.*, 2018). Accordingly, two patterns, each with four stages, were recognized for grey matter ARTAG. One includes a striatal pathway of spreading towards the cortex and/or amygdala, and the brainstem, and an amygdala pathway, which precedes the involvement of the striatum and/or cortex and proceeds towards the brainstem. Astrocytic plaque pathology in CBD follows a predominantly frontal-parietal cortical to temporal-occipital cortical to subcortical to brainstem pathway (four stages). Tufted astrocyte pathology in PSP shows a striatum to frontal-parietal cortical to temporal to occipital to amygdala and to brainstem sequence (four stages).

2.4. Novel perspectives in the neuropathology of 4R-tauopathies

Recent studies suggest that **astrocytes in 4R-tauopathies** might play an underappreciated role in the disease process. Indeed, in a cohort of incidental or presymptomatic CBD cases, Ling et al. found a predominance of astrocytic plaques as compared to neuronal lesions during the early stages of the disease in CBD (Ling *et al.*, 2016). The authors speculated that CBD starts as an astrogliopathy with neuronal lesions gradually taking over as the predominant lesions.

Furthermore, in advanced forms of primary 4R-tauopathies when most of the regions contain both, neuronal and glial tau pathologies, there are still brain areas (such as the occipital cortex) where only astroglial tau pathology can be seen (Kovacs *et al.*, 2017b). That fine granular tau immunoreactivity in astrocytes can represent early forms of astroglial tau pathologies in primary 4R-tauopathies (Kovacs *et al.*, 2017b; Kovacs *et al.*, 2018) provided a link to gray matter ARTAG and suggested these astrocytic immunoreactivities might be indicative of an early preclinical form of primary 4R-tauopathy or ARTAG (Kovacs *et al.*, 2017a). Astroglial tau pathologies might reflect their contribution to disease spreading or clearance of disease-associated proteins, and might lead to astroglial dysfunction and eventually contribute to neuronal degeneration (Kovacs *et al.*, 2017a).

While studies on PSP did not focus on the cellular distribution of tau in incidental cases, they indicate the earliest vulnerable regions for tau pathologies. Accordingly, a pallidonigroluysian distribution together with the pons (Yoshida *et al.*, 2017) and with sparse involvement of the pre-motor cortex (Williams *et al.*, 2007) can be considered as highly and early vulnerable regions. In addition, these observations support the notion that presymptomatic PSP cases might be more frequent than assumed (Evidente *et al.*, 2011; Kovacs *et al.*, 2013; Williams *et al.*, 2007; Yoshida *et al.*, 2017).

For both CBD and PSP areas with predominantly astrocytic tau pathology can be recognized (Kovacs *et al.*, 2017b), suggesting a dissociation of neuronal and astroglial tau pathology. Moreover, astroglial tau pathology in CBD seems to follow a predominantly frontal-parietal cortical to temporal-occipital cortical, to subcortical, to brainstem pathway contrasting PSP where striatum to frontal-parietal cortical to temporal to occipital, to amygdala, and to brainstem sequence has been recognized (Kovacs *et al.*, 2018).

Although tau pathology is interpreted as a primary force of pathogenesis, additional neurodegenerative conditions associated with 4R tauopathies are increasingly recognized (Kovacs, 2019). Accordingly, Lewy body disorder (alpha-synucleinopathy) has been reported in approximately 20% of CBD and PSP cases (Kovacs, 2019). Alzheimer's disease neuropathological change is reported in approximately 11 and 26% of CBD and PSP cases, respectively (Robinson *et al.*, 2018). TDP-43 pathology is observed in 15.4 (Uryu *et al.*, 2008), 24 (Robinson *et al.*, 2018) or 45% (Koga *et al.*, 2018) of CBD, and 6 (Koga *et al.*, 2017) and 16% (Robinson *et al.*, 2018) of PSP cases. In particular, CBD with severe TDP-43 pathology is considered as a distinct clinicopathologic subtype, characterized by PSP-like clinical presentations (Koga *et al.*, 2018). AGD is more frequently seen with ageing, therefore, Alzheimer's disease changes or Lewy body disorder are frequent (Kovacs, 2019).

3. Clinical spectrum

The clinical spectrum of 4R-tauopathies is remarkably diverse and includes predominantly cognitive, predominantly motor or overlap manifestations (Table 1).

In the past, Richardson's syndrome (RS) was considered the archetypical clinical phenotype of PSP. Similarly, corticobasal syndrome (CBS) was thought to be the most characteristic clinical manifestation of CBD. However, over the past two decades, clinicopathological studies associated various other **syndromes with PSP and CBD pathology**. These syndromes include RS, CBS, behavioral variant of frontotemporal dementia (bvFTD), non-fluent/agrammatic variant of primary progressive aphasia (nfaPPA), apraxia of speech, PSP with parkinsonism (PSP-P), and progressive gait freezing (PGF) (Respondek *et al.*, 2017; Respondek *et al.*, 2014). Very rarely, primary lateral

sclerosis (PLS) (Nagao *et al.*, 2012) and late-onset cerebellar ataxia (Kanazawa *et al.*, 2013) have been described in cases with PSP pathology. Current diagnostic criteria for PSP (Hoglinger *et al.*, 2017) and CBD (Armstrong *et al.*, 2013) have incorporated those "variant" clinical phenotypes and thereby expanded the clinical spectrum of 4R-tauopathies.

A number of studies in PSP and CBD have emphasized that the clinical picture relates with the distribution and severity of tau pathology and degenerative changes, which are no less variable (Williams *et al.*, 2005). E.g., compared to PSP-RS, PSP-P and PSP-PGF manifested with less severe and regionally more restricted tau pathology, while CBS had more severe tau pathology in cortical areas (Yoshida, 2014).

There is also increased awareness of the **clinical spectrum of AGD**, but accepted clinical diagnostic criteria for this 4R-tauopathy do not exist. Apart from AD-like dementia, behavioral syndromes, psychosis and depression have been associated with AGD pathology (Rodriguez *et al.*, 2016). Clinicopathological studies on pure AGD are complicated by the fact that AGD pathology is often found associated with other neurodegenerative disorders, such as AD, PSP, CBD, Lewy body disease (LBD), and transactive response DNA-binding protein 43 (TDP-43) proteinopathies (Rodriguez *et al.*, 2016). In addition, eating and appetite disorders are also common neuropsychiatric symptoms, most likely related to the involvement of the hypothalamus (Rodriguez *et al.*, 2016). Furthermore, a subgroup of AGD patients presents as bvFTD (Gil *et al.*, 2019).

GGT has been associated with various clinical syndromes, most often with bvFTD and motor neuron disease features (Ahmed *et al.*, 2013). **ARTAG** mainly occurs in individuals over 60 years of age and has not been associated with any specific clinical syndrome (Kovacs *et al.*, 2016).

The following provides an overview of the most frequent clinical syndromes associated with 4R-tauopathies.

3.1. Richardson's syndrome (RS)

RS is characterized by early postural instability within the first three years after symptom onset, slowing of vertical saccades and vertical supranuclear gaze palsy (Litvan *et al.*, 1996). Other common features include levodopa-resistant and axial-predominant parkinsonism, frontal behavioral and personality changes, executive dysfunction and pseudobulbar signs such as dysphagia and hypokinetic spastic dysarthria (Litvan *et al.*, 1996). Nonspecific ocular signs such as diplopia, photophobia, and lid opening apraxia may occur early in RS (Respondek *et al.*, 2017). Patients with RS most often die of aspiration pneumonia after a mean disease duration of 6 to 8 years (Litvan *et al.*, 1996; Respondek *et al.*, 2014). RS is rather specific for PSP, but may also present in patients with CBD (Armstrong *et al.*, 2013).

3.2. Corticobasal syndrome (CBS)

CBS is essentially defined by various combinations of extrapyramidal motor (rigidity, akinesia, dystonia, or myoclonus) and cortical signs (apraxia, cortical sensory loss, or alien-limb phenomenon) (Armstrong *et al.*, 2013; Hoglinger *et al.*, 2017). Clinical symptoms frequently present asymmetrically (Armstrong *et al.*, 2013; Hoglinger *et al.*, 2017). Postural instability, dysphagia and dysarthria, and slow and/or hypometric saccades may occur (Chahine *et al.*, 2014). The mean disease duration is 5 to 8 years, and thus similar to that of RS (Kouri *et al.*, 2011). In a clinicopathological series of 21 CBS cases, only 5 had CBD pathology, 6 had PSP pathology, and

5 had AD pathology. Other rarer pathological diagnoses of CBS included TDP-43 pathology and Parkinson´s disease (PD) (Ling *et al.*, 2010).

3.3. *Behavioral variant of frontotemporal dementia (bvFTD)*

bvFTD is characterized by prominent behavioral and personality changes. It is a common clinical syndrome of 4R-tauopathies, including PSP, CBD, and AGD. PSP pathology accounts for approximately 5% of bvFTD cases (Josephs *et al.*, 2006), while TDP-43 pathology is found in about 50% (Irwin, 2016). Other differential diagnoses are PiD, for which bvFTD is the most frequent clinical manifestation, and rarely AD (Irwin, 2016).

3.4. *Non-fluent/agrammatic variant of primary progressive aphasia (nfaPPA)*

nfaPPA can be a manifestation of PSP and CBD (Grossman, 2010; Josephs *et al.*, 2006). Signs include grammatical errors and simplifications and poor comprehension of complex sentences. nfaPPA seems to be highly predictive for an underlying 4R-tauopathy, when associated with apraxia of speech, which presents as a slow, effortful and distorted speech (Josephs *et al.*, 2006). Further common pathologies of nfaPPA are PiD, tau-negative FTD and AD (Grossman, 2010).

3.5. *PSP with parkinsonism (PSP-P)*

A parkinsonian phenotype has been described as the second most common clinical manifestation of PSP (Respondek *et al.*, 2014; Williams *et al.*, 2005). PSP-P is characterized by predominant parkinsonism, which is often asymmetric, sometimes even tremor-dominant, and may respond to levodopa during the first years of illness (Respondek *et al.*, 2014; Williams *et al.*, 2005). Postural

instability, oculomotor dysfunction and cognitive deficits may develop after several years (Respondek *et al.*, 2014; Williams *et al.*, 2005).

3.6. Progressive gait freezing (PGF)

PGF is probably highly specific for a 4R-tauopathy and has been associated with PSP and CBD pathology, and less often with pallidonigroluysian atrophy, LBD, and MSA (Factor *et al.*, 2006). The predominant clinical sign during the first years of illness is levodopa-resistant gait freezing (Factor *et al.*, 2006).

4. Genetic contribution to PSP and CBD

Most cases of PSP and CBD arise sporadically. They have a multifactorial etiology, i.e., an interplay of genetic, epigenetic and environmental factors may cause the disease (Fig. 2A). While some studies report a slight male predominance for PSP and a marginal female predominance in CBD, the differences are highly variable across studies and usually do not reach statistical significance (Ling *et al.*, 2010; Nath and Burn, 2000; Respondek *et al.*, 2013; Williams and Lees, 2009).

4.1. PSP

4.1.1. Rare autosomal dominant inheritance

Although familial occurrence of neurodegenerative diseases is found in up to 33% of PSP index patients (Donker Kaat *et al.*, 2009), autosomal dominant inheritance was proven in only a few families (de Yebenes *et al.*, 1995; Rojo *et al.*, 1999). In affected members of these families,

pathogenic mutations were almost exclusively detected in exon 10 of the tau-encoding gene ***MAPT*** (Im *et al.*, 2015). Mutations in exon 10 favor increased tau fibrillogenesis and cause neuropathological alterations (Stanford *et al.*, 2000).

4.1.2. Common multifactorial etiology

Common variation at the ***MAPT*** locus has also been identified as a major factor contributing to the multifactorial cases of PSP (Conrad *et al.*, 1997). Thus, the H1 haplotype clade (specifically haplotype H1c) of *MAPT* is much more common in PSP patients (94%) than in controls (78%) (Baker *et al.*, 1999). A recent study with 802 PSP patients and 1,312 controls identified three MAPT H1 subhaplotypes to be associated with risk of PSP (Heckman *et al.*, 2019). Haplotype clade H1 differs from (potentially protective) haplotype clade H2 by an inversion polymorphism of about 900 kb in chromosome 17q21 that includes *MAPT*. These two haplotype clades can be further differentiated based on specific single nucleotide polymorphisms (SNPs).

The genetic contribution to the common multifactorial cases of PSP was further investigated in a genome-wide association study (GWAS) (Hoglinger *et al.*, 2011), in which the exploratory cohort included 1,114 pathology-proven PSP cases and 3,247 controls, and the confirmatory cohort consisted of 1,051 cases and 3,560 controls. The most significant association was found with two H1 haplotypes of *MAPT* (Hoglinger *et al.*, 2011) confirming previous findings of the important role of *MAPT* in the etiology of PSP (Bennett *et al.*, 1998; Conrad *et al.*, 1997; Pittman *et al.*, 2005). Furthermore, highly significant associations were detected with SNPs in genes encoding myelin-associated oligodendrocyte basic protein (***MOBP***), syntaxin 6 (***STX6***) and eukaryotic translation initiation factor 2-alpha kinase 3 (***EIF2AK3***, also termed ***PERK*** (pancreatic endoplasmic reticulum kinase)) (Fig.2 A). MOBP appears to be required for compacting or

stabilizing the myelin sheath (Kaushansky *et al.*, 2010). The specific SNP in *MOBP* results in somewhat lower expression of *MOBP* and might contribute to the disease by destabilizing myelin. On the other hand, this SNP has an even greater negative effect because it increases the expression of a gene 70 kb distant from *MOBP* (Zhao *et al.,* 2015). This gene, solute carrier family 25 member 38 (*SLC25A38*) encodes appoptosin, which is involved in neuronal cell death (Zhang *et al.*, 2012b) and might increase the caspase cleavage of tau (Zhao *et al.*, 2015). STX6 is involved in intracellular vesicle trafficking (Jung *et al.*, 2012b). PERK plays a role in the unfolded protein response (UPR) (Scheper and Hoozemans, 2015). Interestingly, recent studies suggest that PSP-associated PERK alleles are functional hypomorphs that increase neuronal vulnerability (Yuan *et al.*, 2018) and that activation of PERK mitigates tau pathology (Bruch *et al.*, 2017).

Another GWAS exploring the genetic contribution to different clinical PSP variants (RS vs. non-RS syndromes) revealed tripartite motif-containing protein 11, encoded by ***TRIM11***, to be involved in the pathological mechanism of the disease (Jabbari *et al.*, 2018). Genome-wide significance was found for a polymorphism within an intronic region of *TRIM11* (Jabbari *et al.*, 2018) (Fig.2 A).

The most recent GWAS in PSP identified two additional genes, ***SLCO1A2*** and ***DUSP10*** (Sanchez-Contreras *et al.*, 2018), and confirmed the previously detected associations of *MOBP, STX6* and *EIF2AK3* (Hoglinger *et al.*, 2011). A rational link to the pathology of PSP of these novel genes is not easily conceivable. While *SLCO1A2*, coding for solute carrier organic anion transporter family member 1A2, is associated with intrahepatic cholestasis and aneurysmal bone cysts, *DUSP10*, encoding dual specificity phosphatase 10, plays a role in regulating members of the MAP kinase superfamily. Association of *DUSP10* with a neurodegenerative disease has not yet been documented.

Another joint analysis of the 1,114 autopsy-proven cases from the original PSP GWAS (Hoglinger *et al.*, 2011) and 690 novel, mostly clinically diagnosed cases replicated known PSP susceptibility loci including *MAPT*, *MOBP,* and *STX6* and identified novel susceptibility loci near ***RUNX2*** and ***SLCO1A2*** (Chen *et al.*, 2018).

Recently, a significant genetic overlap was found between CBD and PSP. Common genetic variants in *N*-ethylmaleimide-sensitive factor (***NSF***, tagging the *MAPT* H1 haplotype), *MOBP*, C-X-C chemokine receptor type 4 (***CXCR4***), epidermal growth factor receptor (***EGFR***) and glycine decarboxylase (***GLDC***) showed shared genetic associations (Bonham *et al.*, 2018; Yokoyama *et al.*, 2017). Additionally, an up to 180-fold shared enrichment of associated genes has been reported in PSP and rheumatoid arthritis (Broce *et al.*, 2018).

Apolipoprotein E ε2 allele (***APOE* ε2**) was shown to be associated with increased tau pathology in postmortem brains of PSP patients and the recessive *APOE* ε2/ε2 genotype increased the risk of PSP and CBD (Zhao *et al.*, 2018).

4.1.3. Epigenetic alterations

Hypothesis-driven studies were conducted to search for epigenetic effects in the etiology of PSP. Assuming potential methylation differences, the genes *MAPT*, *MOBP,* leucine rich repeat containing 37 member A4 (*LRRC37A4*), ADP ribosylation factor like GTPase 17A (*ARL17A*) and *ARL17B* were studied in patients and controls. To-date, however, no convincing methylation differences were found in these genes (Allen *et al.*, 2016; Huin *et al.*, 2016).

Recently, hypothesis-free epigenome-wide DNA methylation profiling was performed in PSP (Weber *et al.*, 2018). More than 485,000 CpG sites distributed over the entire genome were interrogated by bisulfite converted DNA, extracted from postmortem prefrontal lobe tissue of a

total of 94 PSP patients and 71 controls without neurological or psychiatric diseases. Significant methylation differences of >1% were detected at 717 sites (617 hyper-, 90 hypomethylated). 62% of hypermethylated and 70% of hypomethylated sites were associated with protein-coding genes. At 34 of the hypermethylated and at 4 of the hypomethylated sites, methylation differences exceeded 5%. Methylation differences were usually found at one or a few CpGs at any given gene/locus. At the gene distal-less homeobox 1 (***DLX1***), however, numerous CpGs including a CpG island were hypermethylated at >5%. Hypermethylation mainly affected the downstream region of *DLX1* and exon 3 of its antisense transcript *DLX1AS* (Weber *et al.*, 2018). While hypermethylation did not affect the expression of *DLX1*, the *DLX1AS* expression was significantly reduced in patients. Consistent with these findings the amount of DLX1 protein was increased in gray matter of PSP brains. Alteration in the amount of DLX1 appears to be an important contributing factor in the etiology of PSP. Interestingly, two specific DLX1 binding sites were identified in the MAPT promoter region and, consistently, DLX1 overexpression changed tau expression (Fig. 2A). Furthermore, pathway analysis taking significantly differentially methylated genes into account suggests that DLX1 affects phosphorylation of tau either by the Wnt signaling pathway or via *gamma*-aminobutyric acid A ($GABA_A$) receptors (Fig. 2A).

Epigenetic effects can also be mediated by miRNAs, i.e., small non-coding RNAs (encompassing 21 to 23 nucleotides) that negatively regulate posttranscriptional expression of target genes. Two groups reported downregulation of **miRNA-132** in PSP (Smith *et al.*, 2011; Tatura *et al.*, 2016). While one group reported statistically significant downregulation (Smith *et al.*, 2011), another study only observed a trend towards reduced expression of miRNA-132 in PSP (Tatura *et al.*, 2016). Interestingly, Smith et al. also found significant downregulation of polypyrimidine tract binding protein 2 (*PTBP2*) which appears to result in retention of *MAPT* exon 10 and thus in

increased fibrillogenesis (Smith *et al.*, 2011). This finding supports the concept that downregulation of miRNA-132 plays a biologically relevant role in the pathogenesis of PSP.

4.2. CBD

4.2.1. Rare autosomal dominant inheritance

Similar to PSP, autosomal dominant inheritance of CBD is extremely rare. One study reports apparently autosomal dominant inheritance of autopsy-confirmed members of a family (mother and daughter). However, no causative mutation has been identified in this family so far (Jung *et al.*, 2012a). In addition, there are a few reports of autosomal dominant co-segregation of PSP, CBD and hereditary FTLD-*MAPT* within single families (Brown *et al.*, 1996; Bugiani *et al.*, 1999; Fekete *et al.*, 2013; Fujioka *et al.*, 2014; Josephs *et al.*, 2006; Spillantini *et al.*, 2000; Tuite *et al.*, 2005).

4.2.2. Common multifactorial etiology

A GWAS of CBD revealed significant genetic overlap with PSP (Kouri *et al.*, 2015). The exploratory GWAS of 152 cases and 3,311 controls found a highly significant association with the ***MAPT*** H1c haplotype as well as with a SNP in the vicinity of ***MOBP***. These findings were confirmed in the confirmatory cohort of 67 CBD cases and 439 controls. The study further demonstrated the association of CBD with a SNP at 8p12, that mainly appears to affect long non-coding RNA kinesin family member 13B (***lnc-KIF13B-1***) and a SNP at 2p22 that might influence the expression of son of sevenless homolog 1 (***SOS1***). Further experiments are required to demonstrate whether any of these genes influence the development of CBD (Kouri *et al.*, 2015).

Shared genetic overlap between CBD and PSP were detected at ***NSF*** (tagging the MAPT H1 haplotype), ***MOBP***, ***CXCR4***, ***EGFR***, and ***GLDC*** (Yokoyama *et al.*, 2017). In CBD and FTD, only SNPs tagging the *MAPT* haplotype were shared by both disorders (Yokoyama *et al.*, 2017). Another recent study investigated the immune-related genetic background in neurodegenerative diseases. This study showed a genetic overlap between CBD and celiac disease (Broce *et al.*, 2018). The validity of these findings needs to be assessed in the future. To the best of our knowledge, no epigenetic effects have been reported in CBD at present.

5. Environmental risk factors

5.1. Global risk factors

To date six case-control studies have been published that investigated environmental and life style risk factors in PSP. While one study found a risk-increasing association of low population density and high educational level with PSP (Davis *et al.*, 1988), another study showed low educational level to be a risk factor (Golbe *et al.*, 1996). An association between the consumption of Annona fruits and the occurrence of PSP-like atypical parkinsonism was found in Guadeloupe (Caparros-Lefebvre and Elbaz, 1999). A study focusing on smoking as risk factor did not find a correlation with the occurrence of PSP (Vanacore *et al.*, 2000). Also, no correlation was found in a study exploring living situation, use of pesticides, smoking, alcohol consumption, and coffee or tea drinking and risk for PSP (Vidal *et al.*, 2009). Another study tested whether well water drinking and lower education were associated with PSP. While there was some initial evidence suggesting such correlation, a multivariate analysis only identified well water drinking as a significant risk factor for PSP (Litvan *et al.*, 2016). In AGD, one study found an association between the disease

and lower socioeconomic status. However, the cause for this observation remained unclear (Rodriguez *et al.*, 2016). Presently, no data suggests the implication of environmental risk factors in the etiology of CBD.

5.2. Geographical clustering

An increased incidence of 4R-tauopathies has been identified in several geographic regions. Among these, **Guadeloupe** is best known for its increased prevalence of atypical parkinsonism. This was epidemiologically associated with the consumption of Annona fruits (Caparros-Lefebvre and Elbaz, 1999; Lannuzel *et al.*, 2007)

Another region with high incidence of PSP has been identified in **northern France,** centered at the communities of Wattrelos and Leers (12.3 times higher than expected) (Caparros-Lefebvre *et al.*, 2015). Toxins from various industries including metal extraction, textile and leather processing have been suspected as risk factors. In addition, contamination of homegrown plants by arsenic and hexavalent chromium has been implicated. However, none of these potential risk factors was unequivocally found to underlie the high prevalence of PSP in this region (Caparros-Lefebvre *et al.*, 2015).

The amyotrophic lateral sclerosis-parkinsonism-dementia complex found in **Guam** is not a pure 4R-tauopathy. Nevertheless, its high prevalence in Guam suggests the presence of a common genetic or environmental factor that could be relevant for other tauopathies. One hypothesis was that *beta*-methylamino-L-alanine (BMAA) found in cycad seeds that were processed to flour could have caused the disease. Another hypothesis was that BMAA, while not being toxic for the animals itself, accumulated in bats which were eaten by humans. However, both hypotheses were never

confirmed (Steele and McGeer, 2008). After all, a genetic or environmental factor has never been identified unequivocally (Lee, 2011).

5.3. Potential molecular risk factors

PSP on Guadeloupe was ascribed to the consumption of **annonaceous acetogenins**, a group of highly potent lipophilic mitochondrial complex I (CxI) inhibitors, found in Annona fruits (Lannuzel *et al.*, 2007). Strikingly, *in vitro* CxI inhibition by annonacin led to increased levels of 4R-tau mRNA in cultured neurons, while 3R-tau mRNA levels were not changed (Bruch *et al.*, 2014). This emphasizes that CxI inhibition is a distinct risk factor, particularly for 4R-tauopathies. Thus, intake of annonaceous acetogenins and particularly annonacin is so far the best studied and most convincing environmental risk factor for 4R-tauopathies (Fig. 2A). Consistently, mesenchymal stem cells from PSP patients showed decreased mitochondrial membrane potential, reduced NADH-dependent respiration, and increased oxidative stress (Angelova *et al.*, 2018). Based on this association, **other CxI inhibitors** were examined for their potential to induce tau pathology, such as rotenone, which was found to induce tau pathology in rodents (Hoglinger *et al.*, 2005). *In vitro,* various potentially ubiquitously present CxI inhibitors of microbial, plant-derived, or synthetic origin induced cell death and tau hyperphosphorylation (Hollerhage *et al.*, 2009). Piericidin A, a microbial CxI inhibitor aggravated tau pathology in tau transgenic mice (Höllerhage *et al.*, 2014). These results suggest that other ubiquitously present CxI inhibitors could contribute to the etiology of 4R-tauopathies. However, convincing evidence from epidemiological studies is missing.

5.4. *Future perspectives*

Apart from the correlation between Annona fruits and the occurrence of atypical parkinsonism on Guadeloupe, no other distinct risk factor for 4R-tauopathies has been conclusively identified so far. The relevance of other toxins including metals or CxI inhibitors remains elusive. The identification of further risk factors is difficult because case-control studies are only able to identify strong risk factors whereas cohort studies are extremely challenging in rare diseases. Ideally, cohort-studies with larger numbers of patients including uncommon phenotypes will help to identify further risk factors for 4R-tauopathies.

6. Intracellular tau pathogenesis

6.1. *Tau isoforms*

The **tau protein** was identified in the 1970s as an essential component for tubulin to form microtubule fibrils (Cleveland *et al.*, 1977a, b; Weingarten *et al.*, 1975). Tau is expressed in the central and peripheral nervous system and to a lesser extent in kidneys, lung and testis (Gu *et al.*, 1996). In the CNS, full-length tau protein is primarily located in axons (Lee *et al.*, 2001; Trojanowski *et al.*, 1989). It is also present in nuclear or somatodendritic compartments of neurons (Tashiro *et al.*, 1997), to a lesser extent in oligodendrocytes (Klein *et al.*, 2002) and extracellularly in the interstitial fluid (ISF) at nanomolar concentrations (Yamada *et al.*, 2011).

Tau is encoded by the ***MAPT* gene** on chromosome 17q21. *MAPT* comprises 16 exons (Neve *et al.*, 1986). Exons 1, 4, 5, 7, 9, 11 and 12 are constitutive whereas others are subject to alternative splicing (Wang and Mandelkow, 2016). **Six tau isoforms** exist (Fig. 2B) generated by alternative splicing of exons 2 and 3 near the N-terminus and of exon 10 for the second repeat sequence in

the MBD near the C-terminus (Lee *et al.*, 2001). By their number of N-terminal exons and repeat domains, the tau isoforms are termed 2N/4R (45.9 kDa), 2N/3R (42.6 kDa), 1N/4R (43.0 kDa), 1N/3R (39.7 kDa), 0N/4R (40.0 kDa), or 0N/3R (36.7 kDa). In a healthy human adult brain the ratio of 3R to 4R is 1:1 and that of 1N:0N:2N tau is 54:37:9 (Brandt *et al.*, 2005). Other splice variations include "big-tau" (Oblinger *et al.*, 1991), that derive from alternative splicing of exon 4a in the spinal cord and the peripheral nervous system (Georgieff *et al.*, 1993; Goedert *et al.*, 1989), and splice variants of exon 6 which contain no MBD and appear only in the CNS and skeletal muscle (Luo *et al.*, 2004) . Their function remains unclear.

Tau is **unfolded** in its natural state in cells. Due to its unusual hydrophilic character and temporary secondary structures (α-helix, ß-strand, polyproline helix), the protein maintains a flexible conformation within the cytoplasm with a low content of secondary structures (Mukrasch *et al.*, 2009; Schweers *et al.*, 1994). Tau also forms a so-called **"paperclip" structure** with the C-terminal end, folding back over the MBD through intramolecular interactions between the C- and N-terminus (Jeganathan *et al.*, 2008). A full-length tau (2N/4R) consists of 80 serine and threonine amino acids, 56 negative (aspartic acid and glutamic acid), 58 positive (lysine and arginine) and 8 aromatic amino acids (5 tyrosine, 3 phenylalanine, no tryptophan) (Mandelkow and Mandelkow, 2012). Fifty percent of the tau protein is made up of 5 amino acids, glycine, lysine, proline, serine and threonine (Bodea *et al.*, 2016). The asymmetry of charges is essential to the fast direct interaction of the tau protein with microtubule-binding partners, the secondary structure and the aggregation propensity of tau (Mandelkow and Mandelkow, 2012; Morris *et al.*, 2011).

Four domains can be distinguished within the tau protein (e.g. 2N/4R, 441 amino acids; Fig. 2F): the **N-terminal projection region** (amino acids 1 - 150), a **proline-rich domain** (amino acids 151

– 243), the **microtubule-binding domain** (MBD, amino acids 244 -369) and the **C-terminal region** (amino acids 370 – 441) (Mandelkow *et al.*, 1996).

The **N-terminal projection domain** does not bind to microtubules directly and projects away from microtubules (Guo *et al.*, 2017). It exerts multiple functions such as regulation of the microtubule dynamics (Chen *et al.*, 1992), inhibition of axonal transport (Kanaan et al., 2011), subcellular distribution of the tau protein (Liu and Gotz, 2013; Paholikova *et al.*, 2015), association with plasma membrane binding protein annexin A2 (Gauthier-Kemper *et al.*, 2011) and the binding to the dynactin complex, which influences axonal transport via interaction of microtubule motor dynein with membranous cargo (Magnani *et al.*, 2007).

The **proline-rich domain** harbors seven adjacent proline regions, which serve as a potential binding site for Src homology 3 (SH3)-containing proteins, such as tyrosine kinase Fyn (Lee *et al.*, 1998), thus suggesting a role in the dendritic compartment and postsynaptic targeting of Fyn (Ittner *et al.*, 2010; Lau *et al.*, 2016; Mondragon-Rodriguez *et al.*, 2012). Moreover, the proline-rich domain has been shown as a DNA and RNA binding site, which could be essential for genomic surveillance under cellular stress (Bukar Maina *et al.*, 2016).

The repeats within the **MBD** mediate interactions between the tau protein, microtubules and tubulin (Mukrasch *et al.*, 2007; Sillen *et al.*, 2007). Data from "single molecule tracking" indicate fast direct interactions between tau isoforms and microtubules via a "kiss-and-hop mechanism" (Janning *et al.*, 2014). Quantitative analysis with photoactive green fluorescent protein-tagged tubulin suggests tau protein as a regulator of tubulin and microtubule equilibrium and to be important for microtubule assembly and recycling (Janning *et al.*, 2014). Other proteins that are associated with the MBD (Guo *et al.*, 2017) imply F-actin (Correas *et al.*, 1990), apolipoprotein E

(Huang *et al.*, 1995) and presenilin (Takashima *et al.*, 1998), histone deacetylase 6 (Ding *et al.*, 2008) and α-synuclein (Jensen *et al.*, 1999).

The function of the **C-terminal domain** of tau remains elusive. Few studies suggested that alterations in the C-terminus might influence other tau regions, interactions with binding partners, availability for phosphorylation and aggregation propensity (Abraha *et al.*, 2000; Berry *et al.*, 2003; Connell *et al.*, 2001; Reynolds *et al.*, 2008).

Altered translation of tau isoforms (Ingelsson *et al.*, 2007; Takanashi *et al.*, 2002; Umeda *et al.*, 2004) might also contribute to the pathogenesis of 4R-tauopathies. A significant shift of 4R/3R ratios is found in postmortem brains of CBD and PSP patients. However, no correlation has been seen between the expression patterns of tau mRNA isoforms and the accumulation of phosphorylated tau (pTau) (Takanashi *et al.*, 2002).

6.2. Post-translational tau modifications

In the adult human brain, tau can be post-translationally modified (Martin *et al.*, 2011; Morris *et al.*, 2015) (Fig. 2C), either enzymatically by phosphorylation (Aerts *et al.*, 2011; Lee *et al.*, 2004), acetylation (Cohen *et al.*, 2013; Cohen *et al.*, 2011; Irwin *et al.*, 2012), methylation (Funk *et al.*, 2014), ubiquitination (Cripps *et al.*, 2006; Lu *et al.*, 2018), sumoylation (Dorval and Fraser, 2006), *O*-linkage to *N*-acetylglucosamine (Arnold *et al.*, 1996; Leney *et al.*, 2017), nitration (Reyes *et al.*, 2008), proteolytic cleavage (truncation) (Mondragon-Rodriguez *et al.*, 2008) and non-enzymatically by glycation (Ledesma *et al.*, 1994), oxidation (Landino *et al.*, 2004), deamination (Miyasaka *et al.*, 2005) and isomerization (Miyasaka *et al.*, 2005). The tau phosphorylation is developmentally regulated (Ksiezak-Reding *et al.*, 1992), but the functional relevance of this regulation is still unclear (Bodea *et al.*, 2016; Goedert and Spillantini, 2017).

Phosphorylations at serine, threonine and tyrosine residues are the most commonly described post-translational modifications. So far, 85 potentially accessible phosphorylation sites have been identified in the 2N/4R isoform (Guo *et al.*, 2017; Mietelska-Porowska *et al.*, 2014). Distinct hyperphosphorylation of tau was seen in postmortem brain tissues of CBD and PSP patients (Feany *et al.*, 1995; Ferrer *et al.*, 2002). *In vitro* experiments show that hyperphosphorylation of tau influences its binding affinity to microtubules and other proteins (Niewidok *et al.*, 2016). Accumulation of hyperphosphorylated tau in dendritic spines diminishes synaptic function and leads to degeneration (Frandemiche *et al.*, 2014; Harris *et al.*, 2012). Moreover, tau phosphorylation is associated with misfolding and pathological aggregation of tau (Jellinger, 2011; Mandelkow and Mandelkow, 2012; Morris *et al.*, 2011; Wills *et al.*, 2010) and alters the interaction of tau with potential binding partners, such as the plasma membrane, DNA or Fyn (Ekinci and Shea, 2000; Hanger *et al.*, 2009). In AD patients, 8.1 mol phosphate/mol protein can be found on average in aggregated, hyperphosphorylated tau, called paired helical filaments (PHF), while in healthy controls, it is 1.9 mol phosphate/mol protein (Ksiezak-Reding *et al.*, 1992). At present, comparative postmortem brain analysis of the phosphate/protein ratio in NFTs of different tauopathies is missing for 4R-tauopathies.

Tau kinases can be divided into three main classes: **1) Proline-directed protein kinases** including glycogen synthase kinase (GSK)-3 α/β, cyclin-dependent kinase 5 (CDK5) and mitogen-activated kinases (Martin *et al.*, 2013). **2) Non-proline-directed protein kinases** such as tau-tubulin kinase 1/2, casein kinase 1α/1δ/1ε/2, microtubule affinity-regulating kinases, phosphorylase kinase, protein kinase N and Ca^{2+}/calmodulin-dependent kinase II (Martin *et al.*, 2013). **3) Tyrosine protein kinases** such Src family kinase members, spleen tyrosine kinase, lymphocyte-specific protein kinase, ABL related gene kinases and Fyn (Martin *et al.*, 2013).

GSK-3β regulates the phosphorylation of 42 known phosphorylation sites in the tau protein (Guo *et al.*, 2017), including 29 hyperphosphorylated sites observed in AD (Hanger *et al.*, 2009; Lovestone *et al.*, 1994). Analyses of AD postmortem brains suggested a GSK-3β colocalization with NFT pathology, a correlation of GSK-3β activity and NFT burden and a correlation of GSK-3β protein and hyperphosphorylation of tau (Hanger *et al.*, 1992; Leroy *et al.*, 2002). However, in postmortem brain tissue of PSP patients, protein levels of all GSK-3 isoforms were unaltered compared to controls (Borghi *et al.*, 2004). Levels of GSK-3β expression in CBD and AGD are not known. Despite the crucial role of tau phosphorylation in the pathoetiology of 4R-tauopathies, data about differences in the phosphorylation pattern of tau among various 4R-tauopathies is pending.

Tau phosphatases include protein phosphatase (PP) 2A (PP2A), PP2B (calcineurin), PP1 and PP5 (Braithwaite *et al.*, 2012). So far, their impact on human 4R-tauopathies has not been sufficiently studied. Although **hyperphosphorylation at serine, threonine** and **tyrosine residues** of the tau protein is a hallmark of 4R-tauopathies, the physiological and pathophysiological functions of these modifications remain unclear (Bodea *et al.*, 2016). The classical concepts of impaired interaction between pTau and microtubules, increased aggregation propensity, as well as the degeneration of neurons (Alonso *et al.*, 1996; Salehi *et al.*, 2003) are based on *in vitro* systems. However, these findings did not perfectly match with the results of animal and human studies (Bonda *et al.*, 2011). Furthermore, knockout mice generated to model a loss of tau function, do not show an apparent phenotype (Elder *et al.*, 1998; Harada *et al.*, 1994; Rao *et al.*, 1998). Moreover, it is an open debate whether hyperphosphorylation of tau is responsible for shifting the tau conformation from α-helix to ß-sheet (Bodea *et al.*, 2016; Goedert and Spillantini, 2017).

Tau protein is subject to extensive **additional post-translational modifications**. Although functional implications remain elusive, acetylation (Min *et al.*, 2010; Yang and Seto, 2008) and methylation (Funk *et al.*, 2014; Park *et al.*, 2018) of tau were shown for PSP, FTLD-*MAPT* and AD, and ubiquitination for AD (Cripps *et al.*, 2006; Mori *et al.*, 1987). It is noteworthy that acetylation of tau at lysine 280 was found in tau aggregates of patients with PSP, AGD, PiD, tangle-predominant senile dementia, FTLD-*MAPT* and AD (Wang and Mandelkow, 2016). Other post-translational modifications of tau such as glycosylation, nitration, oxidation, polyamination, sumoylation and ubiquitination might arise before hyperphosphorylation and tau filament formation (Necula and Kuret, 2004). Abnormal nitration at tyrosine residues 18, 29 and 394 was seen in tauopathies (Reyes *et al.*, 2012). Nitration appears to change the secondary structure of tau and site-specific nitration may either protect or promote tau-fibrillization (Reyes *et al.*, 2012). Notably, glycosylation of tau may facilitate phosphorylation (Liu *et al.*, 2002; Wang *et al.*, 1996) whereas *O*-linkage to *N*-acetylglucosamine (*O*-GlcNAc) may protect from phosphorylation (Liu *et al.*, 2004) and aggregation (Yuzwa *et al.*, 2014).

6.3. Tau fragmentation

Tau contains numerous potential cleavage sites. Various tau fragments were identified in experimental systems (Quinn *et al.*, 2018), but only a few tau truncations have been discovered in neurodegenerative diseases (Fig. 2C,F). Four truncated forms have been found in 4R-tauopathies, i.e., Tau35, 17KDa Tau and Tau224 in both PSP and CBD, and Tau-C in PSP (Table 2).

Immunoblots of brain homogenates from patients with AD, FTLD, PSP, and CBD showed a variable fragmentation pattern as visualized by different antibodies against distinct tau regions (Arai *et al.*, 2001; Matsumoto *et al.*, 2015). Abnormally truncated tau in CBD and PSP is present

in sarkosyl-insoluble and SDS-soluble fractions (Arai *et al*., 2001). This may suggest that the presence of tau fragments in the immunoblot of sarkosyl-insoluble fractions is also associated with the dissociation of proteolyzed aggregates in the presence of SDS in denaturing gel. The presence of different tau fragments may depend on distinct types of cells undergoing degeneration, distinct proteolytic mechanisms and structural differences in aggregates and fibrils (Falcon *et al.*, 2018; Goedert *et al.*, 2017). Tau truncation may trigger tau aggregation or vice versa. Two main hypotheses describe the possible role of tau cleavage in tauopathies.

First, tau **cleavage may be an upstream event** that removes the N- and C-terminal residues of the MBD, which then facilitate tau aggregation by shifting the binding affinity from tubulin-tau to tau-tau interactions (Lai *et al.*, 2016; Simic *et al.*, 2016; Wang *et al.*, 2007). An example of this phenomenon is Glu391-cleaved tau in which sequential proteolysis at Glu391 leads to tau-tau interactions and self-accumulation of tau via binding/digestion cycles *in vitro* (Wischik *et al.*, 1996). In this model, however, factors triggering the activation of proteases which lead to tau pathology have not yet been identified.

In a second scenario, **tau truncation may be a consequence of tau aggregation**, a byproduct of proteolytic degradation of tau fibrils, when cells are trying to eliminate protein aggregates. Increasing evidence suggests that autophagic and proteasomal degradation mechanisms of tau are actively cooperating to protect cells against toxic protein aggregates (Wang and Mandelkow, 2012). Proteases responsible for tau cleavage can be either autophagic-lysosomal, such as cathepsins and asparagine endopeptidase, or cell death-associated, such as caspases and calpains (Wang *et al.*, 2010). This second scenario provides a rational model for tau truncation. However, a potential toxic or protective relevance of the resulting cleavage has presently not been shown.

6.4. Tau aggregation

Self-assembly of monomeric tau produces β-sheet structures, called amyloid fibrils (Nizynski *et al.*, 2017), which are basically formed upon the **interaction of MBDs** (Fig. 2C). Two hexapeptide motifs in the MBD of 4R tau, ^{306}VQIVYK^{311} and ^{275}VQIINK^{280}, have a high propensity for participating in β-sheet formation and are crucial for the generation of early aggregates (Ganguly *et al.*, 2015; von Bergen *et al.*, 2001; von Bergen *et al.*, 2000).

In contrast to other amyloid-forming proteins, **tau fibrillization is not occurring spontaneously at physiological conditions** (Kuret *et al.*, 2005), possibly due to electrostatic repulsion of cationic amino acids and a rate-limiting conformational barrier (Chirita *et al.*, 2005). Generally, the interaction of C- and N-terminal regions with the MBD stabilizes tau monomers and prevents fibrillization. Cleavage of these regions, however, increases the aggregation propensity of tau (Berry *et al.*, 2003; Horowitz *et al.*, 2006). Tau fibrillization can also be induced by diminishing the positive charge by extensive phosphorylation (Alonso *et al.*, 1996) or interactions with anionic compounds (Chirita *et al.*, 2003; Goedert *et al.*, 1996; Holmes *et al.*, 2013; Kampers *et al.*, 1996; Sibille *et al.*, 2006; Wilson and Binder, 1997).

The fibrillization model of tau is, similar to other amyloid-forming proteins, a **nucleation-dependent polymerization** with sigmoidal kinetics. It consists of three steps, including nucleation, elongation and plateau (Lee *et al.*, 2007).

The **nucleation** is the rate-limiting slow step in the fibrillization, kinetically referred to as a lag phase, in which partially folded intermediates and oligomeric structures are formed. Early-stage structures are difficult to detect and characterize due to the variability of aggregated species and the intensive molecular transition in this phase. Nevertheless, granular tau oligomers have been identified by *in vitro* fibrillization of recombinant tau, and in different Braak stages of AD (Maeda

et al., 2007; Patterson *et al.*, 2011). Similarly, various studies reported the presence of tau oligomers in the brain of PSP patients (Gerson *et al.*, 2014; Ward *et al.*, 2013).

Conversion of oligomers to small primary fibrils initiates the exponential **elongation** phase of fibrillization in which progressive growth by incorporation of monomers form mature fibrillar structures (Margittai and Langen, 2004).

Finally, fibrillization reaches the **plateau phase**, when the rate of fibril formation and fibril dissociation are in equilibrium, which is primarily associated with the concentration of monomers and fibrils.

6.5. Gain of toxic function and loss of function

The pathological process from soluble tau protein to insoluble filamentous aggregates leads to a gain of toxic function (Goedert *et al.*, 2017). While the pathological role of tau fibrils is under debate (Busche *et al.*, 2019; Gendron and Petrucelli, 2009; Trojanowski and Lee, 2005), toxic tau oligomers in PSP patients (Gerson *et al.*, 2014) are thought to play a crucial role via different mechanisms, including loss of membrane integrity (Flach *et al.*, 2012), mitochondrial and synaptic dysfunction (Lasagna-Reeves *et al.*, 2011) as well as impairment of axonal transport, long-term potentiation, memory and cognition (Morfini *et al.*, 2009; Ward *et al.*, 2012). A gain of toxic function might also be caused by insufficient clearance of oligomers from the ISF by the glymphatic system during sleep (Holth *et al.*, 2019).

Furthermore, impairment of different degradation mechanisms, as discussed in chapter 7, as well as tau protein sequestration (Luk *et al.*, 2012) and metal homeostasis (Barnham and Bush, 2014) might contribute to the toxic gain of function in 4R-tauopathies.

Also, a loss of physiological functions could contribute to the pathogenesis in 4R-tauopathies resulting from deficient axonal transport, consequently leading to altered mitochondrial and synaptic functions and loss of trophic support.

7. Intracellular tau clearance and repair mechanisms

7.1. Ubiquitin-proteasome system (UPS)

In the context of tauopathies, the UPS plays a pivotal role in the degradation of abnormal tau and in general tau turnover (Sulistio and Heese, 2016). The UPS follows a multi-step process, starting with the transfer of ubiquitin to the substrate protein (monomeric abnormal tau, including, e.g., hyperphosphorylated, misfolded, or partially cleaved tau, etc.) via an enzyme cascade (E1-E3, Fig. 2D). The E1 enzymes activate ubiquitin by forming an active thioester intermediate enabling the transfer of ubiquitin to E2 conjugating enzymes. E3 ligases play a key role within the UPS by recognizing the substrate protein as a sensor. E2 then interacts with E3 and ubiquitin is transferred from E2 to lysine residues in the substrate protein. This process is repeated several times and results in polyubiquitination (Gadhave *et al.*, 2016; Sulistio and Heese, 2016). Substrate proteins tagged with polyubiquitin chains are then recognized by the 26S proteasome, a multisubunit protein complex in which the degradation occurs. In general, the UPS is a highly complex and tightly regulated system required for protein homeostasis. Any impairment of this system is fatal for the disease process of tauopathies. E3 ligases have been in the focus of intense research because they select misfolded tau. Approximately 600 E3 ligases are encoded in the human genome (Lee *et al.*, 2013), making it difficult to identify which specific ligase(s) might play the most crucial role in tauopathies. In addition, several chaperons such as Hsp70 and Hsp90 are interacting with

E3 ligases, such as C-terminus of Hsc70-interacting protein (CHIP), increasing the complexity of the tau degradation process (Khanna *et al.*, 2016). Whereas in AD, intense research unraveled certain mechanisms (Zhang *et al.*, 2017), the UPS remains poorly investigated in 4R-tauopathies. However, in PSP a risk gene was recently discovered, which encodes for the E3 ligase TRIM11 (Jabbari *et al.*, 2018). Furthermore, significant reductions of distinct proteasomal subunits in PSP have been reported (Bukhatwa *et al.*, 2010). In AGD it was found that ubiquitin is present in a majority of argyrophilic grains and coiled bodies and can be co-localized with pTau, suggesting involvement of impaired UPS in the disease pathology (Ferrer *et al.*, 2008). The UPS might be important in the development of 4R-tauopathies and an interesting target for therapeutic strategies.

7.2. Autophagy-lysosome system (ALS)

The ALS is another important mechanism to maintain protein homeostasis in the cell (Khanna *et al.*, 2016). In tauopathies, it is involved not only in the degradation of monomeric abnormal tau but also of larger aggregated tau species (Wang and Mandelkow, 2012). The ALS can achieve this by mainly two major pathways (Fig. 2D), chaperone-mediated autophagy (CMA) and macroautophagy (Ciechanover and Kwon, 2015). CMA is a selective proteolytic mechanism. Two KFERQ homology motifs in tau (Wang *et al.*, 2009) can be recognized by the chaperone heat shock cognate 70 (Hsc70) and subsequently binds to lysosomes via lysosome-associated membrane protein 2A (LAMP-2A) (Massey *et al.*, 2004; Wang and Mandelkow, 2012). Misfolded tau then translocates into the lysosomal lumen where it is degraded. Macroautophagy is a further pathway of the ALS. It degrades misfolded tau and larger aggregated tau species (Wang and Mandelkow, 2012; Wang *et al.*, 2009). In a first step, tau species are bound to adaptors (e.g., p62) and form condensed cargo complexes which interact with light chain 3 II (LC3-II) on the surface

of autophagic double layer membranes, the so-called phagophore (Ciechanover and Kwon, 2015). Phagophoric membranes merge and autophagosomes are formed which finally fuse with lysosomes enabling the degradation of cargoes by the lysosomal enzyme machinery. Notably, the ALS is a highly complex mechanism and involves numerous additional components and processes. Due to the importance of the ALS in neurodegeneration, impairment of the ALS leads to detrimental consequences. It has been shown that in aged neurons the components of the ALS are downregulated, predisposing for neurodegenerative diseases (Ciechanover and Kwon, 2015). Several studies show that failure of macroautophagy might be a part of the AD pathogenesis (Chesser *et al.*, 2013). There is first evidence that the ALS is impaired in 4R-tauopathies as well (Piras *et al.*, 2016), but unlike in AD, the ALS still remains understudied in 4R-tauopathies.

7.3. Unfolded protein response (UPR)

The UPR is a complex mechanism against cellular stress caused by misfolded proteins (Paschen and Mengesdorf, 2005). It uses three main pathways to restore homeostasis (Fig. 2D), the PERK pathway, the activating transcription factor 6 (ATF6) pathway and the inositol-requiring enzyme 1 (IRE1) pathway (Scheper and Hoozemans, 2015). Restoration of homeostasis can be achieved by a global shutdown of protein synthesis or by recruiting factors that act against cellular stress. However, if the cell cannot cope with the stress, the UPR can also drive the cell into apoptosis.

The PERK pathway is of particular interest in 4R-tauopathies, as it was discovered that the PERK gene is a major genetic risk factor for PSP (Hoglinger *et al.*, 2011). Neuropathological studies found that abnormal PERK signaling is involved in tauopathies (Nijholt *et al.*, 2012; Stutzbach *et al.*, 2013). PERK loss of function might lead to tau-positive NFTs (Bruch *et al.*, 2015). So far, only a few studies investigated the involvement of the UPR in 4R-tauopathies. The UPR might

have substantial implications on the development of tauopathies and due to its complexity it is a key challenge to develop new therapeutic strategies (Bruch *et al.*, 2017).

8. Propagation of tau pathology

Accumulating evidence suggests that tau pathology can be transmitted from one cell to another, a process termed **intercellular tau spreading** (Fig. 2E) (Mudher *et al.*, 2017). In 2009, cell-to-cell transmission of tau was reported for the first time *in vitro* (Frost *et al.*, 2009) and *in vivo* (Clavaguera *et al.*, 2009). Intercellular tau spreading is considered a prion-like mechanism (Holmes and Diamond, 2014), but unlike in prion diseases, there is no evidence suggesting infectiousness of 4R-tauopathies from a patient to other humans. An unidentified pathological form of tau, which spreads from cell to cell and seeds the abnormal aggregation of tau in healthy cells, is the major element of the prion-like hypothesis of tau spreading (Goedert and Spillantini, 2017). This process comprises three main steps, including 1) the release of spreading tau species from the diseased cells, 2) uptake of extracellular tau by healthy cells, and 3) formation of new aggregates inside the recipient cells by recruitment of normal endogenous tau (seeding). In the following sections, we will review tau species that have been found in the extracellular environment as well as the mechanisms of tau release and uptake, and finally discuss the various species of tau and their capacity as tau seeds in the propagation of tau pathology.

8.1. Extracellular tau (eTau)

Despite the physiological function of tau as an intracellular microtubule-associated protein, various species of tau have been observed in the extracellular environment, the so-called extracellular tau (eTau) (Fig. 2F).

eTau is found in the culture medium of neuroblastoma cells, normal primary neurons (Karch *et al.*, 2012) and in induced pluripotent stem cell (iPSC)-derived neurons (Bright *et al.*, 2015), but also in the ISF and CSF of mice (Yamada *et al.*, 2011) and in human brain (Magnoni *et al.*, 2012).

In body fluids, tau is present in a **vesicle-free** form but also **within extracellular vesicles** (Dujardin *et al.*, 2014). eTau in extracellular vesicles can be found in the culture medium of tau-overexpressing neuroblastoma cells (Saman *et al.*, 2012), mouse brain (Polanco *et al.*, 2016) as well as human plasma (Stern *et al.*, 2016). However, the percentage of tau in vesicle-associated form in the conditioned medium of tau-overexpressing HEK293 cells, rat cortical neurons and rat primary embryonic cortical neurons was only 1%, 3% and 10%, respectively (Dujardin *et al.*, 2014; Wang *et al.*, 2017; Yan *et al.*, 2016). A recent study with conditioned medium of iPSC-derived neurons and human CSF confirmed less than 1% of tau to be associated with exosomes (Guix *et al.*, 2018).

C-terminal **cleaved eTau** was detected in the CSF of mice (Barten *et al.*, 2011). eTau in CSF of healthy individuals and PSP patients is mainly truncated and only comprises of mid-region residues and lacks the MBD (Barthelemy et al., 2016). The Tau224 fragment was significantly lower in the CSF of PSP and CBD patients compared to controls, while it was increased in AD (Cicognola *et al.*, 2019). eTau is also found in phosphorylated form in the CSF of PSP and CBD patients and healthy individuals (Noguchi *et al.*, 2005). A recent study showed that reduction

in CSF pTau (at threonine 181) is directly correlated with the severity and rate of disease progression in PSP (Rojas *et al.*, 2018).

pTau aggregates are rarely found in the extracellular space of normal neurons (Kanmert *et al.*, 2015) and in the CSF of healthy individuals (Sengupta *et al.*, 2017). However, in brains of PSP patients, tangles outside of cells were present as a remnant of dead neurons, so-called ghost tangles (Arima *et al.*, 1999). Moreover, eTau aggregates such as oligomers and seed-competent species of high molecular weight were reported in the CSF of patients with tauopathy (Sengupta *et al.*, 2017; Takeda *et al.*, 2016).

Concentrations of total tau, pTau and Tau224 fragment in the CSF of PSP patients are lower than in AD, suggesting different pathological mechanisms in 4R-tauopathies as compared to AD (Cicognola *et al.*, 2019; Hall *et al.*, 2012; Wagshal *et al.*, 2015). Kinetics studies of eTau in iPSC-derived cortical neurons revealed a shorter half-life of 4R-tau compared to 3R-tau, which might explain the lower total CSF tau in 4R-tauopathies (Sato *et al.*, 2018). In addition, the differences in the distribution of tau pathology between neurons and glia cells may lead to a lower amount of eTau in PSP and CBD compared to other tauopathies. A more detailed characterization and profile analysis of eTau fragments, modified monomers and aggregates in the CSF and ISF of patients with 4R-tauopathies can provide a better understanding of eTau species under these specific pathological conditions.

eTau may be involved in pathological processes in two ways. First, **eTau is able to induce toxicity and neuronal dysfunction**. Treatment of hippocampal neurons with tau cause cell death by dysregulating calcium homeostasis (Gomez-Ramos *et al.*, 2006). This toxicity resulted from soluble monomeric and oligomeric eTau and from phosphorylated or insoluble PHFs. A subsequent study revealed the role of muscarinic receptors M1 and M3 in eTau-driven

neurotoxicity (Gomez-Ramos *et al.*, 2008). Residues 391 to 407 of tau were identified to interact with muscarinic receptors, with ten times stronger receptor affinity than acetylcholine (Gomez-Ramos *et al.*, 2009). This finding highlights a possible physiological function of eTau as well as a detrimental impact. Consistently, extracellular treatments with recombinant truncated (Florenzano *et al.*, 2017) and oligomeric (Fa *et al.*, 2016; Kaniyappan *et al.*, 2017) tau were shown to induce synaptic dysfunction. Moreover, exogenous exposure to the secretome of iPSC-derived neurons, containing synaptotoxic forms of tau, inhibited long-term potentiation *in vivo* (Hu *et al.*, 2018). Subcortical injection of tau oligomers, but not monomers and fibrils, impaired memory and induced synaptic dysfunction (Lasagna-Reeves *et al.*, 2011). Importantly, clearance of extracellular tau oligomers by specific antibodies protected against locomotor and memory deficits in a mouse model of 4R-tauopathy with the P301L-*MAPT* mutation (Castillo-Carranza *et al.*, 2014).

As a second pathological feature, **eTau plays a role in the intercellular propagation of tau pathology**. Specific tau antibodies revealed the stereotypical stages of tau pathology, successively involving anatomically connected brain areas in a hierarchical pattern, called Braak stages, in AD (Braak and Braak, 1991; Braak *et al.*, 2011), providing a conceptual basis for cell-to-cell propagation of tau pathology during disease progression in human patients. Albeit a staging system has been conceptualized for PSP (Williams *et al.*, 2007), a stringent proof of tau pathology spreading following a hierarchical pattern has not yet been unequivocally demonstrated for 4R-tauopathies.

8.2. Tau release mechanisms

As a prerequisite for its subsequent uptake by adjacent or connected cells, tau has to be first translocated from cytoplasm to extracellular milieu (Fig. 2E). Previously, tau found in the CSF was thought to be a consequence of passive release from dying neurons (Blennow *et al.*, 1995). However, tau is also present in the CSF of healthy individuals (Blennow *et al.*, 1995; Sato *et al.*, 2018; Vandermeeren *et al.*, 1993; Vigo-Pelfrey *et al.*, 1995) and in the brain ISF of wildtype mice and P301S mutant tau transgenic mice (Yamada *et al.*, 2011). Moreover, the presence of tau in the cell culture medium of primary neurons was shown to be independent of cell death (Kanmert *et al.*, 2015).

In the extracellular space, tau is present as vesicle-free eTau not embedded in extracellular vesicles (Chai *et al.*, 2012; Dujardin *et al.*, 2014; Guix *et al.*, 2018; Katsinelos *et al.*, 2018; Kim *et al.*, 2010a; Kim *et al.*, 2010b; Saman *et al.*, 2012; Simon *et al.*, 2012; Wang *et al.*, 2017; Wu *et al.*, 2016; Yan *et al.*, 2016) or as eTau associated with extracellular vesicles such as ectosomes (Dujardin *et al.*, 2014; Yan *et al.*, 2016) and exosomes (Fig. 2E) (Asai *et al.*, 2015; Dujardin *et al.*, 2014; Guix *et al.*, 2018; Saman *et al.*, 2012; Simon *et al.*, 2012; Wang *et al.*, 2017; Yan *et al.*, 2016). It has been suggested that under normal physiological conditions, tau is actively secreted predominantly by ectosomes and a shift toward exosomal secretion appears only upon increasing cellular tau accumulation (Dujardin *et al.*, 2014).

Independent of the vesicle-associated secretion pathways, tau has been suggested to be released from the cells via a non-canonical exocytic **mechanism depending on the chaperone Hsc70** in concert with its co-chaperone DnaJ (Fontaine *et al.*, 2016).

Recently, it was shown **that tau directly translocates through the plasma membrane** via a mechanism mediated by its interaction with phosphatidylinositol 4,5-bisphosphate and sulfated

proteoglycans (Katsinelos *et al.*, 2018). More specifically, this unconventional secretion process is preferably targeting hyperphosphorylated tau due to its increased intracellular abundance upon detachment from the microtubules. Importantly, proteoglycan-associated soluble eTau is able to initiate tau aggregation in adjacent recipient cells. Interestingly, also another recent study described an unconventional mechanism for tau secretion via ATP-independent direct translocation through the plasma membrane (Merezhko *et al.*, 2018). It was demonstrated that also this non-vesicular mechanism is facilitated by heparan sulfate proteoglycans (HSPGs) and predominantly targets phosphorylated oligomeric tau species that are clustered in plasma membrane microdomains. Intriguingly, it was suggested that this particular mechanism of tau secretion might be modulated via altering the properties of the plasma membrane.

eTau levels appear to depend on the intracellular tau composition. eTau levels are changed in relation to the isoform composition of tau protein (Karch *et al.*, 2012). Various tau mutations affect the cellular tau release *in vitro*. Another study suggested that C-terminal tau cleavage enhances tau secretion *in vitro* (Plouffe *et al.*, 2012).

Induction of **neuronal activity** and the **sleep-wake cycle** strongly promotes cellular tau release *in vitro* and *in vivo* (Holth *et al.*, 2019; Pooler *et al.*, 2013; Wang *et al.*, 2017; Yamada *et al.*, 2014) and exacerbates the progression of tau pathology *in vivo* (Wu *et al.*, 2016). Intriguingly, synaptic connectivity has been shown to significantly stimulate the secretion and propagation of tau pathology (Calafate *et al.*, 2015; Wang *et al.*, 2017).

Moreover, **lysosomal dysfunction**, an established feature across many neurodegenerative disorders, and **nutrient deprivation** increase the release of tau from primary neurons (Mohamed *et al.*, 2014). Furthermore, it has been shown that up-regulation of mammalian target of rapamycin (**mTOR**) activity enhanced tau release (Tang *et al.*, 2015). Additionally, Ras-related proteins 1A

and 7A (**Rab1A** and **Rab7A**) are implicated in the modulation of tau secretion via changes in Golgi dynamics and membrane trafficking, respectively (Mohamed *et al.*, 2017; Rodriguez *et al.*, 2017).

8.3. Tau uptake mechanisms

There is currently limited knowledge about the **identity of tau species taken up by recipient cells** (Fig. 2E). Whereas some studies showed the preferred internalization of **small monomeric or oligomeric tau** species (Evans *et al.*, 2018; Frost *et al.*, 2009; Rauch *et al.*, 2018; Usenovic *et al.*, 2015; Wu *et al.*, 2013), others suggested that only **high-molecular weight oligomeric and fibrillized tau** is internalized (Mirbaha *et al.*, 2015; Takeda *et al.*, 2015). Another recent study has demonstrated that the accumulation of intracellular tau depends on the **isoform composition** of the eTau oligomers (Swanson *et al.*, 2017). Also the specific **cellular mechanisms of tau uptake** are subject of debate.

Macropinocytosis, which is a subtype of bulk endocytosis specialized in internalization of fluids and macromolecular structures, has been implicated in cellular uptake of tau fibrils (Holmes *et al.*, 2013). eTau fibrils promoted the dynamic rearrangement of the plasma membrane to induce the formation of macropinosomes. Furthermore, macropinocytosis-mediated cellular tau uptake was found to be dependent on HSPGs on the cell surface where 6-*O*-sulfation was critical for tau-heparan sulfate interaction (Holmes *et al.*, 2013; Mirbaha *et al.*, 2015; Stopschinski *et al.*, 2018; Zhao *et al.*, 2017). In addition, tau uptake has been shown to be decreased in the presence of HSPG inhibitors such as soluble heparin *in vitro* and *in vivo* (Holmes *et al.*, 2013).

More recently, distinct uptake mechanisms with a specific kinetics have been suggested for monomeric or fibrillar tau species. Whereas monomeric tau entered iPSC-derived neurons through a slow macropinocytic mechanism dependent on actin polymerization, both monomeric and aggregated tau species entered neurons via **dynamin-dependent endocytosis** (Evans *et al.*, 2018). Of note, the lack of actin dependence of aggregated tau entry into neurons suggests that it does not enter human neurons via macropinocytosis, as has been reported in non-neuronal cells (Holmes *et al.*, 2013).

In addition, tau may be taken up via **receptor-mediated endocytosis** (Gomez-Ramos *et al.*, 2009; Lasagna-Reeves *et al.*, 2012; Wu *et al.*, 2013). Tau has been demonstrated to cause a receptor-activated increase in intracellular calcium through M1/M3 muscarinic receptor stimulation (Gomez-Ramos *et al.*, 2008) that could, in turn, induce endocytosis of eTau. Takahashi et al. have implied that the extracellular domain of amyloid precursor protein might be involved in the incorporation of tau fibrils into cells (Takahashi *et al.*, 2015). Upon internalization, tau aggregates were shown to co-localize with a marker of fluid-phase endocytosis (dextran) rather than lipid raft marker (cholera toxin B), suggesting the involvement of an active endocytotic process (Guo and Lee, 2014).

In addition, **tunneling nanotubes** between cells (Clavaguera *et al.*, 2015; Tardivel *et al.*, 2016) or **extracellular vesicles** as shuttles (DeLeo and Ikezu, 2018; Guix *et al.*, 2018; Wang and Han, 2018) have been proposed to be involved in the cell-to-cell tau propagation, although little is known about the specific uptake mechanism.

Subsequent to uptake, internalized tau aggregates were capable of inducing fibril formation of intracellular full-length tau in recipient cells (Guo and Lee, 2011; Nonaka *et al.*, 2010).

Human studies indicate an underappreciated role of astrocytes in 4R-tauopathies. Regarding uptake of tau, importantly, astrocytes have been found to highly express an array of phagocytic receptors (Chung *et al.*, 2013; Davis *et al.*, 2014) and astrocytes play an important role in the clearance of toxic α-synuclein (another neurodegeneration-related protein) species from the extracellular space (Lindstrom *et al.*, 2017). However, there is a paucity of observations on the role of astrocytes in the clearance or propagation of tau. On the other hand, experimental studies in tau transgenic mouse model support the notion that astrocytic tau pathology contributes to glial degeneration (Higuchi *et al.*, 2002) and as functional consequence of astrocytic tau pathology neuronal degeneration can be detected even in the absence of neuronal tau inclusions (Forman *et al.*, 2005).

8.4. Vesicle trafficking

The risk of PSP has been found to be associated with a SNP in ***STX6*** (Hoglinger et al., 2011). A subsequent study found that the *STX6*-associated SNP rs1411478 is a strong expression-quantitative trait locus with significantly lower expression of *STX6* mRNA in carriers of the risk allele (Ferrari et al., 2014). The *STX6* gene encodes a tail-anchored soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-class protein that mediates the fusion of vesicles with membranes (Hong, 2005; Ungermann and Langosch, 2005). STX6 localizes to the trans-Golgi network, to early endosomes and secretory vesicles (Bock *et al.*, 1997; Burkhardt *et al.*, 2008; Dulubova *et al.*, 2002; Watson and Pessin, 2000) where it facilitates the fusion of secretory granules (Wendler et al., 2001) and mediates the transport of endosomes (Mills et al., 2001) and autophagosomes (Nozawa et al., 2017) in different cell types. In addition, STX6 regulates caveolin-1 (CAV-1) associated plasma membrane

microdomains; inhibiting STX6, therefore, alters CAV-1-mediated endocytosis of macromolecules (Choudhury et al., 2006) and lipids (Simonsen et al., 1999; Urano et al., 2008). Because of its role in controlling endocytic and exocytic processes and intracellular vesicle trafficking, STX6 may be involved in the endosomal uptake and release of tau and, as a consequence, *STX6* gene mutations may thus interfere with tau cell-to-cell transmission.

A recent study reported a mutation in the **bassoon (*BSN*)** gene in a Japanese family with a PSP-like syndrome. Neuropathological examination revealed a novel mixed 3R/4R-tauopathy with pallidonigroluysian degeneration and hippocampal sclerosis in these patients (Yabe *et al.*, 2018). BSN is a presynaptic scaffolding protein that localizes to synaptic neurotransmitter release sites where it is involved in organizing the presynaptic cytoskeleton and vesicle fusion machinery (Davydova *et al.*, 2014; Ivanova *et al.*, 2016). Due to its role in vesicle trafficking, it is therefore well conceivable that *BSN* mutations could also affect tau uptake and release.

Thus, genetic variants in both *STX6* and *BSN* might confer risk by affecting the intercellular spread of tau.

8.5. Tau seeds

Based on the prion-like theory of pathology spreading, the tau species that seed tau aggregation in the healthy cells are the most attractive targets for developing therapeutic strategies to prevent the progression of the disease. However, many attempts to identify the tau seeds have failed so far. Theoretically, **tau seeds must have two main features**: The first factor is the **ability to transfer from diseased cells to healthy cells**, which depends on the solubility, the molecular weight, and the accessibility to cellular release and uptake processes. High solubility along with low molecular weight or size might provide favorable conditions for high mobility of a transmissible tau species.

The second factor is the **ability to induce aggregation in the host cell**, which requires the presence of the MBD and structural features of a potent seed. Most transcellular spreading mechanisms of tau, described previously, assumed that tau seeds have to be transported via the extracellular environment (ISF or CSF). However, the main species of tau, found in CSF, is mid-region truncated tau, which lacks the MBD (Barthelemy *et al.*, 2016). Despite the high solubility and small size of truncated tau, the absence of MBD suggests that this species would not be able to induce aggregation. Nevertheless, the content of CSF does not completely recapitulate the content of ISF surrounding neurons in the brain parenchyma. The concentration of tau in ISF is 10-fold higher than in CSF (Yamada *et al.*, 2011), suggesting that CSF tau may not be representative for the tau species present in the CNS.

Phosphorylation is a well-studied modification known to be capable of increasing the tendency of tau to aggregate (Despres *et al.*, 2017) and to enhance the secretion of tau (Plouffe *et al.*, 2012). However, still no study has demonstrated that the addition of monomeric pTau to a pool of normal tau monomers can seed the aggregation in cell-free or cell-based assays. The most-studied species of tau as transmissible seeds are fibrillar structures, which are highly capable of inducing aggregation. Structural studies revealed that tau fibrils possess a very active fuzzy-coated surface that makes them highly interactive and adhesive (Wegmann *et al.*, 2013). The high molecular weight, large size, and insolubility of tau fibrils, however, appears to reduce their capacity to be transported over large distances. A study of tau propagation concluded that the cell-to-cell transport is mainly occurring via synapses (Ahmed *et al.*, 2014), suggesting that seeds need to pass through fine neurites to reach the synapses and to be released to the synaptic cleft in order to be taken up by another neuron. For such long distant transport, the tau seed needs to be small and highly mobile or transported via the microtubule-associated motor proteins. Nevertheless, protease

activity in the cells, especially in cells with proteinaceous inclusions, that have a very active autophagy-lysosomal system, may lead to digestion of fibrils in the flanking regions of C- and N-terminus of tau, while the core of fibrils is resistant to protease digestion (von Bergen *et al.*, 2006). The "digested fibrils" that lack flanking regions are still highly capable of inducing aggregation and their size and adhesion are reduced, which predisposes them as a candidate for being a transmissible seed. Physical pressure, specific protease activity or self-disaggregation of fibrils may lead to a break in the core of fibrils, which may form short fibril fragments (Kundel *et al.*, 2018). The "fragmented fibrils" with smaller size and aggregation-inducing potential might also be considered as a potent tau seed species (Jackson *et al.*, 2016). Sonicated fibrils made of the tau MBD (called K18 or K19 fibrils) have also been used as a seed for studying tau spreading, simulating the "fragmented digested fibrils" (Guo and Lee, 2011; Michel *et al.*, 2014; Strang *et al.*, 2018). Intermediate aggregates with different molecular weight and size can be further candidates for tau seeds. Small soluble oligomers of tau with aggregation-inducing capacity can be reasonable candidates for tau spreading, especially since they have been found in the brains of PSP patients (Gerson *et al.*, 2014). Commonly, tau monomers are not considered a tau seed, but a recent study revealed that monomeric tau obtained from an aggregation mixture is capable of inducing aggregation (Mirbaha *et al.*, 2018). This study suggests that in normal, inert monomers the aggregation-prone domain VQIINK/VQIVYK is not accessible, but in seed-competent monomers, this region is exposed. Because of high solubility, small size and aggregation-inducing capacity, this species of tau can be a very potent candidate for being a pathologically relevant tau seed. However, further biochemical characterization and pathological examination are required to confirm the presence of such tau monomers in patients.

Inoculation of brain homogenates from human 4R-tauopathy brain extracts into the brains of mice transgenic for wild-type human tau (line ALZ17) recapitulated the hallmark lesions, in particular the astrocytic tau pathologies (Clavaguera *et al.*, 2013). The same study reported successful injection of brain homogenates into non-transgenic C57BL/6 mice. A further study described the rapid and distinct cell type-specific spread of pathological tau following intracerebral injections of CBD and AD brain extracts in young human mutant P301S tau transgenic (Tg) mice (line PS19) (Boluda *et al.*, 2015). This, together with a study using a cell system showing that different 4R-tauopathies are associated with different sets of strains (Sanders *et al.*, 2014) supported the concept of tau strains, a terminology used in prion disease research. As a further advocate for the presence of strains, CBD-tau- and PSP-tau-injected mice showed spatiotemporal transmission of glial tau pathology, suggesting additionally that glial tau transmission contributes to the progression of tauopathies (Narasimhan *et al.*, 2017). A further support to this concept is the subcellular distribution of astroglial tau pathology, which differs significantly between 4R-tauopathies, indicating different processing of pathological tau.

While many pieces of evidence support the concept of tau propagation in 4R-tauopathies, several issues need still to be resolved. Which form of tau is the pathological spreading tau species? Are different strains of spreading tau species the biological basis of distinct tauopathies? What are the mechanisms of release and uptake of tau seeds? Do seeds propagate as vesicle-free or vesicle-associated entities? Does the tau spreading in the brain mainly take place via diffusion in the ISF or only locally to neighboring cells via synaptic connections? Despite these unresolved issues, eTau appears to be a substantial player in the pathogenesis of 4R-tauopathies, and thus an attractive target for the development of therapeutic strategies.

9. Diagnostics

9.1. PET

The imaging of molecular correlates related to 4R-tauopathies can facilitate the assessment and interpretation of pathological findings *in vivo*. Hypometabolism in the midbrain to **[^{18}F]-fluorodeoxyglucose (FDG)**, which is a surrogate of synaptic dysfunction and neuronal loss, is now considered as being of diagnostic value in PSP patients (Hoglinger *et al.*, 2017). This is based on numerous investigations indicating a value of FDG-PET for early detection and differential diagnosis of PSP and CBD vs. PD and MSA (Amtage *et al.*, 2014; Hellwig *et al.*, 2015; Mille *et al.*, 2017; Niethammer *et al.*, 2014; Tang *et al.*, 2010).

Recent innovations in molecular **PET imaging of tauopathies** include the introduction of **first-generation tau radioligands** based on pyridoindole (Chien *et al.*, 2013) (clinical candidate: ^{18}F-AV1451), arylquinolines (Okamura *et al.*, 2013) (clinical candidate: ^{18}F-THK5351), and benzothiazoles (Maruyama *et al.*, 2013) (clinical candidate: ^{11}C-PBB3). These ligands showed promising results in the detection of 4R-tauopathies (Brendel *et al.*, 2017; Schonhaut *et al.*, 2017; Smith *et al.*, 2017), and a correlation of the tau PET signal with clinical severity in PSP (Brendel *et al.*, 2017; Whitwell *et al.*, 2017). Furthermore, tau PET-distinguished cases of CBD from healthy controls (Kikuchi *et al.*, 2016), and tau tracer uptake correlated with the subsequent histopathological assay of tau in confirmed CBD cases (Josephs *et al.*, 2016). However, the primary emphasis in the development of the tau radioligands has hitherto be placed on the detection of paired helical 3R/4R-tau in AD, which is by far the most prevalent tauopathy, and the largest neurodegenerative disease burden to healthcare. Thus, first generation tau tracers seem to lack selectivity for tau isoform and ultrastructure. This calls for the design of radioligands sensitive and

-even more importantly- specific for 4R-tau, without binding to 3R-tau or combined 3R/4R-tau. The development of selective 4R tracers would enable not only the differentiation of 4R-tauopathies from healthy controls and non-tauopathies, but would as well allow an early inter-tauopathy discrimination at an individual level, which so far is only possible at the group level and by disease-specific regional subanalysis. Furthermore, there is increasing evidence that the PET signals from first-generation tau radioligands (pyridoindoles and arylquinolines) are distorted by off-target binding to the A and B forms of monoamine oxidase (Lemoine *et al.*, 2017; Ng *et al.*, 2017; Vermeiren *et al.*, 2018). The mixed signal is especially problematic, because monoamine oxidase B, a marker of astrocytosis, is known to be overexpressed in all neurodegenerative diseases (Carter *et al.*, 2012). Off-target binding of certain tau ligands to neuromelanin has also been described and can affect image evaluation especially in the midbrain. This is as the substantia nigra owes high concentrations of neuromelanin (Coakeley *et al.*, 2018). Further, unspecific binding of the first-generation tau PET tracers to the choroid plexus (Villemagne, 2018) might compromise their utility especially in detecting tau pathology in deeper brain regions.

Emerging **second-generation tau tracers** have improved specificity, and have shown promising results in AD patients (Betthauser *et al.*, 2019; Wong *et al.*, 2018). It remains to be elucidated if 4R-tauopathies can also be detected and distinguished by these new tracers, but preliminary imaging data show promising results in PSP and CBD (Fig. 3). In addition to meeting the requirements for absent off-target binding and selectivity for 4R-tau detection, ideal future PET tracers for 4R-tauopathies would also function in transgenic mouse models, as already proven for some first-generation tau radioligands (Brendel *et al.*, 2016; Maruyama *et al.*, 2013; Ni *et al.*, 2018). Such tracers would establish a platform for preclinical monitoring of anti-tau drugs and enhance translational research in the field of 4R-tauopathies.

9.2. *Fluid biomarkers*

9.2.1. *Tau*

Most studies have focused on CSF levels of **total tau** or **tau phosphorylated at residue 181 (p_{181}Tau)**. These studies did not find consistent changes in PSP compared to healthy controls, PD and other atypical parkinsonian disorders (Jabbari *et al.*, 2017). CSF total tau and p_{181}Tau levels were unchanged over time in two small studies (Backstrom *et al.*, 2015; Boxer *et al.*, 2014), while a large cohort study has found lower baseline p_{181}Tau as predictor of faster decline in PSP rating scale (PSPRS) scores and higher total tau concentrations as predictor of faster impairment in activities of daily living (Rojas *et al.*, 2018).

A lower CSF **ratio between truncated (33 kDa) and larger (55 kDa) tau** forms was reported in PSP compared to other tauopathies and synucleinopathies (Borroni *et al.*, 2009; Borroni *et al.*, 2008). This study further observed a correlation between the 33 kDa/55 kDa tau ratio and brainstem atrophy as well as the severity of motor symptoms in PSP, while the existence of these tau forms has been challenged by others (Kuiperij and Verbeek, 2012). A preliminary study including 6 PSP patients reported significantly higher plasma total tau and p_{181}Tau levels compared to healthy controls by using an ultra-sensitive immunoassay with immunomagnetic reduction method, while concentrations were not different compared to patients with PD and other atypical parkinsonian disorders (Lin *et al.*, 2018). These results require confirmation in larger cohorts of PSP patients.

Tau fragments outside the mid-domain region, which is targeted by commercially available assays, and other pTau epitopes have also been explored by ELISA (Wagshal *et al.*, 2015). This study found lower N-terminal and mid-domain CSF tau levels compared to healthy controls and

AD. A similar approach to assess several non-phosphorylated tau fragments was used in a large cohort of patients with a clinical diagnosis of FTD (Foiani *et al.*, 2019). Among these, a small subset of seven patients were likely to have tau pathology. CSF concentrations of tau N-mid region and tau N-224 were higher in patients with suspected tau pathology compared to healthy controls, but not compared to the FTLD-TDP-43 group. Findings of these studies were further corroborated by the identification of 18 tau peptides by mass spectrometry in the CSF, with low peptide detection at the N- and C-terminus compared to the central core region of the tau protein (Barthelemy *et al.*, 2016). The 1N and 3R isoforms were the most abundant in this study, while the 4R isoform was low and not different between AD, PSP and control subjects. The expression profiles in the central core region were different between PSP and AD patients.

Finally, the potential of monoclonal antibodies for **3R- and 4R-tau isoforms** have been tested in an immune-PCR assay (Luk *et al.*, 2009). This assay found lower 4R-tau concentrations in PSP compared to healthy controls. However, 4R tau concentrations in PSP were not different from PD dementia and AD, while the 4R-tau/pTau ratio was lower compared to PD dementia (Barthelemy *et al.*, 2016; Rojas *et al.*, 2018).

9.2.2. PMCA / RT-QuIC

Protein misfolding cyclic amplification (PMCA) or **real-time quaking-induced conversion (RT-QuIC)** allows the detection of the abnormal form of the prion protein (Moda *et al.*, 2014; Morales *et al.*, 2012). Beyond prion diseases, the potential of these techniques for the development of a diagnostic test is currently under investigation for PD (Fairfoul *et al.*, 2016; Shahnawaz *et al.*, 2017). These techniques may also prove valuable for the detection of tau aggregates in PSP. In this regard, 3R-tau and 3R/4R RT-QuIC assays using postmortem brain tissue homogenates

showed different seeding activities for PiD, a 3R-tauopathy, AD, a 3R/4R-tauopathy and PSP (Kraus *et al.*, 2018; Saijo *et al.*, 2017).

9.2.3. Neurofilament

Several studies reported increased **CSF levels of neurofilament light (NfL)** and **heavy (NfH) chain** in PSP compared to healthy controls (Backstrom *et al.*, 2015; Bech *et al.*, 2012; Boxer *et al.*, 2014; Brettschneider *et al.*, 2006; Hall *et al.*, 2012; Hansson *et al.*, 2017; Magdalinou *et al.*, 2015; Rojas *et al.*, 2018). In most studies, concentrations were also higher in PSP versus PD, but not compared to other atypical parkinsonian disorders limiting the usefulness of this biomarker for the differential diagnosis of PSP. All except one study reported an increase in CSF-NfL levels with increasing disease duration (Backstrom *et al.*, 2015; Boxer *et al.*, 2014; Constantinescu *et al.*, 2010; Rojas *et al.*, 2018).

The improvement of assay sensitivity has further allowed the measurement of **NfL in blood**. Blood NfL concentrations were higher in PSP patients compared to healthy controls, were correlated with CSF NfL levels and increased over time (Hansson *et al.*, 2017; Rojas *et al.*, 2018; Rojas *et al.*, 2016). Blood NfL concentrations were also higher compared to PD, but not compared to MSA or CBD (Hansson *et al.*, 2017). In addition, higher blood and CSF NfL levels predicted a faster progression of PSPRS scores, as well as a more severe decline of functional and cognitive impairment (Rojas *et al.*, 2018; Rojas *et al.*, 2016). Blood NfL levels further predicted a greater magnitude of whole-brain atrophy and superior cerebellar peduncle (SCP) atrophy at one-year follow-up, while CSF NfL levels were correlated with SCP atrophy and predicted the higher progression of SCP atrophy at one-year follow-up (Rojas *et al.*, 2018; Rojas *et al.*, 2016).

9.2.4. *Others*

Additional markers including the amyloid precursor protein, amyloid beta, chitinase-3-like protein 1, monocyte chemoattractant protein-1, glial fibrillary acidic protein and cytokines have been assessed in PSP (Jabbari *et al.*, 2017; Magdalinou *et al.*, 2014; Starhof *et al.*, 2018b), mostly in small studies requiring the replication of results to better understand their relevance. Several cell-free miRNAs were reported to be differently expressed in plasma and CSF in PSP compared to PD (Starhof *et al.*, 2018a). Future studies are warranted to further assess the value of miRNAs for the diagnosis of PSP. Finally, similar to other neurodegenerative diseases, exploring the potential of plasma exosomes for biomarker development in PSP is an emerging field (Ohmichi *et al.*, 2018).

10. Tau targeting therapies

Based on the disease mechanisms of 4R-tauopathies, as summarized in Figure 2, the following rational strategies to develop disease-modifying therapies are currently being pursued.

10.1. *Reduction of tau expression*

Application of agents that reduce tau levels as a possible treatment strategy of neurodegenerative tauopathies has proven efficacy in several animal models of AD and primary tauopathies (DeVos *et al.*, 2018; DeVos *et al.*, 2017). This approach is based on the assumption that the levels of tau proteins are a driving factor in the pathogenesis of tauopathies and affect the formation of tau aggregates. Genetic polymorphisms leading to increased tau levels have been shown to increase the risk for tauopathies (Chen *et al.*, 2017). One potential way to reduce tau expression is the use of **small interfering RNAs (siRNAs)**, which showed efficacy in animal models of tauopathies

(Xu *et al.*, 2014). Another strategy to prevent the formation of tau aggregates via reduction of tau proteins in humans is **antisense oligonucleotides (ASOs)** (Fig. 2A). ASOs have shown to reduce tau mRNA levels in the brain, spinal cord and CSF of non-human primates (DeVos *et al.*, 2017). Initial studies with tau ASOs are carried out in human patients with mild AD (clinicalTrials.gov identifier NCT03186989). In spinal muscular atrophy, ASOs have already been approved for clinical use (Mercuri *et al.*, 2018).

10.2. Alteration of the 3R/4R-tau ratio

The healthy adult human brain contains approximately equal amounts of 3R- and 4R-tau. In diseased brains this equilibrium between the tau splice variants is often altered, likely because of splicing deficits. Moreover, several intronic mutations associated with FTLD-*MAPT* are known to disrupt exon 10 splicing (Qian and Liu, 2014). In a tauopathy-mouse model, modulation of the 3R/4R ratio led to a significant reduction of tau-associated pathology (Espindola *et al.*, 2018). This raises hope that tau splice modifying strategies might be useful in human patients with 4R-tauopathies. Potential treatment strategies include **ASOs** (Schoch *et al.*, 2016) and **small molecule splice modifiers** (Artigas *et al.*, 2015; Zheng *et al.*, 2009) (Fig. 2B).

10.3. O-GlcNAcase (OGA) inhibitors

O-GlcNAcylation is the covalent attachment of O-linked *N*-acetylglucosamine (O-GlcNAc) moieties to serine and threonine residues of proteins (Fig. 2C). *In vitro* experiments showed that O-GlcNAcylated tau is less prone to form toxic, insoluble, pathological aggregates (Gong *et al.*, 2005; Liu *et al.*, 2004). The underlying mechanisms might be the prevention of tau-phosphorylation via competitive binding of *N*-acetylglucosamine to serine and threonine residues

(Hastings *et al.*, 2017), or an anti-aggregative mechanism of the O-GlcNAc moieties (Yuzwa *et al.*, 2012), similar to the reported effects of this modification on α-synuclein aggregation and toxicity (Levine *et al.*, 2019).

The glycoside hydrolase O-GlcNAcase OGA removes the *N*-acetylglucosamine moiety from tau. In an attempt to stabilize the O-GlcNAcylated forms of tau, inhibitors of the OGA have been developed (**OGA inhibitors**) (Fig. 2C). In 4R-tau-transgenic mouse models, the OGA inhibitor thiamet G significantly increased O-GlcNAcylated tau and hindered the formation of tau aggregates (Yuzwa *et al.*, 2012), reduced pTau species and decreased neuronal cell loss (Graham *et al.*, 2014; Hastings *et al.*, 2017). The OGA inhibitor ASN-561 also referred to as ASN120290 showed a better blood-brain barrier penetration compared to previous substances (Quattropani *et al.*, 2014). Preclinical studies in 4R-tau-transgenic mice revealed a significant reduction of sarkosyl-insoluble paired helical filaments and cerebral tau pathology (Permanne *et al.*, 2015). Phase I studies with ASN120290 are ongoing to prepare for a phase II study in PSP (Medina, 2018; Ryan *et al.*, 2018).

10.4. Tau cleavage inhibitors

Cleaved tau species (Fig. 2C) are found to be toxic in several instances (see above). Cleavage of tau by caspases can generate a C-terminal-truncated tau (1-421), which is neurotoxic *in vitro* (Khurana *et al.*, 2010). A SNP in *MOBP*, which was identified as a risk factor in PSP, results in activated caspase-3 and elevated levels of caspase-cleaved tau via increased levels of appoptosin (Zhao *et al.*, 2015). Inhibitors of caspase-3 abolished the appoptosin-induced synapototoxicity *in vitro* and prevented the neuronal loss/defects and astrogliosis *in vivo* (Zhao *et al.*, 2015). Moreover, calpain-mediated tau cleavage can generate a neurotoxic 17-kD tau fragment (Ferreira and Bigio,

2011). They also reported that the activity of calpain and the level of 17-kD tau fragment is high in postmortem brain lysate of PSP patients. Cumulative findings suggest that inhibiting tau cleavage might be a potent therapeutic strategy for 4R-tauopathies.

10.5. Tau acetylation inhibitors

Acetylation of tau promotes the formation of tau aggregates (Cohen *et al.*, 2011) (Fig. 2C). The inhibition of distinct acetyltransferases is therefore an attractive but challenging therapeutic target (Min *et al.*, 2015; Min *et al.*, 2010). **Acetylation inhibitors** include substances that inhibit the histone acetyltransferase p300 (Min *et al.*, 2015), e.g., salsalate, which is currently tested in a pilot study in PSP (clinicalTrials.gov identifier NCT02422485). Tau acetylation might not be of relevance in AGD (Grinberg *et al.*, 2013).

10.6. Kinase inhibitors

Hyperphosphorylation of tau plays a crucial role in the pathogenesis of 4R-tauopathies. It reduces microtubule binding of tau (Mandelkow *et al.*, 1995) and favors the aggregation of tau to form toxic oligomers and insoluble NFTs (Iqbal *et al.*, 2009) (Fig. 2C). Therefore, tau **kinase inhibitors** attract attention as possible therapeutic targets (Hanger *et al.*, 2009).

In brains of PSP patients, the amount of the **GSK-3β** kinase was found to be increased in NFTs (Sun *et al.*, 2002). Tideglusib, a thiadiazolidinone irreversibly inhibits GSK-3β and yielded promising preclinical results by preventing cell loss and restoring cognitive functions in tau transgenic mice (Dominguez *et al.*, 2012; Sereno *et al.*, 2009). A clinical trial in PSP patients, however, did not detect any effect on disease progression compared to placebo (Tolosa *et al.*, 2014). Yet, magnetic resonance imaging (MRI) of a subgroup of the study population suggested

reduced progression in cerebral atrophy, particularly in the temporal and parietal areas (Hoglinger *et al.*, 2014), suggesting a possible subclinical effect on disease progression. Sodium valproate, another GSK-3β inhibitor, administered to PSP patients for 24 months, led to a significant worsening of the patients compared to placebo, presumably due to side effects (Leclair-Visonneau *et al.*, 2016). Another GSK-3β inhibitor is lithium, which also induces autophagy (Shimada et al., 2012). A clinical trial of lithium in PSP- and CBD-patients was discontinued because of poor tolerability (clinicalTrial.gov identifier NCT00703677).

A further potentially important tau kinase is **CDK5** which is activated by the polypeptide p35. Cleavage of p35 to p25 by calpain results in more stable hyperactive CDK5-p25 complexes leading to tau hyperphosphorylation (Zheng *et al.*, 2010). Preclinical studies testing a selective inhibition of CDK5/p25 activity showed decreased tau phosphorylation and reduced apoptosis in rat cortical neurons (Zheng *et al.*, 2005). Increased expression of CDK5 was suggested in PSP, drawing further attention as a possible therapeutic target (Borghi *et al.*, 2002).

Also, the **rho-associated coiled-coil-containing protein kinases (ROCK 1** and **ROCK2)** appear to be associated with aggregated tau in PSP and CBD brains, warranting further investigations (Gentry *et al.*, 2016).

10.7. Phosphatase activators

Decreased activities of distinct phosphatases result in reduced dephosphorylation of tau **(**Fig. 2C**)**. The phosphatase PP2A has been identified to play a crucial role in tauopathies (Iqbal *et al.*, 2016). PP2A is not only dephosphorylating tau, but it also inactivates certain tau kinases (Iqbal *et al.*, 2016; Voronkov *et al.*, 2011). The development of **phosphatase activators** is a challenging

endeavor, but current research shows promising results that might lead to effective therapies in the future (Tan *et al.*, 2016; Voronkov *et al.*, 2011).

10.8. Aggregation inhibitors

Deposits of aggregated tau protein are a hallmark of tauopathies rendering inhibition of tau aggregation a highly interesting therapeutic target (Fig. 2C). These tau deposits share many features with deposits of aggregated proteins that are characteristic for other neurodegenerative diseases. These features include amongst others, fibril formation with high β-sheet content as well as Thioflavin T positivity and reactivity to Congo red. Not surprisingly, several inhibitors of this type of protein aggregation are effective on various disease-associated protein aggregates, which allows to classify them as broad-spectrum **aggregation inhibitors** (antiaggregants). A number of broad-spectrum antiaggregants underwent clinical evaluation, e.g., leuco-methylthioninium (LMTM, leucomethylene blue), which inhibits aggregate formation of amyloid beta (Irwin *et al.*, 2013), prion protein (Cavaliere *et al.*, 2013), tau (Hochgrafe *et al.*, 2015) and TDP-43 (Arai *et al.*, 2010). LMTM and derivatives (e.g., methylene blue) failed to show clinical benefits in mild to moderate AD and FTD (Gauthier *et al.*, 2016). Another example for a broad-spectrum antiaggregant is epigallocatechin gallate (EGCG), a natural-occurring polyphenol inhibiting the fibrillogenesis of amyloid beta, α-synuclein and tau (Ehrnhoefer *et al.*, 2008). EGCG is currently tested in a clinical trial with MSA patients (Levin *et al.*, 2016). A range of next-generation broad-spectrum antiaggregants are under development aim at improved characteristics, e.g., better blood-brain barrier penetration and more stable pharmacokinetics. For example, NPT088, a bacteriophage capsid protein fused with human IgG1-Fc that inhibits aggregation of amyloid beta and tau (Levenson *et al.*, 2016) and anle138b, an orally bioavailable small molecule with good

brain penetration that inhibits oligomer formation from amyloid beta, α-synuclein, prion protein and tau (Levin *et al.*, 2014; Wagner *et al.*, 2015; Wagner *et al.*, 2013) are in early clinical or late stage of preclinical development, respectively.

10.9. Microtubule stabilizers

Tau has an important function as microtubule stabilizer in neurons (Fig. 2C), enabling axonal transport of cell components. Upon aggregation, tau loses this important function which may contribute to subsequent neuronal dysfunction in 4R-tauopathies (Kadavath *et al.*, 2015). Consequently, therapies aiming to restore this function emerged.

The microtubule-stabilizing agent **paclitaxel** showed improved motor function in tau-transgenic mice and higher density of microtubules in spinal axons (Zhang *et al.*, 2005). However, paclitaxel showed poor blood-brain barrier permeability. **Epothilone D**, a macrolide, showed better blood-brain barrier penetration, led to reduced microtubule dysfunction in young tau-transgenic mice and restored cognitive deficits in aged 4R-tau-transgenic mice (Brunden *et al.*, 2010; Zhang *et al.*, 2012a). The eight amino acid peptide **davunetide** is another microtubule-interacting agent, which reduced tau pathology and enhanced cognitive function in a transgenic mouse model (Matsuoka *et al.*, 2008) and in a *Drosophila* model (Quraishe *et al.*, 2013). A multi-center phase III study in 313 PSP-patients, however, failed to show significant efficacy of intranasal application of davunetide to retard disease progression (Boxer *et al.*, 2014; Khanna *et al.*, 2016). The taxane **TPI-287**, another microtubule stabilizer, has recently been tested in two clinical trials with PSP, CBD and AD patients, however, it did not show significant improvements so far (Medina, 2018).

10.10. Stimulation of tau degradation

The clearance of monomeric abnormal tau or larger tau species is one of the key strategies against tauopathies. Therefore, inducing physiological defense mechanisms against tau, such as the UPS, the ALS, and the UPR, might be feasible therapeutic strategies (Fig. 2D).

The UPS is an important control mechanism to repair or degrade misfolded proteins. The chaperons (e.g. Hsp70 and Hsp90) play a key role in this process as they bind to abnormal tau and induce the degradation process (Khanna *et al.*, 2016). The activation or inhibition of distinct chaperons by small molecule **chaperone modulators** (Fig. 2D) might therefore be a therapeutic target (Tan *et al.*, 2018). It has also been shown that a direct delivery of proteasomes loaded on nanoparticles can increase the efficacy of tau degradation (Han *et al.*, 2014).

The ALS is capable of reducing abnormal tau and larger tau species (Wang and Mandelkow, 2012). **Activation of autophagy** is a possible therapeutic strategy to enhance the tau degradation process (Fig. 2D). Several substances, such as rapamycin and temsirolimus activate macroautophagy via the inhibition of the mTOR pathway (Jiang *et al.*, 2014; Ozcelik *et al.*, 2013). Other substances, e.g., trehalose or lithium, induce autophagy via the autophagy-lysosome formation (Schaeffer *et al.*, 2012; Shimada *et al.*, 2012).

The UPR is a complex cellular stress response mechanism activated by misfolded proteins. In tauopathies and particularly in 4R-tauopathies it might be of high relevance as it was shown that the gene encoding for PERK, a key protein of the UPR, is a risk gene for PSP (Hoglinger *et al.*, 2011). The PERK pathway of the UPR in particular might be a relevant therapeutic target. Thus, the inhibition or activation of distinct proteins in the PERK pathway are in the focus. Recently, a **PERK activator** (Fig. 2D) was shown to mitigate tau pathology (Bruch *et al.*, 2017). Activation of PERK induces the activation of Nrf2, a pathway that can lead to translational regulation of ~250

target genes encoding for proteins with versatile cytoprotective effects (O'Connell and Hayes, 2015). Consistently, also **Nrf2 activators** have been shown to induce degradation of pTau species *in vitro* and *in vivo* (Bruch *et al.*, 2017; Jo *et al.*, 2014; Kim *et al.*, 2016).

In conclusion, stimulation of tau degradation and possible repair mechanisms offer a broad spectrum of promising therapeutic targets for 4R-tauopathies. However, the complexity of these processes impedes the decision for the most viable target. In addition, because these degradation processes are universally located in every cell, influencing distinct mechanisms might also cause detrimental side effects, which needs to be considered for the development of novel therapeutic strategies.

10.11. Tau release blockers

Accumulating evidence suggests that inhibiting the intercellular transfer of tau slows the disease progression in tauopathies. Therapeutic strategies for preventing the interneuronal transfer of tau are thus of paramount interest (Fig. 2E). Inhibition of release of pathogenic tau species from diseased neurons is a hypothetical possibility to halt tau cell-to-cell transmission.

Previously it was demonstrated that tau release is an activity-dependent process (Pooler *et al.*, 2013; Wang *et al.*, 2017; Wu *et al.*, 2016; Yamada *et al.*, 2014), although the specific neuronal factors that couple membrane depolarization with tau release remain to be established. As a consequence, tau release may be blocked by attenuating neuronal activity, e.g., by blocking neuronal ion channels. Another study demonstrated that tau is released by microglia via exosomes and that this exosomal tau release can be decreased by blocking exosome generation through the neutral sphingomyelinase inhibitor GW4869 (Asai *et al.*, 2015).

In summary, these results demonstrate that there are probably multiple target sites to halt tau release. However, since 1) the specific tau species that are transmissible and 2) the molecular mechanism that control tau uptake and release are poorly understood, there are currently no specific compounds available for clinical use and only a few pathways have been defined to specifically inhibit tau release.

10.12. Tau uptake blockers

Tau uptake appears to depend on interaction with HSPGs (Holmes *et al.*, 2013; Mirbaha *et al.*, 2015; Stopschinski *et al.*, 2018; Zhao *et al.*, 2017), since tau uptake has been shown to decrease in the presence of competitive HSPG inhibitors such as the soluble heparin mimic F6 *in vitro* and *in vivo* (Holmes *et al.*, 2013). In addition, uptake of monomeric and aggregated tau appears to depend on dynamin and actin polymerization (Evans *et al.*, 2018). Dynamin-dependent endocytosis has been experimentally blocked by the small molecule inhibitor dynasore, an inhibitor of the GTPase dynamin, and resulted in a 70% reduction of tau uptake. On the other hand, inhibition of actin polymerization with cytochalasin D to interfere with clathrin-independent endocytotic processes, including macropinocytosis has been demonstrated to attenuate the uptake of monomeric tau (Evans *et al.*, 2018). Previous studies reported a decrease in tau uptake in response to treatment with different tau antibodies (Takeda *et al.*, 2015).

In summary, there are currently no compounds available that can specifically inhibit tau uptake without interference with other vital cellular processes.

10.13. Glial phagocytosis enhancers / anti-inflammatory drugs

Microglia and astrocytes can degrade eTau species by phagocytic activity (Majerova *et al.*, 2014; Martini-Stoica *et al.*, 2018). It has been shown that the phagocytic action is reduced in later stages of tauopathies (Perea *et al.*, 2018). **Phagocytosis enhancer** might be a possible therapeutic strategy to reduce pathological eTau species (Martini-Stoica *et al.*, 2018). Although this concept is conceivable, it has to be treated with caution, as it was also demonstrated that microglia might contribute to tau pathology propagation among neurons by phagocytosis and exocytosis of tau proteins (Asai *et al.*, 2015).

Activation of microglia promotes the generation of pro-inflammatory cytokines and leads to tau hyperphosphorylation and aggregation (Tan *et al.*, 2018). **Anti-inflammatory drugs** that interfere with inflammatory pathways might therefore be a further relevant target.

10.14. Anti-Tau monoclonal antibodies

A further approach to prevent the spread of tau pathology is the passive and active immunization against specific Tau epitopes (Fig. 2F). Generally, this strategy is considered to predominantly target eTau, however, interaction of antibodies with intracellular tau species might also be of relevance. Targeting eTau as therapeutic strategy is based on the hypothesis that release of tau species from diseased neurons into the extracellular space and their uptake into previously unaffected neurons are crucial for the propagation of tau pathology along anatomically connected pathways across the brain. Tau-targeting antibodies aim to interrupt this process by binding Tau in the cerebral extracellular space (Bittar *et al.*, 2018; Sigurdsson, 2016).

The antibody **BIIB092** (formerly known as BMS-986168 or IPN007) is a humanized IgG4 monoclonal antibody raised against N-terminal human tau released from familial AD patient-

derived pluripotent stem cells (Bright *et al.*, 2015; Qureshi *et al.*, 2018). In transgenic 4R-tau-overexpressing mice, intraperitoneal administration of the murine analog of BIIB092 (called IPN002) reduced the levels of unbound N-terminal tau in the ISF and CSF (Bright *et al.*, 2015). In cynomolgus monkeys, intravenously administered BIIB092 reduced CSF levels of unbound N-terminal tau by 90% (Qureshi *et al.*, 2018). In a single ascending dose study of intravenous BIIB092 in healthy participants, BIIB092 was well tolerated and reduced unbound CSF N-terminal tau by 67%–97% 28 days after administration (Qureshi *et al.*, 2018).

In a multiple ascending dose phase I study in PSP, repeated administration of BIIB092, at doses up to 2100 mg, were well tolerated and substantially (>90%) reduced unbound N-terminal tau concentrations in CSF at day 85 (Boxer *et al.*, 2018; Boxer *et al.*, 2019). Currently, safety and efficacy of repeated intravenous BIIB092 administrations are being assessed in a 12-month, randomized, placebo-controlled phase II trial in participants with PSP (clinicalTrials.gov identifier NCT03068468).

The antibody **ABBV-8E12** is a humanized IgG4 monoclonal antibody raised against full-length human tau (2N/4R) (Kfoury *et al.*, 2012; Yanamandra *et al.*, 2013), recognizing an N-terminal epitope. The murine version of this antibody blocked transcellular spreading of tau aggregate propagation by trapping tau fibrils in the extracellular space and preventing their uptake (Kfoury *et al.*, 2012). The antibody also blocked the uptake of misfolded tau from brain lysates of 4R-tau transgenic mice into cultured neuronal cells (Yanamandra *et al.*, 2015). *In vivo*, infusion of the antibody into the lateral ventricles of 4R-tau-transgenic mice reduced hyperphosphorylated, aggregated and insoluble tau levels (Yanamandra *et al.*, 2013). A phase I single ascending dose study of intravenously ministered ABBV-8E12 in PSP showed an acceptable safety profile, and plasma half-life and CSF:plasma ratio consistent with other humanized antibodies, in the absence

of signs of immunogenicity (West *et al.*, 2017). Thus, an ongoing randomized placebo-controlled phase II trial (Mendonca *et al.*, 2018) (clinicalTrials.gov identifier NCT02985879) studies the safety and efficacy of repeated intravenous ABBV-8E12 administrations to reduce the disease progression in PSP.

Whereas BIIB092 and ABBV-8E12 target the N-terminal region of tau, the IgG monoclonal antibody **UCB0107** targets the mid-region of tau (amino acids 235–250) adjacent to the MBD, and potently blocked the seeding of human AD and PSP tau in a cell-based assay (Courade *et al.*, 2018), and prevented the tau pathology in brains of tau-transgenic mice following injection of human AD brain extracts (Albert *et al.*, 2019). A phase I study to evaluate safety and tolerability, pharmacokinetics and pharmacodynamics of single ascending intravenous doses in healthy individuals has been completed (clinicalTrials.gov identifier NCT03464227) and a placebo-controlled, double-blind phase II/III study to assess the safety, tolerability and efficacy of UCB0107 in patients with PSP is in preparation.

10.15. Active immunization

While the passive immunization approaches described above rely on regularly repeated infusion of monoclonal anti-eTau antibodies, active immunization approaches (Fig. 2F) rely on exposure to defined tau epitopes as antigens to provoke a specific and hopefully long-lasting immune response against eTau (Bittar *et al.*, 2018). A first in human phase I trial of the tau-like oligopeptide **AADvac1** demonstrated the generation of robust antibody titers (Novak *et al.*, 2017). AADvac1-induced antibodies recognized tau pathology in tissues from AD, PiD, CBD, and PSP brains and all tau isoforms from insoluble brain extracts. A phase I study of AADvac1 in nfaPPA and the first phase II randomized, placebo-controlled, double-blind study of anti tau

immunotherapy addressing the safety and efficacy of AADvac1 in AD are ongoing (clinicalTrials.gov identifiers NCT02579252, NCT03174886).

While the broad spectrum of tau-targeting therapeutic interventions in development is encouraging, it may well be that no individual one of these approaches will be sufficient to prevent disease progression in human patients. As in other diseases, combinational approaches might be required to achieve this goal. While being speculative for the moment, it may also turn out that distinct interventions might be differentially efficacious at different disease stages. For example, tau release or uptake blockers, might be most beneficial in the earliest stages, but may have limited or little impact to most of the affected areas once the disease has spread. Different tau strains might also affect the response to specific therapies, e.g. antibody-based approaches. Planning of future therapeutic trials will have to consider these issues.

11. Concluding remarks

4R-tauopathies represent a new disease concept that is based on grouping diseases, which had previously been considered separate entities, by a common underlying core pathological mechanism, i.e., the pathological modification, aggregation and propagation of 4R-tau isoforms.

This joint consideration of 4R-tauopathies as pathophysiologically defined entity poses new challenges: 4R-tauopathies present a broad clinical spectrum and a high variability in disease progression. Accordingly, their joint recognition early in disease requires major research efforts. Individualized prediction and objective measurement of the disease trajectories will be of utmost importance. Moreover, a major focus will be the development of imaging techniques or fluid biomarkers that substantially facilitate the preclinical detection of 4R-tauopathies.

The concept of 4R-tauopathies offers new opportunities for research and patient care: Past and ongoing research into disease mechanisms have identified 4R-tau as an attractive molecular target for a broad spectrum of investigational new disease-modifying therapies. Thus, 4R-tauopathies will continue to emerge as attractive research area with the ultimate goal of developing causal therapies for currently devastating diseases.

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Figure legends

Fig. 1. Molecular classification of tauopathies and histopathological findings in 4R-tauopathies. Tauopathies are classified by the predominant aggregation of tau isoforms with 3 repeats (3R, yellow boxes), or 4 repeats (4R, blue boxes) of the microtubule-binding domain, or mixed 3R/4R isoforms (green boxes). Secondary tauopathies are associated with other diseases or etiologies (dashed lines). These include e.g. Alzheimer's disease (AD), chronic traumatic encephalopathy (CTE) or anti-IgLON5-related tauopathy. Primary tauopathies have no such associations (straight lines). The images represent molecular pathological characteristics, evidenced by anti-phospho-tau antibodies or Gallyas silver impregnation of the following tauopathies from top to bottom: Progressive supranuclear palsy (PSP): tufted astrocyte (A), globose neurofibrillary tangle in the caudate nucleus (B), oligodendroglial coiled bodies in the internal capsule (C); corticobasal degeneration (CBD): astrocytic plaque in the frontal cortex (D), neuronal corticobasal bodies in the substantia nigra (E), threads and coiled bodies in the parietal white matter (F); argyrophilic grain disease (AGD): classical Gallyas-positive argyrophilic grains (G), tau-positive grains and neuronal cytoplasmic immunoreactivities (pretangles) in the hippocampus (H), coiled bodies in the hippocampal white matter (I); glial globular tauopathy (GGT): globular astroglial inclusions (J), spherical neuronal inclusions in the frontal cortex (K), globular oligodendroglial inclusions in the hippocampal white matter (L). For aging-related tau astrogliopathy (ARTAG) images represent

from left to right subpial (M), subependymal (N), white matter (O), perivascular (P) and gray matter (Q) ARTAG. Scale bars = 25 µm.

Fig. 2. Pathophysiologic changes underlying 4R-tauopathies and investigational disease-modifying therapeutic concepts (grey boxes). (A) Rare and common genetic variants, environmental factors and epigenetic modifications affect gene expression of *MAPT*, encoding the tau protein. *DLX1* hypermethylation, observed in PSP patients' brains, appears to affect *MAPT* expression via indirect pathways (WNT or GABA signaling) and via a direct pathway by altered expression of *DLX1* antisense, increasing DLX1 protein, which acts as a transcription factor at the *MAPT* promoter. (B) *MAPT*-mRNA contains 16 exons, of which exons 2, 3 and 10 are alternatively spliced, encoding the N1, N2, and R2 protein domains, respectively. The resulting 6 different tau protein isoforms are named by their number of N-terminal (N) and microtubule-binding-repeat (R) domains. (C) Physiologically, tau is associated with microtubules. Several posttranslational modifications of tau can positively or negatively affect the affinity of tau to microtubules. Under pathological conditions, tau detaches from microtubules, leading to their destabilization. Unassociated tau aggregates and forms neurofibrillary tangles. OGA inhibitors prevent the removal of O-linked *N*-acetylglucosamine (Gly) and stabilize tau, as indicated by the green line. (D) Aggregated tau and abnormal monomeric tau can be degraded by different cellular pathways. In the ubiquitin-proteasome system, abnormal monomeric tau is recognized by E3 ligases, polyubiquitinated and degraded by the proteasome. The autophagy-lysosome system recognizes abnormal monomeric tau by chaperone Hsc70 via two KFERQ homology motifs and transfers it to lysosomal degradation. Abnormal monomeric tau and tau aggregates can bind to the surface of a phagophore to be incorporated in autophagosomes, which fuse with lysosomes to degrade the

tau species. Abnormal tau can also activate the PERK pathway of the unfolded protein response to induce neuroprotection or to drive the cell into apoptosis. (E) Cell-to-cell spreading of tau pathology requires the release of spreading-competent tau species from diseased cells and their uptake by adjacent unaffected cells. This might involve different cellular and molecular mechanisms. Preventing release and uptake of tau, as well as promoting tau clearance in the extracellular space may be therapeutic strategies to reduce the propagation of pathology. Affecting tau spreading via modulation of neuroinflammation may also protect neurons from degeneration. (F) Various tau species have been detected in the extracellular space *in vitro* and *in vivo.* Different types of tau fragments have been reported inside and outside of cells. The currently known cleavage sites of tau are illustrated on full-length tau protein (2N/4R, 441 amino acids). Spreading-competent extracellular tau (eTau) species are considered therapeutic targets. Green lines indicated stimulation/ activation; red lines indicated inhibition/ reduction.

Fig. 3. PET images using the second-generation tau tracer ^{18}F-PI-2620 in 4R-tauopathies. Distribution volume ratio (DVR) images, normalized by a cerebellar (CBL) reference region and overlaid upon a MRI template, are shown in an axial plane at the level of the parietal cortex (axial 1), of the basal ganglia (axial 2) and of the midbrain (axial 3), as well as in a midsagittal plane (sagittal). In contrast to a representative healthy control (HC, 78 years, female), increased tracer retention in a representative patient with probable progressive supranuclear palsy (PSP, clinical Richardson syndrome, negative Aβ-status, PSPRS: 42, MoCA: 28, 73 years, female) is observed in the caudate nucleus, globus pallidus and putamen (CPP), and the ventral midbrain (VM). Increased tracer retention in a representative patient with probable corticobasal degeneration (CBD, clinical CBS, negative Aβ-status, PSPRS: 21, MoCA: 27, 76 years, female) is shown in

CPP and VM plus in the central, parietal, and frontal cortex (Cx). Importantly, the cortical (Cx) binding in probable 4R-tauopathies is lower when compared to a clinical AD case (positive Aβ-status, MoCA: 11, 56 years, female) with probable underlying 3R/4R-tau, which likely reflects both the lower affinity to 4R-tau in comparison to 3R/4R-tau and the lower density of tau in 4R-tauopathies when compared with AD. The DVR data were derived from kinetic modeling of dynamic 0 to 60 min post-injection PET data by the multilinear reference tissue model 2 (MRTM2) approach. Extra-cerebral PET voxels are masked. Tau-PET imaging and data analyses were performed after informed written consent of the patients and human control, and approved by the local ethics committees of the University of Munich (19-022) and of Austin Health, Victoria Australia (HREC/17/Austin/433).

Tables

Table 1

Clinical phenotypes of 4R-tauopathies

Phenotype		4R-tauopathy	Differential diagnoses
Predominantly cognitive phenotypes			
bvFTD	Behavioral variant of FTD	PSP, CBD, AGD	**TDP-43 positive FTLD**, PiD
nfaPPA	Non-fluent/agrammatic variant of primary progressive aphasia	**PSP**, CBD	PiD, Tau-negative FTLD, AD
svPPA	Semantic variant of primary-progressive aphasia	GGT	**Tau-negative FTLD,** PiD
AOS	Apraxia of speech	**PSP**, **CBD**	TDP-43 positive FTLD
AS	Amnestic syndrome of the hippocampal type	CBD, GGT, AGD, PART	**AD**, Tau-negative FTLD
Cognitive and motor overlap phenotypes			
RS	Richardson's syndrome	**PSP**, CBD	
CBS	Corticobasal syndrome	**CBD, PSP**	**AD**
Predominantly motor phenotypes			
PSP-P	PSP with parkinsonism	**PSP**	PD
PGF	Progressive gait freezing	**PSP**, CBD	PNLD, LBD, MSA
LOCA	Late-onset cerebellar ataxia	PSP	**MSA**, SCA
PLS	Primary lateral sclerosis	PSP	**MND**, Tau-negative FTLD
ALS	Amyotrophic lateral sclerosis	GGT	**MND**, Tau-negative FTLD

AD, Alzheimer's disease; AGD, argyrophilic grain disease; CBD, corticobasal degeneration; FTD, frontotemporal dementia; FTLD, frontotemporal lobar degeneration; GGT, globular glial tauopathy; LBD, Lewy body disease; MSA, multiple system atrophy; PART, primary age-related tauopathy; PiD, Pick's disease; PNLD, pallidonigroluysian degeneration; PSP, progressive supranuclear palsy; SCA, spinocerebellar ataxia. Pathological diagnoses that are most likely associated with each of the clinical phenotypes are highlighted in bold.

Table 2
Disease-associated tau fragments

Cleavage site	Associated fragment	Disease	Biomarker (Tau antibody)	Sample type	Reference
D25	20-22 kDa	AD, OD	CCP-NH2 4268	CSF	[1]
A152	Tau-A	AD	Tau-A	Serum	[2,3]
Between P182-R194	Tau35	AGD, CBD, FTD, PSP	-	Brain homogenate	[4,5,6]
45-230	17 kDa	AD, CBD, PSP	-	Brain homogenate	[7]
K224	Tau224	AD, CBD, PSP	Tau_C224	CSF	[8]
D314	ΔTau314	AD	H1485	Brain homogenate	[9]
N368	37 kDa	AD	Tau-N368	Brain homogenate	[10]
E391	unspecified	AD	MN423	Brain tissue sections	[11]
D402	TauΔCasp6	AD	TauΔCasp6	CSF and brain tissue sections	[12,13]
D421	Tau-C	AD, Tau-FTD, PSP	Tau-C3	Serum (only in combination with Tau-A) and brain tissue sections	[14,15,16]

1, (Amadoro *et al.*, 2014); 2, (Henriksen *et al.*, 2013); 3, (Neergaard *et al.*, 2018); 4, (Arai *et al.*, 2004); 5, (Ikeda *et al.*, 2016); 6, (Wray *et al.*, 2008); 7, (Ferreira and Bigio, 2011); 8, (Cicognola *et al.*, 2019), 9, (Zhao *et al.*, 2016); 10, (Zhang *et al.*, 2014), 11, (Basurto-Islas *et al.*, 2008), 12, (Foveau *et al.*, 2016); 13, (Ramcharitar *et al.*, 2013); 14, (Gamblin *et al.*, 2003); 15, (Henriksen *et al.*, 2015); 16, (Zhao *et al.*, 2015). AD, Alzheimer's disease; CBD, corticobasal degeneration; FTD, frontotemporal dementia; PSP, progressive supranuclear palsy; OD, other dementias.

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