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REVIEW

Tau-targeted treatment strategies in Alzheimer's disease

Jürgen Götz[1], Arne Ittner[1,2] and Lars M Ittner[2]

[1]*Alzheimer's and Parkinson's Disease Laboratory, Brain & Mind Research Institute, University of Sydney, Camperdown, NSW, Australia, and* [2]*Laboratory for Translational Neurodegeneration, Brain & Mind Research Institute, University of Sydney, Camperdown, NSW, Australia*

Correspondence
Jürgen Götz, Alzheimer's and Parkinson's Disease Laboratory, Brain & Mind Research Institute, University of Sydney, 100 Mallett St, Camperdown, NSW 2050, Australia. E-mail: jgoetz@uq.edu.au

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With populations ageing worldwide, the need for treating and preventing diseases associated with high age is pertinent. Alzheimer's disease (AD) is reaching epidemic proportions, yet the currently available therapies are limited to a symptomatic relief, without halting the degenerative process that characterizes the AD brain. As in AD cholinergic neurons are lost at high numbers, the initial strategies were limited to the development of acetylcholinesterase inhibitors, and more recently the NMDA receptor antagonist memantine, in counteracting excitotoxicity. With the identification of the protein tau in intracellular neurofibrillary tangles and of the peptide amyloid-β (Aβ) in extracellular amyloid plaques in the AD brain, and a better understanding of their role in disease, newer strategies are emerging, which aim at either preventing their formation and deposition or at accelerating their clearance. Interestingly, what is well established to combat viral diseases in peripheral organs – vaccination – seems to work for the brain as well. Accordingly, immunization strategies targeting Aβ show efficacy in mice and to some degree also in humans. Even more surprising is the finding in mice that immunization strategies targeting tau, a protein that forms aggregates in nerve cells, ameliorates the tau-associated pathology. We are reviewing the literature and discuss what can be expected regarding the translation into clinical practice and how the findings can be extended to other neurodegenerative diseases with protein aggregation in brain.

Abbreviations

Aβ, amyloid-β; AD, Alzheimer's disease; AgD, argyrophilic grain disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CBD, corticobasal degeneration; CDK5, cyclin-dependent kinase 5; CFA, complete Freund adjuvant; FAD, familial AD: FTLD, frontotemporal lobar degeneration; FUS, Fused-in-Sarcoma; GSK-3β, glycogen synthase kinase-3β; HSP, heat shock protein; MAP, microtubule-associated protein; MARK1, MAP/microtubule affinity-regulating kinase 1; MBR, microtubule-binding repeat; NFT, neurofibrillary tangle; NSC, neural stem cell; PSD95, post-synaptic density protein 95; PSEN, presenilin; PSP, progressive supranuclear palsy; SAD, sporadic AD; SOD, superoxide dismutase; TDP-43, TAR DNA-binding protein 43; UPS, ubiquitin proteasomal system

Alzheimer's disease (AD) and related neurodegenerative disorders represent a serious social and economic threat to most societies. The cost of caring for an increasing number of people with dementia continues to rise. At present, there are an estimated 30 million people with dementia worldwide, with numbers expected to further increase to 80 million by 2040. The countries or regions with the largest numbers of affected individuals are China and the developing western Pacific, Western Europe and the Unites States (Ballard *et al.*, 2011).

AD is a progressive neurodegenerative disease that is characterized by the functional impairment and loss of neurons that results in a progressive decline in memory and other cognitive functions, leading to dementia. Frontotemporal lobar degeneration (FTLD) is a related group of disorders, more heterogeneous in nature, that includes Pick's disease (PiD), frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), argyrophilic grain disease (AgD), corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP). These disorders are characterized by a

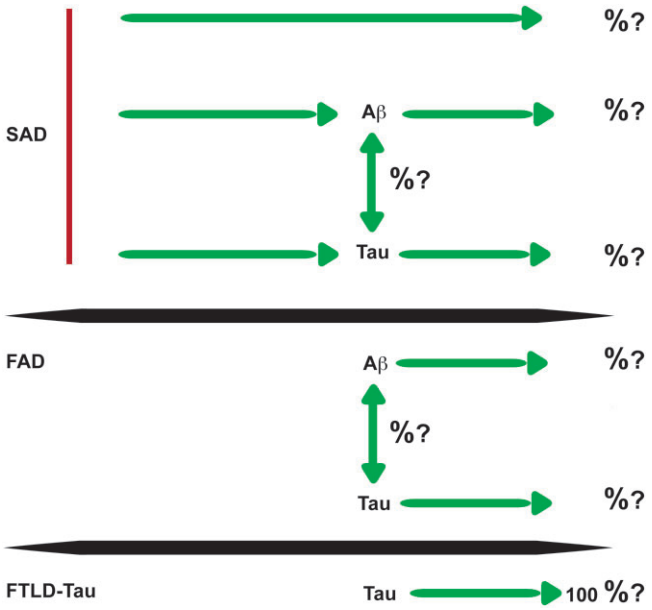

Figure 1

Relative contribution of the key players in AD and FTLD-Tau in toxicity. What causes SAD is not known. Most likely neuronal dysfunction and the loss of neurons are initiated by a range of triggers, such as toxins or oxidative stress that use Aβ, tau or an unknown mediator in executing their toxic functions. Aβ and tau dysregulation have direct consequences on neuronal function. There is also a significant crosstalk between Aβ and tau, in that Aβ is upstream of tau (as formulated by the amyloid cascade hypothesis), but at the same time Aβ toxicity is tau-dependent. For the rare FAD cases, the situation is more defined in that the known FAD mutations (that are all localized in the *APP*, *PSEN1* and *PSEN2* gene, respectively) are linked to Aβ formation, but again there is a crosstalk between Aβ and tau. Finally, in FTLD-Tau, tau dysfunction and NFT formation occur in the absence of a contribution of Aβ. A central question in the field and important as regards treatment strategies is what the relative contribution (%) of the Aβ-and tau-dependent as well as -independent mechanisms are in AD. This is also relevant (see subsequent figures) for the cellular compartments in which tau and Aβ exert their toxic functions and the cellular mechanisms (such as transport, signal transduction or mitochondrial function) they are believed to impair.

broad spectrum of clinical symptoms including behavioural changes, language abnormalities and motor dysfunction (Liscic *et al.*, 2008). FTLD is the second most common form of dementia presenting before the age of 65 (Liscic *et al.*, 2008).

Histopathologically, the AD brain is characterized by the deposition of the amyloid-β (Aβ) peptide and the microtubule-associated protein tau respectively (Figure 1). Aβ is derived from the Aβ precursor protein (APP) and is the major constituent of Aβ plaques, while hyperphosphorylated (i.e. abnormally phosphorylated) forms of tau constitute the major proteinaceous component of the neurofibrillary tangles (NFTs) (Selkoe, 1997). Both lesions can be visualized with special dyes such as thioflavin S or by silver impregnation techniques as both tau and Aβ form filamentous aggregates. The majority of AD cases are sporadic (SAD), with familial (FAD) cases likely accounting for less than 1%. In three genes, autosomal-dominant mutations have been identified, in *APP* itself, as well as in *presenilin 1* (*PSEN1*) and *2* (*PSEN2*) that both encode a component of the enzyme complex that is required to generate Aβ (Bertram and Tanzi, 2008). Aβ deposition is closely associated with the onset of AD, but it is the tau pathology that correlates better with the severity of dementia (Braak and Braak, 1995). At the onset of AD, tau pathology is restricted to distinct brain areas. As disease progresses, additional brain regions are affected in a stereotyped order of appearance, a process known as spreading. This is the basis for the pathological staging of AD into the so-called Braak stages I–VI (Braak and Braak, 1995). In FTLD, tau pathology is found in the absence of overt Aβ formation (Ballatore *et al.*, 2007). In FTDP-17, a subset of FTLD, pathogenic mutations have been identified in the *MAPT* gene encoding tau (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998). The familial forms of AD and FTLD allowed for expression of the mutant genes in transgenic mice and hence reproducing Aβ plaque and NFT formation *in vivo* (Gotz, 2001; Gotz and Ittner, 2008; Ashe and Zahs, 2010).

A second, major subset of FTLD is characterized by tau-negative and ubiquitin-positive lesions. In this subset, the transcription and splicing factor TDP-43 (TAR DNA-binding protein 43) has been identified as the aggregating protein, and consequently, this form of FTLD has been named FTLD-TDP (Neumann *et al.*, 2006). Similar to tau, TDP-43 in the aggregates is hyperphosphorylated and fragmented, a process believed to be linked to toxicity (Neumann *et al.*, 2006; Zhang *et al.*, 2007; Dormann *et al.*, 2009; Igaz *et al.*, 2009). Finally, in a third subset of FTLD, FTLD-FUS, the nuclear protein FUS (Fused-in-Sarcoma), has been identified as aggregating protein (Urwin *et al.*, 2010). While both proteins also form aggregates in subsets of amyotrophic lateral sclerosis (ALS), the mechanisms causing disease are believed to differ between FTLD and ALS (Dormann and Haass, 2011).

Current treatment strategies in AD

Since the 1990s, cholinesterase inhibitors are available for the symptomatic treatment of AD (Ballard *et al.*, 2011), with donepezil, rivastigmine and galantamine licensed for mild-to-moderate AD. Their effects on cognition though are moderate. Treatment with cholinesterase inhibitors provides moderate improvements in mood (particularly apathy) and social interaction; however, the outcome measures used in randomized clinical trials do not translate well into the day-to-day practice (Birks and Harvey, 2006; Loy and Schneider, 2006; Birks *et al.*, 2009; Wilkinson *et al.*, 2009). Memantine is an NMDA receptor antagonist that is licensed for moderate-to-severe AD. This drug was shown to improve cognitive performance and function over a 6 month period compared with placebo (McShane *et al.*, 2006; Gauthier *et al.*, 2008), and there is preliminary evidence suggesting that it might also be beneficial in the prevention and treatment of agitation and aggression (Wilcock *et al.*, 2008). There also seem to be additive benefits of combining a cholinesterase inhibitor and memantine (Lopez *et al.*, 2009). In addition, AD patients are treated with antipsychotic drugs that are used to combat agitation, aggression and psychosis, but the benefits are moderate, with severe side effects such as sedation and Parkin-

sonism (Ballard and Howard, 2006; C. Ballard *et al.*, 2009). Severe depression adds to the impairment and disability in people with AD, yet the benefit of antidepressant therapy has not been established (Weintraub *et al.*, 2010). Finally, anticonvulsants are used off licence as there is preliminary evidence from small randomized controlled trials that this might be an effective treatment of agitation or aggression in AD (C.G. Ballard *et al.*, 2009). Together, this indicates that there is a need for alternative strategies. Amongst the proposed disease-modifying treatments is immunotherapy as discussed in detail below, along with secretase inhibitors, amyloid aggegation inhibitors, copper or zinc modulators, tau aggegation inhibitors, lithium as an inhibitor of the tau kinase GSK3 as well as natural products and vitamins (Ballard *et al.*, 2011). There is a role for lifestyle factors such as diet, physical activity, cognitive training and a stimulating environment, but the relevance for disease onset and progression is far from being resolved (Daffner, 2010).

In the following, we will be discussing the protein tau in more detail and how tau transgenic mice have been exploited for immunization strategies.

Functions of tau under physiological and pathological conditions

Tau was initially isolated from porcine brain (Weingarten *et al.*, 1975). It was identified as a heat-stable protein essential for microtubule assembly. Specifically, it was found to be present in association with tubulin purified by repeated cycles of polymerization. Tau belongs to the family of microtubule-associated proteins (MAPs) that includes MAP2. As neurons develop, tau segregates into axons, and MAP2 into dendrites (Matus, 1990) (Figure 2). The years following tau's identification mainly focused on its tissue distribution and functions in microtubule assembly and stabilization, using the tools available at the time. The focus shifted radically when tau was identified in a highly phosphorylated form as the filamentous core of the neurofibrillary tangles (Grundke-Iqbal *et al.*, 1986; Goedert *et al.*, 1988). Insoluble tau aggregates are present in two dozen tauopathies, where they occur in the absence of overt Aβ deposition. We subsequently generated the first tau transgenic mouse model, reproducing aspects of the tau pathology (Gotz *et al.*, 1995). As referred to above, a major advance was achieved with the identification of pathogenic mutations in familial cases of frontotemporal dementia (FTD) in the *MAPT* gene that encodes tau (Clark *et al.*, 1998; Hutton *et al.*, 1998; Poorkaj *et al.*, 1998). We used this information to express mutant forms of tau in mice; thus, we not only reproduced neurofibrillary tangle formation but also placed tau downstream of Aβ in a pathocascade – a central concept in the field (Gotz *et al.*, 2001a,b).

Tau is however not simply an innocent bystander in Aβ toxicity (Figure 2). The 'axonal' protein Tau is found in dendrites of CNS neurons (Ittner *et al.*, 2010). Tau targets the Src kinase Fyn to the dendritic compartment, where it phosphorylates the NMDA receptor (NMDAR), thereby mediating its interaction with the post-synaptic density protein 95 (PSD95). This complex is then employed by Aβ to exert its toxicity in APP transgenic (APP^{tg}) APP23 mice, as evidenced by increased excitotoxicity, pronounced memory deficits, and premature mortality. The effects are even more dramatic in double transgenic APP^{tg}/tau^{tg} mice that show a particularly early mortality: increased levels of tau in FTD mutant tau transgenic mice (tau^{tg}, such as the pR5 mice) (Gotz *et al.*, 2001b) result in the accumulation of tau in the neuronal soma and dendrites, and consequently an increase in post-synaptic Fyn levels. The opposite is found for mice that express truncated tau (Δtau) (i.e. tau lacking microtubule-binding properties and not localizing to dendrites); in these, Δtau is enriched in the soma, where it interacts with Fyn. In a dominant-negative manner, Δtau acts on the physiological tau/Fyn interaction, preventing Fyn from accessing dendrites; this consequently protects APP^{tg}/Δtau mice from Aβ toxicity. Depletion of tau by a gene knockout ($tau^{-/-}$) prevents the tau-dependent localisation of Fyn to dendrites and hence also protects $APP^{tg}/tau^{-/-}$ mice from Aβ toxicity. Similarly, mutant APP expression on a Fyn null background prevented Aβ-mediated defects (Chin *et al.*, 2004; 2005). Mechanistic differences in retaining Fyn in the soma of $tau^{-/-}$ and Δtau neurons may explain the additive protective effects in $APP^{tg}/tau^{-/-}$/Δtau mice (Ittner *et al.*, 2010). We subsequently proposed a tau-axis hypothesis that explains how progressively increasing levels of dendritic tau make neurons vulnerable to amyloid-β (Ittner and Gotz, 2011).

Tau isoforms and expression pattern

All members of the tau/MAP2 family contain a carboxy-terminal microtubule-binding repeat (MBR) domain and an amino-terminal projection domain (Spillantini and Goedert, 1998). In the human CNS, tau exists as six major splicing isoforms ranging from 352 to 441 amino acids in size. They are generated by the presence or absence of two amino-terminal inserts (0N, 1N, 2N) and the presence or absence of the second repeat region, resulting in either three- (3R) or four-repeat (4R) isoforms (Lee *et al.*, 2001). The functional implications of having six major isoforms in the human brain are not understood. That the 3R forms are predominantly expressed during early development, while in the adult, 3R and 4R forms are expressed at equimolar ratios, has been explained in terms of the high degree of plasticity that is needed as the brain undergoes maturation. Once this is completed, the equimolar ratio becomes critical for the normal functioning of the brain since a distortion of this ratio (e.g. by a shift to 4R as caused by intronic mutations in FTLD) leads to neuronal degeneration (Hutton *et al.*, 1998). In a pathological context, 3R has been shown to inhibit the assembly of 4R tau (Adams *et al.*, 2010). Even less than for 3R/4R tau is known about the specific physiological functions of the amino-terminal insert variants (0N, 1N, and 2N). One study suggests that exon-2-containing mRNAs (1N and 2N) are associated with cells undergoing axonal sprouting, while exon-3-containing mRNAs (2N) are expressed in mature neurons that have already established their connections (Collet *et al.*, 1997). Tau has been found at high levels in the brain, where it is mainly expressed in neurons (Binder *et al.*, 1985). In tauopathies such as PSP and CBD, tau forms glial inclusions that have been faithfully modelled in transgenic mice (Higuchi *et al.*, 2002; Higuchi *et al.*, 2005) (Figure 2). This firmly estab-

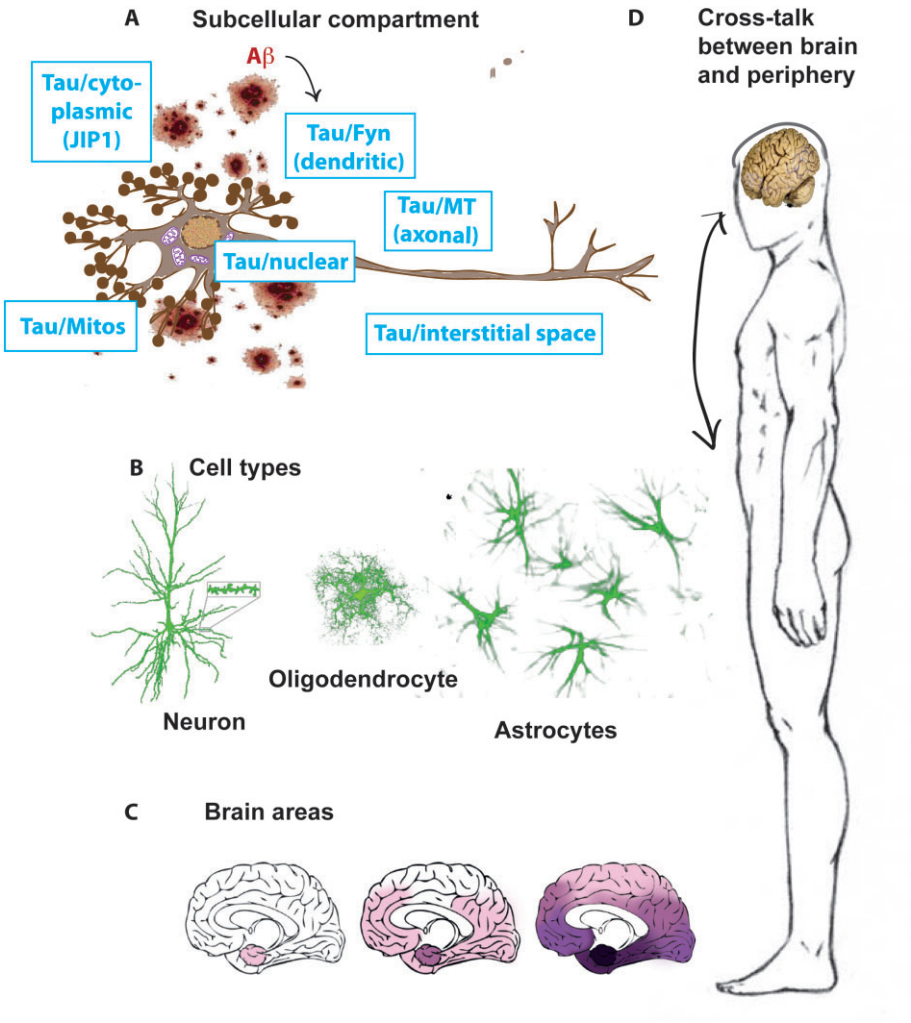

Figure 2

The compartmentalization of tau as a key player. (A) Tau's established physiological function is in the axon, where it is bound to and believed to stabilize microtubules. We found recently that under physiological conditions tau is also in dendritic spines where it has a function in targeting the PSD95 kinase Fyn to the post-synaptic density. In addition, tau has a role in the nucleus, either directly or indirectly, by altering the nucleo-cytoplasmic distribution of splicing factors. Pathological tau affects mitochondrial functions, specifically by targeting complex I of the mitochondrial respiratory chain. Furthermore, under pathological conditions, as tau is re-localized from the axon to the somatodendritic domain, it traps proteins (such as Jip1) in the soma and prevents them from executing their physiological function in the axonal compartment. Finally, new evidence suggests that tau is secreted into the interstitial space, potentially allowing for removal of interstitial tau by tau-targeted vaccination approaches. (B) An additional layer of complexity is added by the finding that although tau is perceived as a neuronal protein, under pathological conditions, such as in PSP or in CBD, tau forms fibrillar aggregates in astrocytes and oligodendrocytes. (C) As AD progresses, tau spreads in a stereotyped fashion through the brain. This observation has led to the definition of the so-called Braak stages. It can be envisaged that tau's toxic functions vary depending on the brain area where tau accumulates. (D) There is an increasing appreciation that the periphery, and in particular the immune system, has a decisive role, in a cross-talk with the brain, in the AD pathology.

lished that tau has an additional pathological function in glia. In peripheral tissues, tau expression has been identified in heart, skeletal muscle, lung, kidney and testis, and at low levels, adrenal gland, stomach and liver (Gu *et al.*, 1996).

Tau-interacting proteins and functional domains

Subcellular fractionations in both primary neuronal cultures and cell lines suggest that only about 20–50% of total tau is bound to microtubules (Drubin *et al.*, 1985; Ferreira *et al.*, 1989). Additional interactions with both cellular organelles and proteins have been reported, and the majority of these have been established in cell-free assays (Brandt and Leschik, 2004). Whether these are *bona fide* interactions, remains to be established *in vivo*. Using crude bovine brain extracts, tau and the kinase glycogen synthase kinase-3β (GSK-3β) have been shown to be part of an approximately 500 kDa microtubule-associated tau phosphorylation complex (Sun *et al.*, 2002). Subsequent work by the same group revealed that a dimer composed of 14-3-3 zeta simultaneously binds to and bridges

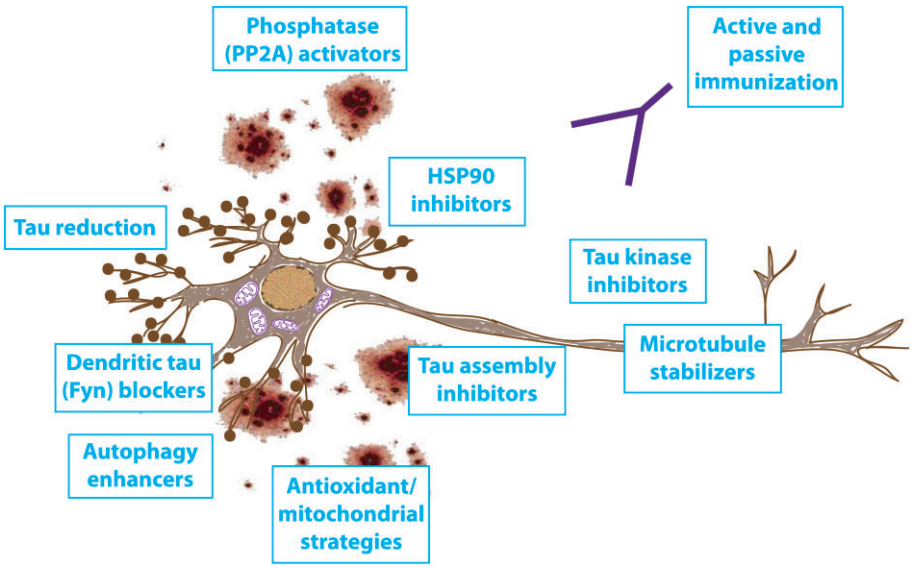

Figure 3

Tau-targeted therapeutic strategies. A host of strategies have been devised in tau-transgenic animal models to ameliorate biochemical changes due to tau over-expression (such as insolubility and hyperphosphorylation), histological alterations (such as NFT formation and somatodendritic localization) as well as behavioural (both memory and motor) impairments. To combat AD rather than only targeting Aβ, a combinatorial tau/Aβ strategy is likely the better approach.

tau and GSK-3β, stimulating tau's phosphorylation (Agarwal-Mawal *et al.*, 2003).

Via its MBR domain, Tau stabilizes microtubules and promotes their nucleation and growth *in vitro* (Cleveland *et al.*, 1977); it promotes the outgrowth of neurites and determines neuronal polarity (Kosik and McConlogue, 1994). Further interactions of the MBR domain include the microfilaments (actin), organelles such as mitochondria or the tau phosphatase PP2A, an enzyme whose physiological functions we have studied in detail (Gotz *et al.*, 1998; Kins *et al.*, 2001; 2003). The projection domain of tau interacts with Src kinases, including Fyn (Brandt *et al.*, 1995; Lee *et al.*, 1998; 2004; Brandt and Leschik, 2004). This domain determines microtubule spacing and is thought to link the axonal microtubules to the plasma membrane in a phosphorylation-dependent manner, with the less phosphorylated Tau isoforms enriched in the membrane fraction (Brandt and Leschik, 2004). In extending these studies, we found that a Fyn/tau interaction (Chin *et al.*, 2004; 2005; Bhaskar *et al.*, 2005) is needed to target Fyn to the dendrite (Ittner *et al.*, 2010). The projection domain also binds to the carboxy-terminus of the p150 subunit of dynactin that has an essential role in axonal transport (Magnani *et al.*, 2007).

Phosphorylation of tau

Tau contains an unusually high content of putative phosphorylation sites (45 serines, 35 threonines and 4 tyrosines), and for many of these, specific antibodies are available. Under physiological conditions, there are on average 2–3 moles of phosphate per mole of tau, whereas under pathological conditions, this ratio is increased to 7–8 moles (Kopke *et al.*, 1993). This has been termed 'hyperphosphorylation' (Pettegrew *et al.*, 1987): some sites are phosphorylated to a higher degree in the diseased than in the healthy brain; others are *de novo* phosphorylated. Hyperphosphorylation is critical for tau to detach from microtubules and is believed to be a prerequisite for it to aggregate (Avila *et al.*, 2006). As cytoplasmic tau levels increase further, tau aggregates and forms insoluble filaments. With increasing cellular tau levels, tau also aberrantly interacts with a host of cellular proteins, preventing these from executing their normal functions (Figure 2). One example is the kinesin adapter protein JIP1, and interestingly, tau needs to be highly phosphorylated to pathologically interact with JIP1 (Ittner *et al.*, 2008; 2009). Disruption of the tau/JIP1 interaction may be a strategy to block the toxic effects of accumulating tau (Figures 2 and 3).

Therapeutic approaches targeting tau

Several approaches to therapeutically target tau pathology have been pursued in animal models in recent years (Brunden *et al.*, 2009) (Figure 3). In more generic terms, transplantation of cells with the potential to differentiate *in situ* either into neuronal or glial cell types can be envisaged (Ferrari *et al.*, 2000). Such an approach has been successfully applied to mice with a combined tau and Aβ pathology in which neural stem cell (NSC) transplantation improved cognition via brain-derived neurotrophic factor (BDNF) (Blurton-Jones *et al.*, 2009). Interestingly, the spatial learning and memory deficits were rescued without altering the Aβ or tau pathology. Also, antioxidant strategies may be feasible as both tau and Aβ cause mitochondrial dysfunction and increased levels of reactive oxidative species (Eckert *et al.*, 2008).

Tau aggregation

More specific approaches aim to inhibit the formation of tau oligomers and fibrils (Figure 3). Although blocking tau/tau aggregation with small-molecule drugs is generally thought to be difficult because of the large surface areas that are involved in such interactions, there is growing evidence that tau multimerization can be disrupted with low-molecular-mass compounds (Brunden *et al.*, 2009). The dye methylene blue, for example, has been reported to inhibit tau aggregation. However, while phase II data presented at the International Conference on Alzheimer's Disease (ICAD) in 2008 suggested that this compound had a positive therapeutic effect (Wischik and Staff, 2009), work in zebrafish revealed that it was biologically active but failed to rescue any aspect of the pathology in tau transgenic fish (van Bebber *et al.*, 2010). What is therefore eagerly awaited is a phase III clinical trial. Several groups have performed high-throughput screenings to find 'aggregation breakers'. Screening of a 200 000 compound library identified a few inhibitors of fibrillization using as a read-out fluorescence caused by the intercalating dye, thioflavine S (Pickhardt *et al.*, 2005). This led to the identification of a number of anthraquinone inhibitors of tau fibril formation, including daunorubicin and adriamycin. Furthermore, it was shown that an anthraquinone analogue could reduce the formation of tau inclusions in neuroblastoma cells that overexpressed a human 4R tau fragment. A number of N-phenylamine tau fibrillization inhibitors identified from this screen were later shown to be also active in the cell culture model (Khlistunova *et al.*, 2006). More recently, a rhodanine series of tau fibril inhibitors was identified that disaggregated preformed tau fibrils and also prevented tau aggregate formation in neuroblastoma cells (Bulic *et al.*, 2007). Several research teams have since identified more chemical entities that inhibit fibrillization (Brunden *et al.*, 2009). Obviously, the preferred compounds are likely those that prevent the initial stages of tau/tau interaction, so that they lead to an increase of tau monomers and not an accumulation of intermediate multimeric structures, which could have detrimental biological effects (Brunden *et al.*, 2008). Another facet of tau aggregation is that in the course of disease tau becomes cross-linked by reactive carbonyl compounds, leading to the formation of advanced glycation end-products (AGEs) (Kuhla *et al.*, 2007). This type of modification renders tau resistant to proteolysis, thus assisting in its aggregation (Ledesma *et al.*, 1994). Interestingly, in P301L tau transgenic mice, the methylglyoxal-detoxifying enzyme glyoxalase I is up-regulated, possibly to combat AGE formation (Chen *et al.*, 2004b).

Targeting microtubule stabilization

Impaired axonal transport and microtubule function are a central pathomechanism in tauopathies (Gotz *et al.*, 2006). In tau transgenic mice with axonopathy, amyotrophy and, consequently, a motor phenotype, a reduction in microtubule density and fast axonal transport was found (Ishihara *et al.*, 1999; 2001). When these mice were treated with the microtubule-stabilizing drug paclitaxel, they showed a significant improvement of fast axonal transport and microtubule density compared with vehicle-treated mice. Furthermore, their motor function markedly improved. Because paclitaxel does not cross the blood–brain barrier (BBB) easily, one would conclude that it is the uptake at peripheral neuromuscular junctions and the subsequent retrograde transport to spinal motor neurons that caused the observed improvements (Zhang *et al.*, 2005). The octapeptide NAP containing the amino acid sequence NAPVSIPQ on the other hand crosses the BBB easily and promotes microtubule assembly (Gozes and Divinski, 2004). NAP was found to show efficacy using various routes of delivery (Matsuoka *et al.*, 2007; 2008). An important goal is obviously to keep peripheral levels of microtubule-stabilizing drugs as low as possible.

Targeting tau folding

Cells have devised mechanisms to get rid of un- or misfolded proteins that when generated under diverse conditions must be either refolded by molecular chaperones such as the heat shock proteins (HSPs), or eliminated by the ubiquitin proteasomal system (UPS) through an energy-dependent process and concerted action of a number of molecules, including specific ubiquitin ligases (Petrucelli *et al.*, 2004). In AD, protein aggregation provides a cellular stress that can initiate or feed into autophagy, a cellular degradation pathway, that involves the lysosomal machinery (Higgins *et al.*, 2010). Interestingly, proteins with a role in the UPS are altered in their expression level in P301L mutant tau transgenic mice (David *et al.*, 2006); in a second P301L tau transgenic model, a slowed turnover of tau and an enhanced stress response was found (Dickey *et al.*, 2009), and in AD, there is increasing evidence that both systems, autophagy and UPS, are affected (Keck *et al.*, 2003; Nixon *et al.*, 2005). Natively unfolded tau can be directly processed by the 20S proteasome without a requirement for ubiquitylation (David *et al.*, 2002). The involvement of the UPS in the degradation of hyperphosphorylated tau, however, has been shown through the use of inhibitors of HSP90 (Dickey *et al.*, 2006; 2007; Luo *et al.*, 2007). HSP90 is a major cellular chaperone, which assembles large complexes with a variety of co-chaperones, to maintain protein quality control and assist in protein degradation via proteasomal and autophagic-lysosomal pathways. Tau is a client protein for these HSP90 complexes. Large immunophilins such as FKBP51 and FKBP52 are also co-chaperones of HSP90–tau complexes (Salminen *et al.*, 2011). FKBP52 binds directly and specifically to tau, especially in its hyperphosphorylated form. Overexpression of FKBP52 in differentiated PC12 cells prevents the accumulation of tau suggesting a role for FKBP52 in therapeutic intervention (Chambraud *et al.*, 2010).

Targeting tau phosphorylation

The identification of phosphorylation sites crucial for tau's dissociation and aggregation is believed to help identify kinases and phosphatases involved in its hyperphosphorylation and dephosphorylation respectively (Ferrari *et al.*, 2003;

Hoerndli *et al*., 2004; Pennanen and Gotz, 2005; Steinhilb *et al*., 2007) (Figure 3). These enzymes present themselves as excellent targets for a therapeutic intervention in AD and related dementias (Iqbal and Grundke-Iqbal, 2008). Several kinases have been shown to phosphorylate tau *in vitro*, MAP/microtubule affinity-regulating kinase 1 (MARK1) phosphorylating two serine residues that are located within the (Ser^{262}/Ser^{356}), and cyclin-dependent kinase 5 (CDK5), GSK3, ERK2 and p38, among others, that all phosphorylate various epitopes outside of the MBD region (Dolan and Johnson, 2010). Tau hyperphosphorylation is compartmentalized as are the kinases (Gotz and Nitsch, 2001). A small number of kinase inhibitors have been tested for efficacy in tau transgenic models. These include the GSK3 inhibitor lithium chloride that reduced levels of insoluble tau, hyperphosphorylated tau and behavioural impairment in various tau transgenic mouse models (Perez *et al*., 2003; Noble *et al*., 2005; Engel *et al*., 2006; Caccamo *et al*., 2007). Another good example is the non-specific kinase inhibitor K252a (for CDK5, GSK3 and ERK1) that also reduced levels of hyperphosphorylated tau and ameliorated the motor phenotype that characterises this particular mouse strain (Le Corre *et al*., 2006). Interestingly, NFT numbers were not reduced, suggesting that the main cytotoxic effects of tau are not exerted by NFTs, but by lower molecular mass aggregates. We have recently shown that a small orally delivered compound, sodium selenate, induces dephosphorylation of tau in a protein phosphatase 2A (PP2A)-dependent manner in two tau transgenic mouse lines, pR5 and K3 (Pennanen *et al*., 2004; Ittner *et al*., 2008), causing a reduced phosphorylation of tau, reduced insolubility, and reduced behavioural impairment in memory and motor functions as well as preventing neuronal loss (van Eersel *et al*., 2010). To date, only three tau-directed drugs have progressed into human clinical trails, but results on their efficacy are not yet available (Brunden *et al*., 2009; Hampel *et al*., 2009). Given this and the central role of tau in disease, there is a need for new therapeutic approaches targeting tau pathology (Figure 3).

Aβ-based immunization strategies in AD

There is a cross-talk between Aβ and tau as shown by the finding that in tau transgenic mouse models, NFTs can be induced either by intracranial injection of Aβ (Gotz *et al*., 2001b) or by breeding tau mutant mice with mice that produce Aβ deposits (Lewis *et al*., 2001). Not surprisingly, immunizing against Aβ has also effects on the tau pathology in co-expressing mice (Oddo *et al*., 2004).

Aβ-directed vaccinations have been pioneered by Schenk and colleagues who initially vaccinated young FAD mutant PDAPP mice with the $Aβ_{42}$ peptide, that prevented the development of neuritic Aβ plaques, and in older mice significantly reduced them (Schenk *et al*., 1999). When in a second APP mutant strain, older mice were immunized, these were protected from behavioural impairment as revealed by memory testing (Morgan *et al*., 2000). Subsequently, an Aβ-directed passive vaccination approach was found to be similarly effective (Bard *et al*., 2000). Furthermore, vaccination reduced age-dependent learning deficits, which correlated with reductions in both soluble Aβ and tau (Oddo *et al*., 2006). While the argument can be raised that the APP mutant mice with Aβ plaque formation lack a number of critical features of AD, including hyperphosphorylated tau pathology and significant neuron loss, they resemble an early preclinical phase of the disease, which may be the optimal phase in which to initiate therapy that prevents the disorder (Morgan, 2011). Importantly, efficacy of the vaccine is also found in a nonhuman primate, the Carribean vervet (Lemere *et al*., 2004).

The success of immunotherapy came as a surprise in light of the dogma that the brain is an immune-privileged organ with minimal immune surveillance. The normal range of CSF IgG (5–50 $mg \cdot L^{-1}$) is roughly 0.1% of the plasma IgG concentration, with an average concentration of 0.1 $\mu mol \cdot L^{-1}$ (Morgan, 2011), and studies in rodents with iodinated anti-Aβ antibodies confirm that 0.1% of the circulating antibody enters the CNS (Pan *et al*., 2002). This has led to several proposed mechanisms as to how the Aβ-based immunotherapy works (and these concepts can be easily applied to the tau-based vaccination approaches) (Morgan, 2011).

(i) Opsonization and phagocytosis: This mechanism depends on sufficient quantities of antibody entering the brain, that then bind to Aβ to trigger phagocytosis by either resident microglia, or infiltrating monocytes /macrophages. Some studies revealed that amyloid plaques are decorated by antibodies (Wilcock *et al*., 2004). Other studies refute the idea that anti-Aβ-antibodies are taken up by neurons (Winton *et al*., 2011).

(ii) Peripheral sink: This mechanism does not need a penetration of the CNS by the antibodies; instead, it suggests that the presence of circulating antibodies creates a 'peripheral sink', which alters the equilibrium across the BBB for Aβ, favouring an efflux (DeMattos *et al*., 2001).

(iii) Catalytic modification of conformation: Antibodies act catalytically to modify the secondary structure of the Aβ monomers into a conformation that is less likely to form aggregates. In support of this concept, stoichiometries of antibodies as low as 1:10 effectively block Aβ fibril formation *in vitro* (Solomon *et al*., 1996; 1997).

Encouraged by the efficacy in mice, a clinical trial was launched with AN-1792 containing pre-aggregated synthetic $Aβ_{42}$ and the adjuvant QS-21 (Orgogozo *et al*., 2003). The phase IIa trial was halted prematurely as 6% of the patients who had received the vaccine developed meningoencephalitis; however, since some patients developed Aβ-antibody titres that correlated with a slowed cognitive decline (Hock *et al*., 2003), this encouraged the development of over a dozen antibody fragments and humanized Aβ-specific antibodies, which are currently in various stages of clinical trials (Morgan, 2011). Whether these approaches will lead to the anticipated outcome remains to be seen as for those human cases from the first trial that were followed up no cognitive benefits of the amyloid clearance could be detected (Holmes *et al*., 2008).

Tau-based immunization approaches

The first tau-based immunization approach used full-length recombinant human tau to immunize C57BL/6 wild-type

mice. The immunogen was emulsified in complete Freund adjuvant (CFA) supplemented with *Mycobacterium* tuberculosis. Pertussis toxin (PT) was administered i.p. the same day and 48 h later. An additional tau injection in CFA was administered 1 week later (Rosenmann *et al.*, 2006). Anti-tau antibodies were detected in the serum of tau-immunized mice that developed neurological symptoms including tail and hind limb paralysis. Tau-related abnormalities were visualized by Gallyas silver impregnation and were detected in both neurons and glial cells in brain stem and spinal cord. To confirm the presence of tau aggregates, the phosphotau-specific antibodies AT8 (Ser^{202}/Thr^{205}) and AT100 (Thr^{212}/Ser^{214}) were employed, the first being a physiological and the second a pathological epitope. Again, tau-related abnormalities were found in both neurons and oligodendrocytes. Axonal damage and inflammation was revealed without concomitant demyelination. Because the axonal damage in the tau-immunized mice occurred in close contact with cellular infiltrates, it was assumed that a local disruption of the BBB facilitates the passage of serum anti-tau antibodies. The authors concluded that these results together provide a link between tau autoimmunity and tauopathy-like abnormalities, indicating potential dangers of using tau for immunotherapy.

While the vaccination with full-length tau caused encephalitis (Rosenmann *et al.*, 2006), subsequent active immunization approaches using a tau phospho-peptide showed efficacy by preventing a pathology in tau transgenic models, in the absence of obvious side effects (Asuni *et al.*, 2007; Boimel *et al.*, 2010; Boutajangout *et al.*, 2010).

Asuni and colleagues used a 30-amino-acid peptide that comprised the PHF1 phospho-epitope of tau (Ser^{396}/Ser^{404}) in aluminium adjuvant to immunize 2 month-old P301L tau transgenic JNPL3 mice (Asuni *et al.*, 2007). Monthly immunization for up to 8 months strongly reduced tau phosphorylation and led to a 1.7-fold increased tau solubility. Total tau levels though were not reduced. MC1 immunoreactivity revealed aberrantly aggregated tau, but Gallyas staining to visualize NFTs has not been performed. In the behavioural dimension, the immunization increased the time the animals were able to stay on the RotaRod, reduced the number of foot slips on the narrow beam, and conferred a higher maximum velocity in the locomotor activity test. The tau antibodies generated in the animals recognized pathological tau on brain sections, and levels correlated inversely with the pathology. Interestingly, age-dependent auto-antibodies that recognized recombinant tau protein but not the immunogen were detected in the JNPL3 mice. While the immunotherapy was performed from 2 to 8 months of age (JNPL3 mice develop NFTs within this time frame), it remains to be established whether an immunization at a later age would also remove existing NFTs and the concomitant tau pathology (treatment vs. prevention).

Similar results were obtained by immunizing mice that express all six human tau isoforms on an $MAPT^{-/-}$ background, the htau model (Andorfer *et al.*, 2005), together with M146L mutant PS1 (Boutajangout *et al.*, 2010). At 3–4 months of age, the mice received peptide that comprised the PHF1 phospho-epitope of tau (Ser^{396}/Ser^{404}) i.p. in aluminium adjuvant as above, with the first three injections every 2 weeks, until 7–8 months of age. Subsequent administration was at monthly intervals. The immunotherapy strongly reduced tau pathology throughout the brain. Tau solubility was not affected at statistically significant values, but there was a trend towards a reduction in the PHF1-immunized group. The finding of a similar degree of microgliosis and astrogliosis in PHF1- and control-immunized mice suggests that the gradual removal of tau aggregates is not associated with gliosis. Gallyas staining was not performed. In the radial arm maze, the closed field symmetrical maze and novel object recognition test, cognitive impairment could be prevented by the vaccination. As in the above study, autoantibodies were found in the controls, and it is thus likely that they are also present in the immunized mice (Boutajangout *et al.*, 2010). What their pathogenic role is remains unclear, although naturally occurring autoantibodies have been suggested as a treatment approach in AD (Dodel *et al.*, 2011).

In the fourth study (Boimel *et al.*, 2010), 3 month-old K257T/P301S double mutant tau-expressing mice were immunized with a mix of three short peptides comprising the phosphorylation sites Ser^{202}/Thr^{205} (PHF1), Thr^{212}/Ser^{214} (AT100) and Thr^{231} (AT180) respectively. Tau phosphorylation and NFT formation, as determined by immunohistochemistry and Gallyas silver staining, was significantly reduced. Again, treatment was started before the onset of NFT formation and efficacy of the immunization approach after NFTs have formed is eagerly awaited. As in the third discussed study (Boutajangout *et al.*, 2010), immunization of the K257T/P301S mice did not result in astrocyte activation; there was however a slight increase in the number of lectin-positive microglia, but these were not activated (Boimel *et al.*, 2010). Infiltration with peripheral monocytes was not reported. Therefore, to which degree the glial compartment contributes to the effects of tau-targeted immunization remains to be determined. In Bi *et al.* (2011), we found that immunization of P301L tau mutant pR5 mice with the PHF1 peptide slowed the progression of tau pathology, by decreasing tau phosphorylation, reducing NFT numbers and – in aged mice – causing astrocyte activation.

Finally, a passive immunization approach has been applied by two research teams: The first study immunized 2 to 3 month-old JNPL3 mice weekly with either PHF1 antibodies (250 μg per 125 μL) or pooled mouse IgG for a total of 13 injections (Boutajangout *et al.*, 2011). Three behavioural tests were performed: the traverse beam, RotaRod and locomotor activity. The treated mice performed better in one of these tests, the traverse beam test. Levels of insoluble tau were reduced (particularly of CP13-tau), while those of soluble tau stayed unaffected. PHF1 immunoreactivity in the dentate gyrus was reduced twofold in the immunized compared with the control group. There was no evidence of an increased astrogliosis. The second study employed antibodies for the PHF1 epitope Ser^{396}/Ser^{404} as above, and in addition for the early conformational epitope MC1 (for a list of epitopes, see Chen *et al.*, 2004a), and a control mouse IgG (Chai *et al.*, 2011). Two mouse models were tested: in the JNPL3 study, antibodies were administered at 15 $mg \cdot kg^{-1}$ three times a week for 2 months and then at 10 $mg \cdot kg^{-1}$ twice a week for the remaining 2 months, whereas in the P301S study, the antibodies were administered at 15 $mg \cdot kg^{-1}$ twice weekly. While vaccination with the two tau-specific antibodies caused reduced levels in hyperphosphorylated tau (such as of

the 64 kDa species), total transgenic tau levels (HT7) were not affected. The treatment delayed the onset of motor function decline (as determined on the RotaRod) and weight loss (in both strains). This was accompanied by a concomitant reduction in neurospheroids in the spinal cord. Interestingly, both therapeutic tau antibodies, despite recognizing different pathological epitopes, produced very similar levels of phenotypic improvement (Chai *et al.*, 2011). Vaccination had however different effects on different phospho-epitopes in the two treated groups. Also, therapeutic long-term effects on motor neuron degeneration still need to be established.

As discussed above, the mechanisms, by which tau-directed antibodies ameliorate the tau-associated pathology are far from being resolved, and further investigations beyond these studies are needed (Sigurdsson, 2009). While one study revealed intra-neuronal antibodies upon tau-targeted immunization (Asuni *et al.*, 2007), another study showed antibodies in brain vessels, but not neurons or brain parenchyma (Boimel *et al.*, 2010). We have found previously in an active immunization trial of P301L tau mutant pR5 mice using the Aβ peptide, that anti-Aβ antibodies bound to the intracerebrally injected Aβ aggregates (Kulic *et al.*, 2005). As discussed above, there is an ongoing discussion as to whether and to which extent antibodies enter the brain and in particular, the cytoplasm of neuronal and glial cells (Winton *et al.*, 2011).

Regarding tau, a new facet has emerged very recently – the concept that tau is secreted and is spreading (Figure 3). Tau pathology in AD starts in the medial temporal lobe, but with disease progression, tau pathology manifests throughout the brain, in a particular sequence of affected brain areas (Braak and Braak, 1995). The molecular mechanisms underlying this spreading are only beginning to emerge. Expression of wild-type human tau in ALZ17 mice results in the accumulation of hyperphosphorylated tau in neurons, in the absence of NFT formation (Probst *et al.*, 2000). However, when ALZ17 mice are intracerebrally injected with brain extracts from NFT-bearing P301S tau transgenic mice (Allen *et al.*, 2002), NFTs develop in ALZ17 brains, with the pathology spreading along neuronal connections (Clavaguera *et al.*, 2009). Importantly, the newly formed NFTs in ALZ17 are made up of wild-type human tau, suggesting that non-NFT forming tau has been converted to NFT-forming tau. That tau can be released from and be taken up by cells has been shown in multiple experimental systems (Frost *et al.*, 2009; Kim *et al.*, 2010). This implies that the sink hypothesis could also apply to tau, with tau being sucked away from the cytoplasm into the interstitial space. As has been suggested, it is likely that tau antibodies can target pathological tau both extra- and intracellularly (Sigurdsson, 2009): The extracellular clearance can be envisaged to occur similar to what is thought to take place with antibodies targeting Aβ; antibody binding may directly promote disassembly and as well signal microglia to clear the antibody–protein complexes. Intracellular clearance may involve direct antibody uptake. The site of antibody–tau interaction within the cell is likely to take place in the endosomal/autophagy-lysosomal system (Sigurdsson, 2009). This view is supported by the observed changes in cathepsin D and L levels, which points to a lysosome-mediated degradation in the immunized K257T/P301S double mutant tau-expressing mice (Boimel *et al.*, 2010). On the other hand, total tau levels remained unchanged upon immunization of JNPL3 mice (Asuni *et al.*, 2007), arguing against an overtly increased degradation of tau. It may however well be that there is not one mechanism, but that different mechanisms are combined in action and that their relative role differs depending on the mouse strain and the immunogen used.

Conclusions and outlook

At present, the tau-targeted therapies that are in clinical trials target tau phosphorylation by GSK3 (lithium), microtubule stability and aggregation (Morris *et al.*, 2011). It is fair to say that vaccination (initially targeting mainly Aβ) is a promising therapeutic strategy in AD, but how it translates into clinical practice remains to be seen. In light of the finding that Aβ pathology depends on the presence of tau (Roberson *et al.*, 2007; Ittner *et al.*, 2010), and that many tauopathies are characterized by tau pathology in the absence of Aβ deposition, it makes absolute sense to pursue a tau-targeted treatment strategy, either alone or for AD in combination with an Aβ-targeting approach (Figure 1). As it stands, the studies in mice are promising, and it will only be a matter of time until these findings will be challenged in human trials. Tau will function as a door opener for other neurodegenerative diseases with intraneuronal or intraglial protein aggregation. Amongst these is superoxide dismutase (SOD) that forms aggregates in ALS, protein such as FUS, TDP-43 and, importantly, α-synuclein that forms insoluble aggregates in Parkinson's disease. In fact, it is already 6 years ago that α-synuclein-transgenic mice have been successfully vaccinated with α-synuclein (Masliah *et al.*, 2005).

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Conflicts of interest

There are no competing interests.

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