Biochemical and structural characterization of tau oligomers and cerebrospinal-fluid-derived tau in murine models of tauopathy

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Index Thesis

I)Preface

The following dissertation was written by the author. The “Results” section consists of a published manuscript and a second one in revision. In the co-first-authorship publication (Skachokova, Martinisi et al., 2019) the author significantly contributed to experiments, analysis, and writing process. In the first-authorship manuscript in revision (Martinisi et al., 2020) the author significantly contributed to experiments, analysis, and writing process. Please refer to the Authors’ contribution section for further details.

II)Acknowledgments

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And last for the best, I would like to thank Nonna Rosaria, Nonno Alfonso and Nonno Clemente to watch over me even if I lost you during this Ph.D. Only God knows how much I want you to be there now, for everything. But I hope you'll be proud of me nonetheless.

III) Abstract

Tau is a microtubule stabilizing protein that forms aggregates in Alzheimer's disease (AD). Tau protein abnormal aggregation is one of the main pathological features in AD patients' brain, particularly in the forms of insoluble tau fibrils. However, in recent years, a more prominent role in pathogenesis and diagnosis of AD has been increasingly given also to soluble tau oligomers and tau fragmentation pathways as well.

It was previously shown that fragmentation is linked to oligomeric stress in murine models of tauopathy, as co-expression of fragmented and full-length tau in tau transgenic mice results in the formation of oligomeric, non-fibrillary tau species and causes severe paralysis. This paralysis is fully reversible once expression of the tau fragment is halted, even though mutant tau expression is maintained. Whereas various strategies to target tau aggregation have been developed, little is known about the long-term consequences of reverted tau toxicity.

Tau fragments constitute also the majority of the protein reaching the cerebrospinal fluid (CSF) compartment. CSF-derived tau is a key diagnostic biomarker for AD; however, its features are largely unknown. Particularly, it is an unresolved question whether CSF-derived tau possesses a seeding competence. Previous *in vivo* studies on CSF-derived Aβ proved that this other biomarker does not have a seeding potential; however, *in vitro* studies on CSF-derived tau pointed towards another direction. Therefore, it was investigated whether CSF collected from patients diagnosed with probable AD or mild cognitive impairment (MCI) likely due to AD harbors a prion-like tau seeding potential.

The present thesis shows that tau species can be neurotoxic in the absence of seeding-competent tau aggregates, and mice can clear these tau forms permanently without tau seeding or spreading effects. These observations suggest that early targeting of non-fibrillar tau species may represent a therapeutically effective intervention in tauopathies. On the other hand, the absent seeding competence of early toxic tau species also warrants caution when using seeding-based tests for preclinical tauopathy diagnostics.

The results of the thesis provide also first evidence for *in vivo* prion-like properties of AD patients' CSF, accelerating tau pathology in susceptible tau transgenic mice. This demonstrates that biologically active tau seeds reach the CSF compartment in AD. Further studies may help to evaluate strain specific properties of CSF-derived tau bioseeds, and to assess their diagnostic potential.

In conclusion, the results presented in the thesis follow therefore nascent lines of research where oligomers are questioning the importance of established hallmarks in neurodegenerative diseases, and oligomerization might be indeed linked to fragmentation of protein at the center of neurological proteinopathies and be a suitable target for therapeutic strategies; furthermore, these results also

call for the importance and specificity of tau biomarkers for AD diagnosis, and this newfound seeding competence of CSF-tau could therefore improve the diagnostic tools at our disposal

1) Introduction

1.1) Alzheimer's disease (AD)

Dementia is a deteriorating process which involves memory, thinking, behavior and the ability to perform daily activities (WHO, 2015). In 2015, dementia cases have been accounted up to almost 50 million people in the world; among these, 60-70 % are hypothesized to be caused by the neurodegenerative disease known as Alzheimer's disease (AD) (WHO, 2015). It has been estimated that, by 2050, the number of people affected by AD will triple compared to present days (Hebert et al, 2013). Nowadays, AD is the sixth leading cause of death in US (Heron, 2016) and the mortality rates of the disease are also increasing constantly (Taylor et al, 2017). This neurological disorder is usually divided in late onset AD (LOAD) and early onset AD (EOAD), with the first form being accounted for more than 90 % of cases (Blennow et al, 2006). AD is phenotypically characterized by a slow, but progressive and unstoppable cognitive decline, starting from early mnemonic difficulties, difficulties in word-finding, and complex behavioral changes (Mucke, 2009).

Histologically, the disease is characterized mainly by two types of pathological hallmarks. The first ones are amyloid-beta (Aβ) plaques, cleaved pathological products of the amyloid precursor protein (APP), and those are specific traits for AD (Murphy & LeVine, 2010). The second ones are neurofibrillary tangles (NFT), a pathological type of tau filaments which are distinctive of a group of diseases called tauopathies (Goedert & Spillantini, 2006).

AD is a multifactorial disease, and its pathogenesis is far from being clarified, but there is a general better understanding of risk factors (Ballard et al, 2011). A significant increase of the presence of the apopoliprotein E4 allele (ApoE4, on chromosome 19) has been proven in LOAD patients and it has been deemed as the highest known genetic risk factor for developing the disease (Sadigh-Eteghad et al, 2012). Other known mutations in EOAD include those regarding genes coding for APP on chromosome 21), presenilin 1 (PSEN1, on chromosome 14) (Campion et al, 1999) and 2 (PSEN2, on chromosome 1) (Lanoiselée et al, 2017), with the last two forming half of the gamma-secretase complex which helps cleaving APP into the smaller Aβ fragments (Kaether et al, 2006).

The specific pathogenicity of Aβ, the EOAD-causing mutations on APP and the enzymes which cleave it, and the higher occurrence of AD in people affected by Down syndrome, caused by a trisomy on the same chromosome 21 which contains the APP gene, all led to the amyloid hypothesis (Hardy & Alisop, 1991) which considers amyloid deposition as the primary event in AD etiology.

1.2) Amyloid hypothesis and Aβ role in AD

As stated before, the reasons and the mechanisms behind AD development are not fully understood; however, the amyloid hypothesis, postulated in 1991 (Hardy & Alisop, 1991) and based on the fundamental role of the cleaved fragment Aβ from its precursor protein (APP), has been generally debated as the most likely and detailed (although not complete or conclusive) theory on the cause of AD (Selkoe & Hardy, 2016), therefore giving rise to an enormous field of research on Aβ and its progenitor APP.

APP is a single-pass transmembrane glycoprotein with a large N-terminal extracellular domain and a shorter C-terminal cytoplasmic tail (O'Brien & Wong, 2011). It is important to report that its role is unknown, and that APP loss by itself has not been proven as deleterious (O'Brien & Wong, 2011). APP undergoes through a proteolytic processing which branches in two different pathways, amyloidogenic and non-amyloidogenic, with the first one giving birth to the neurotoxic Aβ cleaved fragment (O'Brien & Wong, 2011). The amyloidogenic pathway needed for the cleavage of APP into Aβ involves processing by β-secretase 1, also known as β-site APP-cleaving enzyme 1 (BACE-1) in the extracellular domain, at the residue corresponding to the β-cleavage site, generating the soluble APP-β (sAPPβ) ectodomain, that starts with the N-terminus of Aβ and a 99-residues membrane-associated peptide (C99) (Thinakaran & Koo, 2008). A second cleavage is executed by γ-secretase, on variable sites of the sAPPβ (usually after 40 or 42 residues from the beginning of it), leaving the Aβ fragment (Thinakaran & Koo, 2008). The most fibrillogenic isoforms of Aβ are the 40 and 42 residues long, particularly the latter (Murphy & Levine, 2010), whose C-terminus allows for a faster formation of pathogenic oligomers (Bitan et al, 2003). Aβ has been shown to aggregate and spread through connected brain regions, and spread the amyloid deposition (Langer et al, 2011).

It is widely accepted that Aβ has at least a proven correlation with AD, and it is central for the comprehension of the pathophysiology of AD (Herrup, 2015). However, there are many evidences against the amyloid hypothesis and the centrality of Aβ for the development of AD (Ricciarelli & Fedele, 2017). One of the main concerns is that amyloid deposition does not seem to correlate well to the AD and dementia phenotypes, as histological tests proved (Murphy & LeVine, 2010). Particularly, amyloid deposition has been shown not to be parallel to memory and cognitive impairment (Villemagne et al, 2011), but mostly related to functional network disruption, as per Electroencephalography (EEG) and Magnetic Resonance Imaging (MRI) measurement (Pievani et al, 2011). Later, positron emission tomography (PET) studies further confirmed that Aβ deposits do not correlate well with the degree of cognitive impairment (Landau et al, 2016). Furthermore, even though Aβ has been shown to be cytotoxic *in vitro* (Ono et al, 2009), no neuronal cell death has been seen in mouse models carrying EOAD-causing mutations (Ricciarelli & Fedele, 2017).

It is important to report that drugs targeting Aβ and the enzymes processing it, such as beta-site APP cleaving enzyme (BACE) inhibitors, γ-secretase inhibitors and antibodies against Aβ have all proven ineffective, sometimes even worsening cognition (Mullard, 2019a). In fact, more than 100 drugs directed against Aβ have failed to elicit a significant clinical improvement (Becker & Greig, 2012). More in detail, some of these drugs have managed to decrease Aβ levels in the brain, but none of these showed clinical efficacy (Giacobini & Gold, 2013) with the researchers incurring into failures from different directions (Mehta et al, 2017). As an example, anti-aggregant drugs against amyloid plaque formation, such as tramiprosate, reduced the plaque load in animal models but failed to meet clinical endpoints when administered to patients (Aisen et al, 2011). Also acting on the secretases processing APP was not successful: a few clinical trials involving BACE inhibitors ended in failure due to excessive liver toxicity (Barão et al, 2016), while the ones involving γ-secretase inhibitors did not manage to have the drugs marketed, due mainly to very low blood-brain barrier (BBB) penetrance (Green et al, 2009; Coric et al, 2012). Finally, also monoclonal antibodies against Aβ failed in clinical trials: among many cases, emblematic is Lilly's Solanezumab, which targeted Aβ central epitope, necessary for its oligomerization (Watt et al, 2014). This monoclonal antibody did not improve the cognitive decline in clinical trials and resulted in possible complications such as edema (Honig et al, 2018). The drug was even resurrected for further trials once it seemed to slow down disease progress, but it was later definitively dropped after failing again to meet the desired clinical endpoints (Knopman, 2019).

A possible explanation for this series of failure for drugs targeting Aβ is that the amyloid burden is not parallel to cognitive impairment, as many diagnostic techniques have confirmed (Pievani et al, 2011; Landau et al, 2016). More in detail, the missing correlation of Aβ with cognitive decline in symptomatic phase of dementia (Mehta et al, 2017) could be meaning that the drugs against Aβ are administered too late in time, because Aβ might start the pathogenic cascade of events leading to AD, but that same cascade continues independently of it (Selkoe & Hardy, 2016). Another explanation behind the failure of drugs acting on the amyloid cascade is the involvement of β and γ-secretases in pathways different from APP processing (Barão et al, 2016), therefore suggesting that they might be unspecific targets for treating AD (Ricciarelli & Fedele, 2017).

Consequently, other targets for possible drugs against AD have been hypothesized, and the role of Aβ in AD development has been questioned by researchers, who consider that tau protein could have a more prominent role for the pathological mechanism behind AD, compared to Aβ (Makin, 2018), especially due to the better correlation of the other main pathological hallmark of AD, NFTs, with the disease progression and cognitive impairment (Braak & Braak, 1991).

1.3) Tau protein

Tau is a microtubule-associated protein encoded by chromosome 17, with the role of stabilizing the microtubules and promote axonal outgrowth (Wang & Mandelkow, 2016), through a complex equilibrium between phosphorylation and dephosphorylation (Pasinetti et al, 2010). It is a very hydrophilic and highly soluble protein (Wang & Mandelkow, 2016). Overall, the protein is basic, possessing an acidic N-terminus and a neutral C-terminus, separated by a proline-rich motif (Wang & Mandelkow, 2016). There are six possible isoforms in humans, generated by alternative splicing of exons 2,3 and 10 out of the 16 included in the MAPT gene encoding tau (Wang & Mandelkow, 2016) The protein is formed by two near amino-terminal inserts and four repeat domains; the six isoforms are formed by a variable number of near amino-terminal inserts (0N,1N or 2N) and the presence or absence of the second repeat domain (Wang & Mandelkow, 2016).

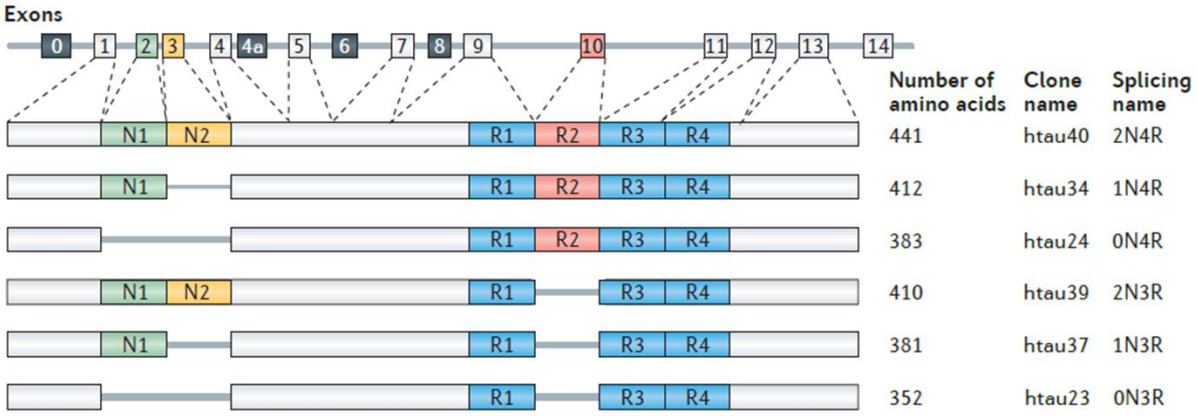

Fig.1: tau isoforms and structures. Differential splicing of exons 2 and 3 and exon 10 yields the 6 different isoforms of human tau, with 0,1 or 2 near amino-terminal inserts and the expression or not of the second repeat domain. Adapted from Wang & Mandelkow, 2016.

Tau is expressed mainly in the neurons of the central nervous system (CNS), but also in astrocytes and oligodendrocytes (Shin et al, 1991). After its discovery in 1975, tau has been almost immediately characterized as an intrinsically disordered protein (Cleveland et al, 1977). Although it is natively unfolded, its natural state shows little tendency for aggregation; however, paired helical filaments (PHF) and the previously cited NFT are pathological hallmarks common to many neurodegenerative diseases usually known as tauopathies, which include chronic traumatic encephalopathy (CTE), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), Pick's disease, and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Goedert & Spillantini, 2006). Generally, tauopathies occur sporadically, but the mechanisms behind the accumulation on each disease are poorly understood (Rojas & Boxer, 2016). Tau pathological role has been clearly elucidated by the mutations causing FTDP-17, which resulted in tau filamentous lesions without amyloid plaques (Goedert, 2015), and from there research shifted

towards a more prominent attention on the microtubule stabilizer. It has also been reported that tau lesions are common features in Lewy bodies, pathological hallmarks of the group of diseases known as synucleopathies, which comprise Parkinson's disease (PD), the second most frequent neurodegenerative disease in the world behind AD (Moussaud et al, 2014). Therefore, tau is getting increasingly important as a possible therapeutic target in neurodegenerative disorders.

1.3.1) Tau function

As mentioned before, Tau was discovered in 1975 as part of the family of microtubule-associated proteins (Weingarten et al, 1975). It is a heat-stable protein, and later it has been characterized as a microtubule stabilizer, essential for microtubule assembly from tubulin (Cleveland et al, 1977). More in detail, stability of the microtubules is obtained by a complex equilibrium of phosphorylation and dephosphorylation of tau (Pasinetti et al, 2010) with tau detaching from microtubules when phosphorylated (Billingsley & Kinkaid, 1997). It has been estimated that 99 % of tau is bound to microtubules in mature neurons (Congdon et al, 2008). Tau interacts with the microtubules through the repeat domains and flanking regions (Wang & Mandelkow, 2016) at the interface of tubulin heterodimers (Kadavath et al, 2015); more in detail, the repeat domains are microtubule-binding domains, while the flanking regions play a regulatory role in this interaction (Mukrasch et al, 2007). Different aminoacidic sequences among the repeat domains regulate the different affinity of the microtubule-binding domains to the microtubules (Guo et al, 2017). By stabilizing said microtubules, it allows cytoskeleton re-organization (Feinstein & Wilson, 2005), and it might not be the only function tau accomplishes while bound to microtubules, as it is thought to regulate axonal transport through various mechanisms (Wang & Mandelkow, 2016). Among these mechanisms, the common feature is that tau slows down axonal transport by competing for binding sites on microtubules with kinesin (mostly) and dynein (Stamer et al, 2002), which would then act as motor protein in this process. However, most of these mechanisms have been shown *in vitro*, but have been unparalleled so far *in vivo*, with tau deletion not affecting axonal transport in murine models (Yuan et al, 2008).

Tau does not bind to microtubules only; *in vitro* studies showed tau interactions with the ribonucleoproteome, chaperones, the proteasome, histone complexes, and members of the 14-3-3 protein family (Gunawandana et al, 2015) but also neural DNA (Sultan et al, 2011). These interactions show other possible functions of the protein: as an example, tau has been proven to be play a role for translation both *in vitro* and *in vivo*, as a negative regulator. (Papanikolopoulou et al, 2019) Another tau function which has gotten increasing attention during recent years is tau protective role for neural DNA, as proven per *in vitro* studies (Sultan et al, 2011). In fact, tau has been shown to protect neural DNA from heat-induced stress in neuronal cultures (Sultan et al, 2011). Interactions of tau pave the way also for eventual hypotheses of the pathological mechanisms behind tauopathies (Wang.X et al, 2019). Tau has been proven to interact to non-myosin muscle proteins, and this link brought to proposed roles of Tau in maintaining dendritic spines and mitochondrial fission biology,

which have been proven to be disrupted in presence of mutated P301L tau *in vitro* (Wang.X et al, 2019).

More complicated functions of tau in neurological pathways have also been prospected, based on *in vivo* observations. Experiments in *Drosophila* led to the association of tau to long-term sensorial memory, particularly to negative regulation of olfactory memory and facilitation of habituation (Papanikolopoulou et al, 2019), a common neural plasticity phenomenon which sees a decrease of innate responses once a stimulus is repeated over time (Zuo et al, 2017). Altogether, tau first proven role remains the most studied and clarified over the years, but more studies must be executed to better understand the other less highlighted functions of the proteins.

1.3.2) Tau post-translational modifications

As it happens many other proteins, tau undergoes through a number of post-translational modifications (PTM), with the most frequent being phosphorylation (Neddens et al, 2018), just like it happens for the majority of the proteins (Khouri et al, 2011). Tau phosphorylation has a clear role for the development of tauopathies, through heterogeneous spreading and misfolding pathways (Dujardin et al, 2018) and will be described separately. However, tau can also be modified by the means, among others, of glycosylation, acetylation, deamidation, isomerization, nitration, methylation, ubiquitylation, and sumoylation (Martin et al, 2011). PTMs on tau can have variable outcomes: proving this concept, N-glycosylation can promote tau filamentous aggregation and hyperphosphorylation (Congdon & Sigurdsson, 2018), while O-GlcNAcylation, catalysed by the O-GlcNAc transferase (OGT) (Gorelik et al, 2019) acts in a protective way against fibrilization of tau, negatively regulating phosphorylation due to occupation of the phosphorylation sites (Gong et al, 2016). Furthermore, the interplay between these modifications can have an impact on AD pathology (Losev et al, 2019), as they normally co-exist in a dynamic equilibrium (Yuzwa et al, 2008), which is disrupted in AD (Losev et al, 2019).

Among the many PTMs occurring to tau protein, tau acetylation is one of the most studied PTMs (Cohen et al, 2013), with controversial results over the years: some researchers believe in its protective potential (Carlomagno et al, 2017), while other studies brought up its contribution to tau aggregation (Cohen et al, 2013). While deacetylation catalysed by histone deacetylase 6 (HDAC6) leads to aggregation (Cook et al, 2014), the p300 acetyltransferase usually acts on the same sites of HDAC6, thus suggesting another interplay between enzymes which can regulate tau propensity to aggregate (Cook et al, 2014). Tau acetylation on Lys321 also negatively switches off tau hyperphosphorylation *in vitro* (Carlomagno et al, 2017); however, previous studies have found out that p300 acetyltransferase could also increase tau aggregation by possibly impairing tau ubiquitylation (Min et al, 2010), therefore delaying its degradation. Also acetylation on Lys174 by

p300 acetyltransferase is correlated with cognitive decline (Min et al, 2015), so the impact of acetylation on development of tauopathies depends on the acetylated site (Wang & Mandelkow, 2016). It is curious to report that tau can auto-acetylate itself, as it possesses acetylation properties (Cohen et al, 2013). Altogether, these findings suggest that the role of the various PTMs on tau is far from being elucidated, thus leading the researchers to study the more established tau hyperphosphorylation phenomenon as a possible target for therapeutic intervention.

1.3.2.1) Tau phosphorylation

As previously mentioned, the most common form of post-translational modification occurring to tau is phosphorylation, with the protein exhibiting more than 70 possible phosphorylation sites (Neddens et al, 2018). The phosphorylation sites of tau reside mainly in the flanking regions of the proteins, and most of these easily accessible to kinases, especially serine/threonine kinases (Martin et al, 2011), due to its natively unfolded structure (Wang & Mandelkow, 2016). Other phosphorylation sites in or near the repeat domains are targeted for phosphorylation by microtubule affinity-regulating kinases (MARKs) cyclic AMP-dependent protein kinase (PKA) and calcium or calmodulin-dependent protein kinase II (CaMKII), among others (Hanger et al, 2009). Generally, the kinases acting on tau phosphorylation sites can be summarized in three broad groups (Martin et al, 2011). The first one is formed by proline-directed protein kinases (PDPKs), which are serine/threonine kinases and include glycogen synthase kinase-3 (GSK-3), cyclin-dependent kinase-5 (Cdk5) and mitogen-activated protein kinases (MAPKs) (Martin et al, 2011). The second one is formed by non-PDKs, which are serine/threonine kinases including cAMP-dependent protein kinase A (PKA), casein kinase 1 (CK1) and microtubule affinity-regulating kinases (MARKs) (Martin et al, 2011). Finally, the last one is the group of protein kinases specific for tyrosine residues including Src, Fyn, Abl, and Syk (Martin et al, 2011)

As said before, tau regulates microtubule stability keeping a complex balance between phosphorylation and dephosphorylation (Pasinetti et al, 2010), with tau dephosphorylation able to enhance microtubule assembly and tau phosphorylation decreasing the affinity of the protein for microtubules (Martin et al, 2011). Tau phosphorylation has also been proven to detach the protein from actin (Whiteman et al, 2009) and it also modifies the protein interactions with cytoplasmic membrane, DNA, Fyn and other binding partners, which can result to a range of impaired signaling pathways (Hanger et al, 2009). Other physiological functions of tau phosphorylation, besides its role for balancing microtubule assembly, are far from being fully clarified, but this post-translational modification has been linked also to induced hypothermia and lethargic status (Luppi et al, 2019). A possible explanation for this phenomenon is that phosphatases are more sensitive to temperature changes than kinases, as hypothermia inhibits phosphatases exponentially (Planel et al, 2004).

There are also few suggestions on tau phosphorylation being possibly neuroprotective: as an example, phosphorylation on Ser422 is known to impair tau truncation at Asp 421 (Guillozet-Bongaarts et al, 2006), which is one of the most studied pathological cleavages occurring at C-terminus of tau (Ozcelik et al, 2016). There are also tau phosphorylation sites, like Thr 205, which have been shown to protect against Aβ toxicity in murine models of AD (Ittner et al, 2016). However, tau phosphorylation is still mostly and predominantly known for its pathological contribution to neurodegeneration (Neddens et al, 2018).

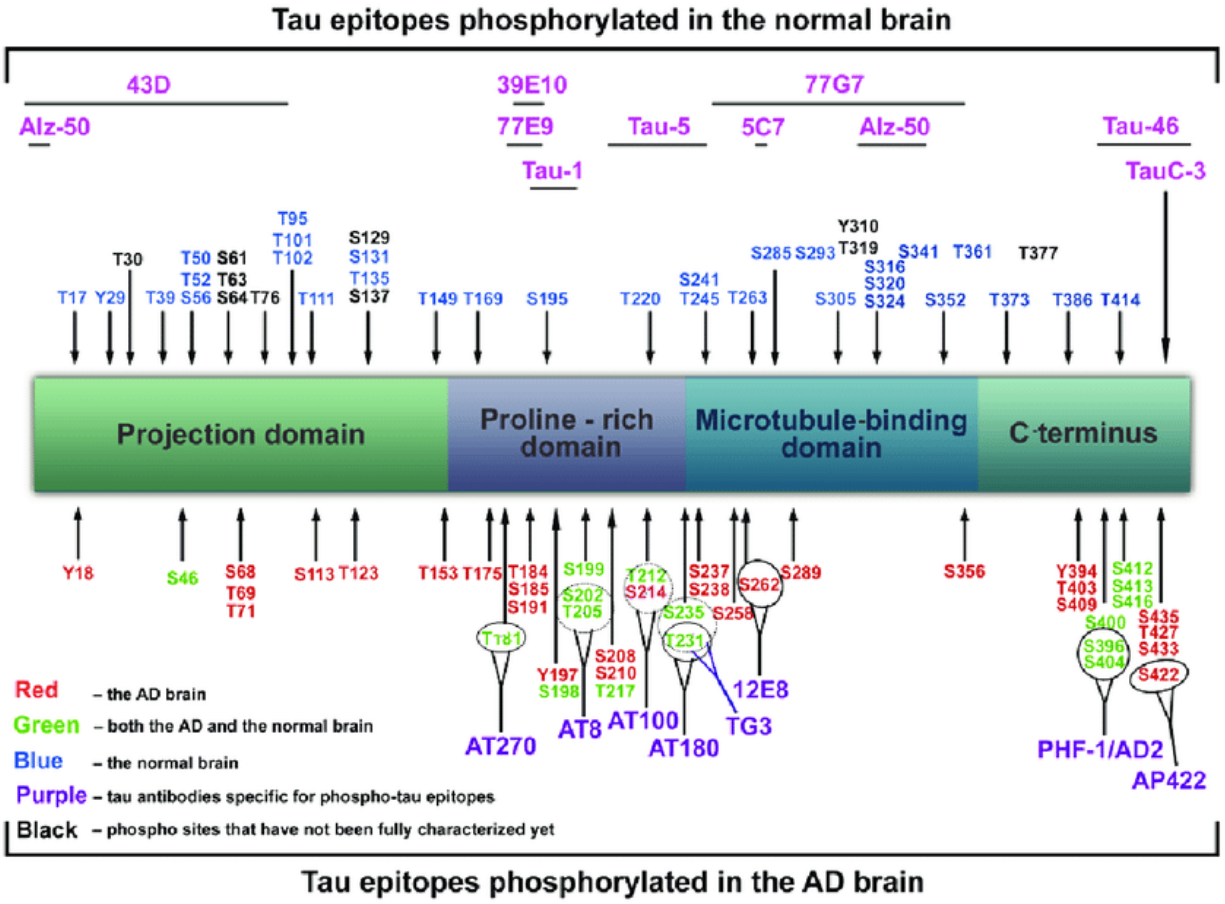

Fig.2: tau phosphorylation sites. The figure shows all putative phosphorylation sites and also the epitopes of the most used antibodies for detection of these phosphorylation sites. (Simic et al, 2016)

The role of tau hyperphosphorylation in pathological conditions has been extensively studied throughout the years, as the link between tau hyperphosphorylation and its ability to self-assemble has been thoroughly established (Guo et al, 2017). In fact, it is known that hyperphosphorylation of tau can provoke self-assembly of the protein into filamentous, pathological forms, which are at the center of tauopathies (Guo et al, 2017). Generally, it has been established as a 3-fold minimum increase of tau phosphorylation compared to controls (Neddens et al, 2018), and in some of the phosphorylation sites it occurs only in pathological situations, often anticipating NFT formation

(Wang.J et al, 2013). In AD, particularly, tau hyperphoshorylation has been shown to result in 8-10 phosphorylated sites per tau molecule (Wang.J et al, 2013).

The exact process behind the formation of NFT has not been clarified yet, even though it is known that tau hyperphosphorylation alone is not sufficient to its aggregation (Wang & Mandelkow, 2016), as there are also phosphorylation events which protect against tau accumulation, such as phosphorylation at Thr205 (Ittner et al, 2016). The aggregation into filamentous tau is suspected to be provoked by unknown cofactors (Wang & Mandelkow, 2016), and also other PTMs are thought to contribute to formation of fibrils, such as the aforementioned N-glycosylation (Congdon & Sigurdsson, 2018). Nonetheless tau hyperphosphorylation is necessary for the aggregation into filamentous forms, as it detaches tau from microtubules and releases soluble tau that can aggregate afterwards (Martin et al, 2011).

Phosphorylation sites constituted by Ser-Pro or Thr-Pro motifs in the flanking regions are usually hyperphosphorylated in tauopathies and they get targeted for phosphorylation by several signal-transducing proline-directed serine/threonine kinases (Wang & Mandelkow, 2016), the first of the three aforementioned groups of kinases acting on tau phosphorylation sites. These phosphorylation events can detach tau from microtubules (Wang & Mandelkow, 2016), as it happens with phosphorylation of the sites in the repeat regions by non-PDKs group of kinases (Hanger et al, 2009). However, phosphorylation sites differ significantly in their impact on tau affinity for microtubules: phosphorylation at Thr231 or Ser262, as an example (Alonso et al, 2010) are known to greatly reduce tau affinity for microtubules and therefore are more likely to release soluble tau which could then form fibrils, while other phosphorylation sites do not have the same impact on the microtubule affinity of the protein (Ando et al, 2016).

From a clinical point of view, there are also phosphorylation sites with a higher relevance: as an example, Thr181 is the most frequent phosphorylated site measured in clinical AD samples (Lathuilière et al, 2017). Other clinically relevant phosphorylation sites are Ser202 and Thr205, which together constitute the AT8 antibody epitope, recently established for immunohistochemical post-mortem analysis of possible AD patients (Braak et al, 2006). This epitope is also important for the understanding of structural changes related to hyperphosphorylation, as tau undergoes through a conformational change defined by phosphorylation of Ser202 and Thr205 altogether which is thought to lead to higher aggregation propensity (Despres et al, 2017). However, as mentioned before, phosphorylation of Thr205 alone has been shown to protect against formation of plaques (Ittner et al, 2016).

In summary, tau hyperphosphorylation remains a hot topic in the development of neurodegenerative disorders, but the impact of the single phosphorylation events can greatly vary, and altogether the mechanisms leading from hyperphosphorylated tau to NFT are yet to be fully clarified.

1.3.3) Tau mutations

More than 80 mutations have been identified referring to tau protein (Wang & Mandelkow, 2016). Many of these are pathogenic, linked to tauopathies such as FTDP-17 (Goedert & Spillantini, 2000), CBD (Kouri et al, 2014) and PSP (Coppola et al, 2012). It has been hypothesized that tau mutations can become pathogenic by changing the ratio between 3R and 4R tau, possibly increasing tau phosphorylation (Lacovich et al, 2017). However, it is not rare that many missense mutations change the ratio of tau isoforms without resulting in pathogenic tau mutated forms, effectively being "benign" mutations (Guerreiro et al, 2010). It is important to report that, while AD is the most common tauopathy occurring in the planet (Goedert & Spillantini, 2006), there is no tau mutation conclusively linked to the AD phenotype (Ghetti et al, 2015) although many mutations in MAPT gene have been occasionally observed in AD patients (Van Giau et al, 2019).

The majority of tau mutations happen in the microtubule-binding regions and tend to decrease tau affinity to the microtubules (Wang & Mandelkow, 2016). Many missense mutations tend instead to have an effect on tau alternative splicing (Ghetti et al, 2015), and the same outcome occurs with intronic mutations, usually clustered at the intron following exon 10 of MAPT gene (Ghetti et al, 2015). There are also mutations which affect tau in a more subtle way, as it happens with the ones hindering the interaction with the dynactin complex (Magnani et al, 2007): these mutations take place at exon 1 of MAPT gene, and they are thought to disrupt axonal transport (Magnani et al, 2007). It has been hypothesized that many of tau mutations lead to loss of function of tau, which would then trigger neurodegenerative processes (Ghetti et al, 2015). Generally, despite the common acceptance of tau mutations affecting post-translational modification, interaction with other proteins, and a variety of intracellular processes, the full complexity of the mutations occurring to tau has yet to be untangled (Guo et al, 2017). The next section will therefore be dedicated to elucidate tau most known pathogenic mutations and the associated neurodegenerative diseases, commonly grouped as tauopathies.

1.3.4) Tauopathies

Tauopathies are the group of diseases characterized by tau inclusions in neurons and glia (Götz et al, 2019), with the main common consequence resulting in dementia and neurodegeneration (Kovacs, 2015). Tauopathies are categorized by the brain regions affected by tau inclusions, the dominant tau isoform that is part of the inclusions, the morphology of said inclusions and the cell types involved in the neurodegenerative processes (Götz et al, 2019).

From a clinical point of view, tauopathies have been divided into primary and secondary sub-types, with the first one including all the tau-related diseases which are independent of amyloid plaques or

other clinical features (Crary et al, 2014). Primary age-related tauopathies (PARTs) therefore do not include AD, but only those tauopathies who depend solely on tau inclusions (Crary et al, 2014) and are a subgroup of frontotemporal lobar degeneration (FTLD) dementia forms (Van Mossevelde et al, 2018). Among the PARTs, the most characterized ones are progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), Pick's disease (Goedert & Spillantini, 2006), and globular glial tauopathy (GGT) (Chung D.E.C et al, 2019). It is definitely worth mentioning that FTDP-17 was the first dementia form directly linked to defined pathological tau mutations (Goedert & Spillantini, 2000), and proving that tau dysfunction can independently cause neurodegeneration, with more of 30 tau mutations identified (Goedert & Jakes, 2005). However, dementia forms caused by these mutations have been now considered as genetic sub-categories of PARTs (Forrest et al, 2018), and so the term FTDP-17 has been mainly discontinued.

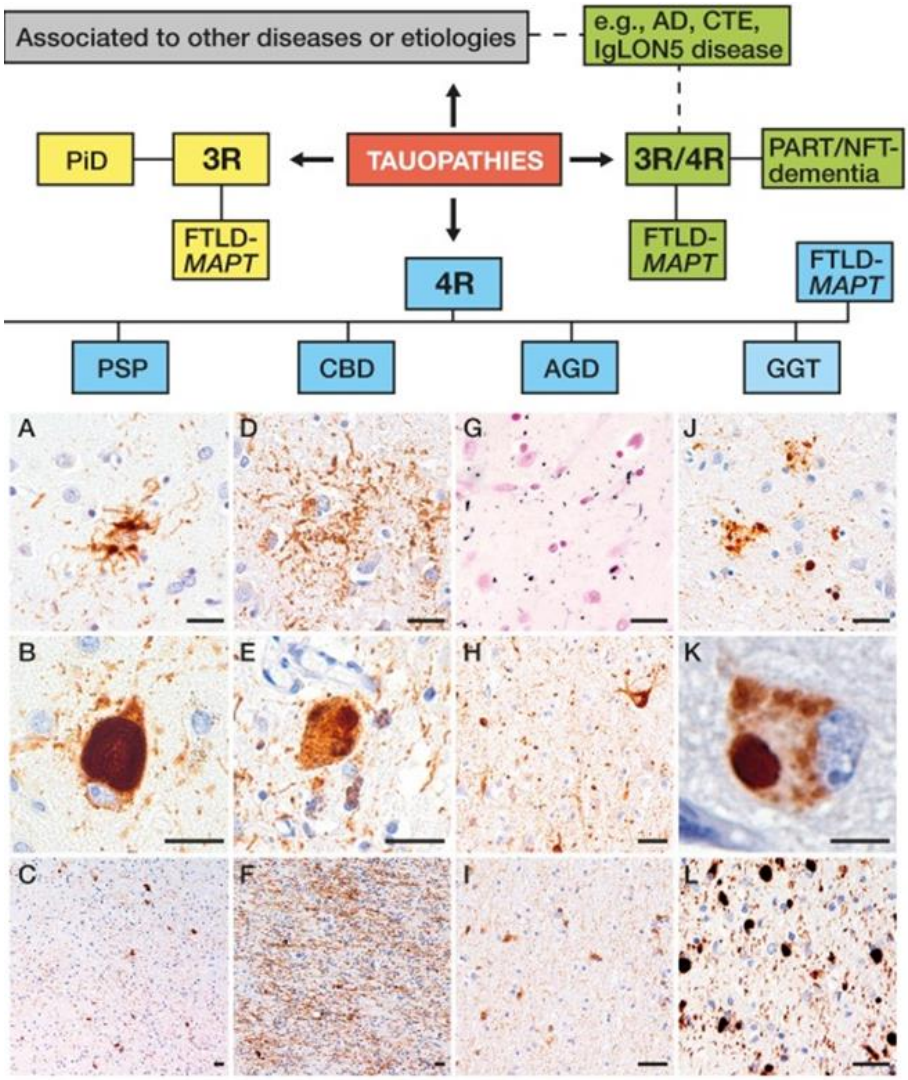

Fig.3: list of main tauopathies affecting human brains, divided by pathological tau isoform, pathological hallmarks and eventual associations with other pathological hallmarks. Tauopathies in the yellow boxes predominantly show 3R tau inclusions, while in the blue boxes they show 4R tau inclusions. Green boxes indicate tauopathies with mixed tau isoforms

as predominant ones. Straight lines indicate primary tauopathies, while dotted lines show secondary ones (Rösler et al, 2019)

Among tau inclusions, neurofibrillary tangles are the most prominent and known ones, but the range of tau inclusions is heterogeneous (Guo et al, 2017). A very important feature they possess is that they can be experimentally transmitted (Clavaguera et al, 2009). Injections of brain homogenates of mice containing tau fibrils in transgenic murine models proved that tau pathology can be seeded in a prion-like manner, as tau aggregates spread in brain regions anatomically connected to the site of the injection even in wild type mice (Clavaguera et al, 2009). The experiment was also replied with the injection in mice of human brain homogenates of various tauopathies-affected patients, and tangles were again spreading from the site of injection towards the closer brain regions (Clavaguera et al, 2013), thus proving that tau is self-propagating, and its seeding competence is manifested in a prion-like manner (Clavaguera et al, 2013). There is extensive research being carried out to discover the features of tau seeding, particularly related to the most seeding-prone sequences in the protein (Stöhr et al, 2017), and some of the most self-aggregating tau sequences seem to be concentrated in the third repeat region of tau (Stöhr et al, 2017); however, tau seeding competence is probably independent of strain isoform compositions and the 3R/4R ratio, but it is instead intrinsic to unique pathological conformations (He et al, 2020).

Among the many pathological conformations of tau coming from tauopathies, the globular 4R tau inclusions coming from GGT have shown the stronger seeding-competence, compared to other brain lysates coming from tauopathies such as AD, CBD and PSP (Chung D.E.C et al, 2019). These inclusions typical of GGT can be found in oligodendrocytes and astrocytes, and they are composed of highly hyperphoshorylated tau inclusions affecting frontal and temporal lobes (Götz et al, 2019). Tau inclusions in astrocytes are the main features of PSP and CBD, which respectively show tufted astrocytes and astrocytic plaques, both composed of twisted fibers of hyperphosphorylated 4R tau (Kovacs, 2015), with pathological tau structures of CBD affecting mostly the forebrain and the ones of PSP affecting mainly the hindbrain (Dickson, 1999). AGD inclusions are also mostly composed of hyperphosphorylated 4R tau (Götz et al, 2019) but they are mainly pre-tangle stage grains in the oligodendrocytes of limbic regions (Tolnay & Clavaguera, 2004). The grains distinctive of AGD can be considered as immature tangles and they have been shown to be less seeding-competent compared to the brain lysates of the other tauopathies (Clavaguera et al, 2013). The only tauopathy which sees a prevalence of 3R tau inclusions is Pick's disease, characterized by Pick's bodies, seeding-competent rounded structures mainly found in frontal and temporal lobes (Saijo et al, 2017).

Tauopathies are therefore variegated and constitute an unsolved puzzle for researchers, as the processes behind the neurodegeneration still escape them. This is even more evident for the

secondary tauopathy, AD, which also sees Aβ playing a role in the disease; however, tau is assuming a more prominent role in present studies, and it will be described in the next section.

1.3.4.1) AD and tau involvement

Alzheimer's disease (AD) can be described as a secondary tauopathy, as it is distinguished not only by fibrillary tau inclusions but also by amyloid plaques (Götz et al, 2019). However, it is important to report that tau pathology is more closely related to cognitive deficits and brain atrophy than Aβ plaques, as proven recently by PET experiments (La Joie et al, 2020). It is also important to report that senile plaque involvement advances in a different way compared to the progression of pathological tau in the brain (Braak & Braak, 1991) and does not correlate to cognitive decline as well as the progression of tau pathology (Bejanin et al, 2017).

1.3.4.1.1) Tau tangles

Tangles are the most common neuronal inclusions in AD, and they are composed of insoluble twisted fibrils of both hyperphosphorylated 3R and 4R Tau (Götz et al, 2019). Punctate Tau aggregates are at first deposited in the cell bodies and dendrites, where they convert into pre-tangles (Götz et al, 2019), a premature form of NFTs which consists of granular cytoplasmic deposits (Kinoshita et al, 1998). These then mature into aforementioned NFTs and then transform into extracellular tangles over decades (Götz et al, 2019). It is curious to report that tangles have been known as one of the main pathological hallmarks of dementia for more than a century (Alzheimer, 1906), but tau was identified more than 80 years later as the protein composing the harmful tangles (Grundke-Iqbal et al, 1986), more than a decade after being identified as a microtubule stabilizer (Weingarten et al, 1975). Tau pathology can propagate in AD patients from one brain region to another in specific patterns (Braak & Braak, 1997); moreover, injection of recombinant tau fibrils has been proved to provoke formation of NFTs propagating from the injection site to connected brain regions in a time dependent manner (Iba et al, 2013). Therefore, even if the process originating the formation of NFTs is still unknown (Umeda et al, 2014), their spreading potential is established and contributes to the worsening of the disease (Braak & Del Tredici, 2016).

The progression of AD is classified into six stages, known as Braak stages, based on the distribution of tangles in the brain (Braak & Braak, 1991). These six stages can be further categorized into three categories, corresponding to the regions in which the tangles are distributed: transentorhinal (I and II), limbic (III and IV) and neocortical (V–VI) stage groups, corresponding to normal cognition, cognitive impairment and dementia (Braak & Braak, 1991). This explains mnemonic decline in early

stages of Alzheimer's disease, as tau accumulates first in the hippocampus and entorhinal cortex, and then spreads to neighbouring regions (Lace et al, 2009). Damages to the hippocampus have been shown to affect memory (Bird & Burgess, 2008), though it is not known exactly how this brain region is related to the mnemonic processes (Bird & Burgess, 2008), but the expression of new memories is correlated to the hippocampal-entorhinal connectivity (Takehara-Nishiuchi, 2014). Tau proposed spreading pattern is thought to start from subcortical areas, continuing to the transentorhinal cortex, limbic system and neocortex (Braak & Del Tredici, 2016). Particularly, limbic system is involved in attention and emotional processes (Li. X et al, 2016), explaining also behavioural problems in AD patients (Mittelman et al, 2006).

1.3.4.1.2) Tau oligomers

Tau fibrils are however not the only pathogenic conformation of tau in AD, as oligomeric tau species contribute significantly to the toxicity of tau in neurodegenerative disorders (Cowan et al, 2010; Gerson et al, 2014) and they have been proven to cause severe neurotoxicity in transgenic tau murine models without the presence of tangles (Oczelik et al, 2016). Tau oligomers have been proven to appear in the brain of AD patients (Lasagna-Reeves et al, 2012). These oligomers have been shown to correlate well with neuronal death in aged mice (Lasagna-Reeves et al, 2011), unlike tangles (Gomez-Isla et al, 1997; Andorfer et al, 2003). Moreover, oligomers are generally thought to initiate disease development in tauopathies (Lasagna-Reeves et al, 2012) and even to appear before the first clinical symptoms (Maeda et al, 2006). Tau oligomers have also shown the capability to spread tau pathology from affected regions of the brain to the unaffected ones (Wu et al, 2013).

Antibodies specifically targeting tau oligomers proved effective in murine models, reducing locomotor and memory deficits without affecting tangles (Castillo-Carranza et al, 2014). Particularly, preventing early tau aggregation from monomers into oligomeric species was shown to be a possibly efficient therapeutic approach (Castillo-Carranza et al 2014). Altogether, these observations lead researchers to believe that tau oligomers are the main responsible behind neuronal dysfunction (Polanco et al, 2018).

Tau oligomers have been shown to anticipate NFT formation (Takashima, 2013). However, their toxicity is relevant also regardless of tangles, as studies have proven that in murine models tau toxicity can be either halted in presence of NFTs (Santacruz et al, 2005), or it can be elicited in absence of tangles (Cowan et al, 2010; Ozcelik et al, 2016). It is of particular interest that the aforementioned severe neurotoxicity caused by tau oligomers, even without the presence of tangles, is obtained in murine models by the interaction between tau fragments and full-length mutant tau, and even by tau fragments interacting with full-length wild type tau (Ozcelik et al, 2016). It is also important to report that this neurotoxicity is reversible, and that the suppression of tau fragment

overexpression in murine models can stop the formation of oligomers and the related severe motor dysfunction (Ozcelik et al, 2016). This brings more attention to the possible role of tau fragmentation for the development of tauopathies, and it will be treated in depth in the following chapters.

1.4) Fragmentation in neurodegenerative diseases

Neurodegenerative disorders are mostly associated with abnormal, pathological protein aggregation processes, thus they have been categorized as proteinopathies (Walker & LeVine, 2012). So far, the mechanisms behind this pathological aggregation processes in proteinopathies have been only partially explained, with many different factors suspected to participate and lead to pathological phenotypes (Walker & Levine, 2012). Proteins which are already biologically prone to aggregation have an increased tendency to cause pathological folding and form pathological aggregates: mutations, such as those commonly found in hereditary forms of proteinopathies, can increase the probability of a protein becoming aggregation-prone (De Baets et al, 2015). In sporadic diseases, instead, the induction of pathological aggregation processes has remained only partly understood. In many neurodegenerative diseases, protein fragments, derived by different cleavage patterns, could play a prominent role in their development and further advancement. There are many examples of small aggregation-prone fragments that can have a pathogenic role for neurodegenerative disorders such as Alzheimer's diseases (AD)(Hardy & Alisop, 2001); familial British and Danish dementia (FBD, FDD) (Ghiso et al, 2001; Rensink et al, 2003); Parkinson's diseases (PD) (Li.W et al, 2005); TDP-43 related disorders (Li.Q et al, 2015) and triplet expansion disorders such as Huntington disease (HD) (Wellington et al, 2002).

The causes behind the fragmentation of the proteins generating the aggregation-prone fragments are generally debated, both in role and mechanism; there are mutations resulting in altered cleavage processes in hereditary variants of neurogenerative diseases, such as presenilin mutations in AD (Campion et al, 1999; Lanoiselée et al, 2017), but in sporadic cases the mechanisms behind protein fragmentation remain to be fully understood. Furthermore, the neurotoxicity of these processes in proteinopathies is still debated, as the contribution of cleaved fragments to the neurodegenerative disorders remains difficult to establish; particularly, it has still to be determined whether the protein fragmentation is one of the steps causing the diseases, or it is just a side effect. However, their presence in these types of disorders is proven, although in different forms.

Fragments participate to the protein aggregates in neurodegenerative disorders in different ways, either alone, or in complex with the full-length protein from which they derive. The first case is represented by amyloid-Dan (ADan) in FDD (Vidal et al, 2000) or amyloid-Bri (ABri) in FBD (Vidal et

al, 1999); the second case is exemplified by α-synucleinopathies (Dufty et al, 2007) or TDP-43 related disorders (Zhang. Y et al, 2009). Tau belongs to the second category (Ozcelik et al, 2016) and undergoes through many different cleavage processes which will be illustrated in the next section.

1.4.1) Tau fragmentation

Tau possesses a variety of cleavage sites which can yield truncated forms of the protein (Martin et al, 2011). Proteolytic processing of tau by a variety of endogenous proteases has been studied for its possible involvement in development of tauopathies (Rissman et al., 2004). Studies on tau aggregation revealed that truncated forms of tau are part of the core of paired helical filaments (PHFs) (Fitzpatrick et al, 2017). Furthermore, fragmented tau has been reported to increase the propensity of tau to convert into fibrils (Abraha et al., 2000); however, tau fragments have also been shown to provoke the formation neurotoxic oligomers, in concert with mutant tau or wild-type tau (Ozcelik et al, 2016). Therefore, tau fragmentation is getting an increasing importance for AD and tauopathies' development.

1.4.1.1) Tau fragmentation in AD

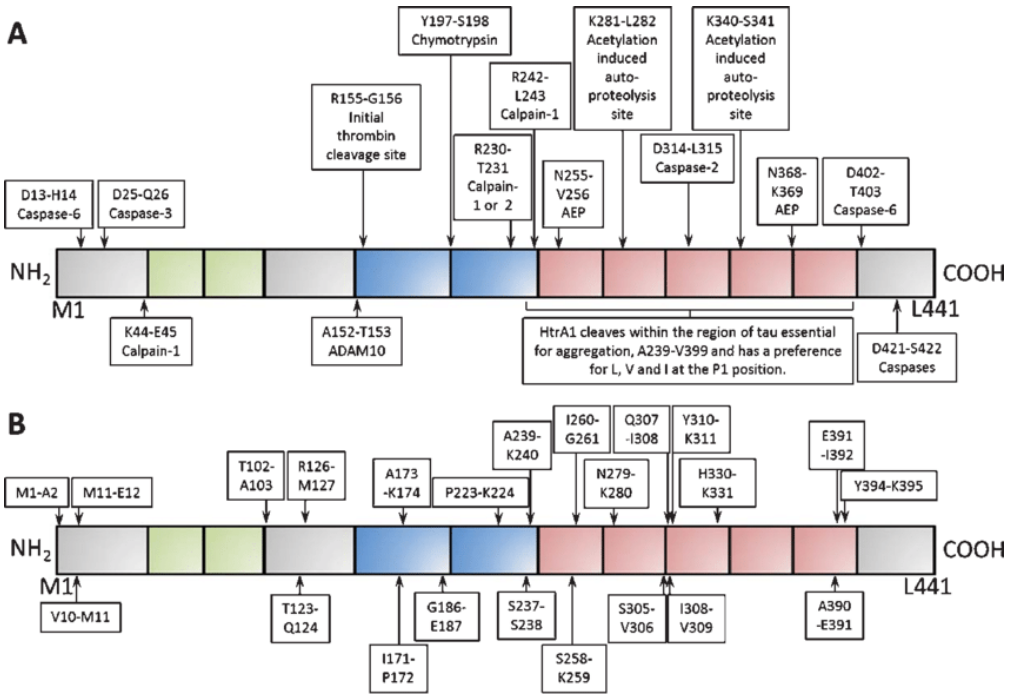

Fig.4: tau cleavage sites. Figure shows all identified truncation sites; figure A shows the sites for which the proteolytic enzyme has been identified, while figure B shows all the sites for which the cleaving enzyme has yet to be identified (Quinn et al, 2018).

Recently, it has been proven that the plaques alone are not sufficient for tau conversion into its pathological form, and tau fragmentation has been identified as the other necessary factor for the plaque-guided conversion of tau into its pathological aggregated form (Li.T et al, 2016). Furthermore, the expression of tau truncated form has been established to induce highly (but reversibly) neurotoxic in presence of full-length tau (Ozcelik et al, 2016), thus strongly implying that tau cleavage could play a crucial role in tauopathies and AD progression, influencing both plaques and NFT pathogenicity.

Tau fragmentation occurs in various and different processes among the various cells and brain tissues, as it can be cleaved in different sites by many proteolytic enzymes such as caspases, calpains, thrombin, cathepsins and PSA (puromycin-sensitive aminopeptidase) (Hanger & Wray, 2010). Cleavage sites were predicted at both C-terminal and N-terminal domains of tau, and the truncation was catalyzed mainly by caspases (Delobel et al, 2008). C-terminal proteolytic processing of tau in human AD brains was first observed at Glu391, with no enzyme being clearly linked to its cleavage (Novak et al, 1993). This tau fragment has been studied in the mice models Tau E391 and it is present in both NFT and PHF, while also driving pathological changes in pre-tangle stages (McMillan et al, 2011), and more generally it is linked to clinical dementia (Basurto-Islas et al, 2008). The recently obtained cryo-EM structural characterization of PHF in AD brains also confirmed that tau truncated at Glu391 is part of the structural core of these pathological hallmarks (Fitzpatrick et al, 2017). Also of note for tau fragmentation pathogenicity, this same study has confirmed that PHF and straight filaments (SF) are mainly composed by tau fragments corresponding to repeat domains 3 and 4 (Fitzpatrick et al, 2017), and that same tau 4R domain has been shown to induce Aβ plaque-guided conversion of tau in seeding-prone form (Li.T et al, 2016).

Nowadays, one of the most studied cleavage processes is the one occurring at Asp421 (D421), associated with caspases 3 (Rissman et al, 2004) and 6 (Guo et al, 2004). This tau fragment has been studied in the mouse model Tau 62/48 (Ozcelik et al, 2016). Many roles in neurodegenerative processes have been proposed for tau cleaved at D421: it is part of NFTs (Zhang et al, 2014), and it also induces severe, but reversible neurotoxicity in AD mouse models when co-expressed with full-length tau (Ozcelik et al, 2016). Tau cleaved at D421 is not an exclusive pathological feature of AD, but it is also found in other tauopathies, such as Pick's disease (Mondragon-Rodriguez et al, 2008). More generally, when transfected *in vitro*, this tau fragment has been shown to induce cell death significantly more than full length tau (Chung C.W. et al, 2001), while *in vivo* accumulation of it correlates with AD and disease progression in mouse models of tauopathies and non-AD patients, e.g in FTDP-17T patients (Basurto-Islas et al, 2008).

N-terminal cleavage of tau is less clarified, at least *in vivo*; *in vitro*, caspase 6 cleaves tau at Asp13 (D13) site (Chung C.W. et al, 2001), which is likely to be physiologically significant for AD progression, as N-terminal tau epitopes get increasingly absent in NFT development in the early

stages of the disease (Horowitz et al, 2004) and cleaved forms of tau at D13 site are increasingly present in more severe stages (Ghosal et al, 2002). It has also been proposed that tau forms cleaved at D13 temporally correlate with the appearance of tau species cleaved at D421 (Horowitz et al, 2004). It has to be said that, although caspase cleavage sites are not fully explained, their activity and the processing of tau leading to its truncated forms clearly precede NFT formation in mouse model Tg4510 (De Calignon et al, 2012). This has led to theories that greatly emphasize the role of tau truncation by caspases over tangle formation, with truncated tau (particularly at D421) recruiting full-length tau to misfold and successively participate to aggregates (De Calignon et al, 2012). Recently, it has been shown that the major tau species in human cerebrospinal fluid (CSF), and more generally in extracellular fluids, are N-terminal truncated forms, mainly cleaved at the end of the mid-domain between residues 222 and 225 (Sato et al, 2018). In the same study, tau production has been directly linked to progression of amyloid disease (in form of amyloid plaques burden) in AD brains, so it is likely that N-terminal truncated tau forms elicit an important role in AD progression *in vivo* too (Sato et al, 2018).

Altogether, tau fragmentation remains a possible explanation for many pathological developments of AD by probably being involved in the formation of filamentous tau (Wang. Y et al, 2009) and, more generally, leading to a possible toxic gain of function (Guo et al, 2017). There are still many unexplained points on how tau fragmentation participates to AD pathological mechanisms, but the evidence is generally growing towards a more prominent role of tau fragments in contributing to neurotoxicity. Further studies will be required to clarify more in detail what tau cleavage process add to the development and progression of neurodegenerative disorders.

Furthermore, it can also be crucial for AD diagnosis, as CSF tau is among the most reliable biomarkers for AD diagnosis (Simonsen et al, 2017) and tau fragments constitute the majority of CSF tau conformations (Sato et al, 2018), with some researchers going as far as telling that full-length tau is practically absent in the CSF of AD patients (Meredith Jr et al, 2013). AD diagnosis and the related importance of CSF biomarkers will be analyzed more in depth in the following chapters.

1.5) AD diagnosis

Since the first histological observation of the disease in 1906, the diagnosis of AD has remained controversial as, even nowadays, it can be established with 100 % certainty only with post-mortem analysis of the brain, thanks to the identification of neurofibrillary tangles and amyloid plaques (DeTure & Dickson, 2019). Even worse, due to clinical similarities with other forms of dementia, AD

can be wrongly suspected in life or, to the contrary, escape detection of clinical experts (Reed et al, 2007). A first official, reliable and organized list of criteria for a likely AD diagnosis was published in 1984 (McKhann et al, 1984) by the Alzheimer's Association, and it has been revised several times recently (Dubois et al, 2007; McKhann et al, 2011).

Altogether, AD diagnosis pre-mortem has been established with a good certainty, and it has been officially established according to cognitive tests, brain imaging, and biomarkers (McKhann et al, 2011). A battery of cognitive tests has been devised over the years. One of most established one to test cognitive impairment is the Mini-Mental State Examination (MMSE) (Tsoi et al, 2015); however, Mini-Cog test and the ACE-R have also a good reliability among the various dementia tests (Tsoi et al, 2015). It is important to mention also the Montreal Cognitive Assessment (MOCA) test, which has extensively been compared to the MMSE and seems to screen better for cognitive impairment (Ciesielska et al, 2016), and the Clinical Dementia Rating (CDR) scale, which is the most used numerical scale assessing dementia (Rosenberg et al, 2013).

Imaging techniques applied to diagnose AD are mainly Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) (Simonsen et al, 2017). MRI detects brain atrophy, typical morphological change of AD patients (Long et al, 2017) which strongly correlates with Braak stages (Long et al, 2017) and it is a useful tool also for early diagnosis of AD (Long et al, 2017). PET instead can be useful to detect amyloid plaques *in vivo*, but it does not have complete precision for a diagnosis of AD because patients can also have a high amyloid burden without being affected by AD (Ossenkoppele et al, 2015). New PET tracers also gave the opportunity to detect tau pathology, and it is also strongly correlated with AD pathology, though not perfectly (Ossenkoppele et al, 2016).

Finally, data from the clinical research increasingly support CSF biomarkers Aβ42, total tau (t-tau), and phosphorylated tau (p-tau) as biomarkers strongly related to AD pathological development, and they contribute with diagnostically relevant information (Blennow & Zetterberg, 2018). Other biomarkers, such as plasma tau and neurofilament light protein (NFL) have been also proposed to be helpful in AD diagnosis (Olsson et al, 2016), but the reliability of CSF biomarkers is much more established (Olsson et al, 2016), and the precision of the diagnosis based on the combination of the three core biomarkers is extremely high, detecting almost all the really affected patients analysed post-mortem (Shaw et al, 2009). It is known that CSF tau levels significantly increase in AD patients, while CSF Aβ levels are significantly lower (Blennow et al, 2001).

The importance of biomarkers in AD diagnosis is debated, as sometimes ambiguous or indeterminate results can lead to confusion (McKhann et al, 2011). Particularly, it is important to report that AD biomarkers are more suited for diagnosis rather than screening (Frisoni et al, 2017), thus not helping the patients before the first insurgence of clinical symptoms. This is strengthened by the fact that Aβ deposits seem to appear much earlier than the first clinical symptoms (Selkoe & Hardy, 2016), and to lead progressively to neurodegeneration (Selkoe & Hardy, 2016). However, it

has been difficult to establish biomarkers in order to follow the hypothesized biochemical events that happen before the clinical manifestation of AD, particularly distinguishing aging-related mild cognitive impairment (MCI) from prodromal AD (Van Giau et al, 2019).

Nonetheless, the reliability of biomarkers for AD diagnosis is nowadays increasing, with many clinical studies showing the levels of the aforementioned biomarkers being strongly related to the progression of the disease (Cohen et al, 2019; Scholl et al, 2018), with researchers going so far to propose even blood-tests related to said biomarkers, for the future (Zetterberg & Burnham, 2019). Altogether, evidence supports the claim that biomarkers will be increasingly important for AD diagnosis, even though more established guidelines have to be set.

1.5.1) CSF biomarkers: Aβ

As mentioned before, among the most important biomarkers for AD diagnosis there is Aβ42 CSF levels (Blennow & Zetterberg, 2018). Studies on the reliability of the biomarkers also tried to establish Aβ42 levels in plasma as a useful biomarker for AD diagnosis, but negative results stopped the attempt (Rival et al, 2009). As said before, CSF Aβ levels strongly decrease in AD patients compared to controls (Blennow et al, 2001), and particularly it is the earliest biomarker to change during the clinical course of the disease (Blennow & Zetterberg, 2018). The transmission of Aβ to the CSF compartment has been long established (Seubert et al, 1992), but the logic for CSF Aβ being significantly lower in AD patients escaped researchers at first (Andreasen et al, 1999). The pathophysiological basis for the reduction of CSF Aβ42 in AD was hypothesized to rely behind the fact that hydrophobic peptide aggregates are sequestered in plaques, with lower amounts remaining to be secreted to the extracellular space and the CSF, resulting in lower CSF levels of Aβ42 (Andreasen et al, 1999). Post-mortem analysis of AD patients confirmed that a high burden of amyloid plaques was correlated with a low amount of Aβ42 in the CSF compartment (Strozyk et al, 2003), and the inverse proportion between Aβ42 in the CSF and Aβ plaques was also later confirmed as well by brain imaging techniques (Fagan et al, 2006).

Among the other isoforms of Aβ, the one which is 40 residues long (Aβ40) is the most abundant, with a concentration 10 times higher than Aβ42 (Olsson et al, 2016); even though it is so concentrated in the CSF, it shows no significant changes between controls and AD patients, thus being useless for the diagnosis (Olsson et al, 2016). However, it seems that the Aβ42/Aβ40 ratio could help the diagnosis of the disease in an even more relevant way than the simple CSF Aβ42 levels (Lewczuk et al, 2017). The reason behind it is still unclear, and the ratio is still not considered officially (Dubois et al, 2007), but it seems that Aβ40 high load differentiates between patients having low CSF Aβ42 levels due to simple low amyloid production and patients that have low CSF Aβ42 levels because of the plaques sequestering hydrophobic aggregates (Blennow & Zetterberg, 2018).

As mentioned before, Aβ is able to propagate amyloid aggregates through the brain (Langer et al, 2011). Seeding capability of Aβ in the CSF compartment were also studied and seem to be weak, if non-existent (Fritschi et al, 2014; Skachokova et al, 2015). In a study comparing the seeding potential of CSF derived from AD patients to the diluted brain extracts from AD patients, the latter proved to spread amyloid deposition through the brain of APP transgenic mice, while the CSF-derived Aβ was unable to do it (Fritschi et al, 2014). Similarly, CSF derived from those APP transgenic mice failed to provoke amyloid spreading in young pre-plaques stage APP transgenic mice (Fritschi et al, 2014), even after long period of concentration (Skachokova et al, 2015). The reason behind this lack of seeding capability was hypothesized to be the lack of N-terminally truncated amyloid-β species and the presence of smaller amyloid-β-positive particles (Fritschi et al, 2014). However, even with the lack of seeding capabilities and the possible improvements derived from the reliability of the Aβ42/Aβ40 ratio, CSF Aβ remains a good diagnostic tool in concert with the other CSF biomarkers, particularly CSF tau (Olsson et al, 2016).

1.5.2) CSF biomarkers: tau

CSF tau levels are currently being used for clinical diagnosis of AD in conjunction with cognitive tests, brain imaging and CSF amyloid-β level measurements (Simonsen et al, 2017), with said levels significantly increased in AD patients compared to controls (Blennow et al, 2001). Pathologically modified tau forms are present in the extracellular space and can transfer between cells in the brain (Guo & Lee, 2014; Wu et al., 2013). Phosphorylated tau can be actively secreted via exosomal release, and reaches the CSF compartment (Saman et al, 2012). Ante mortem cerebrospinal fluid tau levels correlate with postmortem tau pathology in frontotemporal lobar degeneration (Irwin et al, 2017); more in detail, increased tau levels in the CSF correlate best with cognitive decline in AD patients (Wallin et al, 2006).

Experiments *in vitro* hinted at a possible seeding capability of CSF-derived tau *in vitro*: particularly, *in vitro* seeding activity of tau CSF has also been reported to induce high molecular tau aggregation in cell cultures (Takeda et al, 2016), with ventricular CSF samples collected post-mortem at autopsies of AD patients inducing tau aggregation in HEK293 cells expressing a P301S tau repeat domain (Takeda et a,. 2016). Furthermore, recent *in vitro* seeding assays demonstrated the ability of CSF tau to induce conformation changes in *in vitro* tau substrates (Saijo et al, 2017). More in detail, CSF tau derived from Pick's disease patients has been found to induce real-time quacking induced conversion (RT-QuIC) of 3R tau fragment typical of Pick's disease (Saijo et al, 2017). However, to our knowledge, no information on seeding properties of CSF tau *in vivo* are available until now.

The detection of CSF tau levels in possible AD patients is one of the most reliable biomarker measurements in AD diagnosis nowadays, even compared with other biomarkers (Olsson et al, 2016), but it lacks high specificity and is unable to help prognosis of the disease at early or even presymptomatic disease stages (Olsson et al 2016). Both total and phosphorylated tau are increased in the CSF of AD patients at later stages and can help predicting the scope of pathologic processes occurring in AD (Bateman et al., 2012). However, it is not clear whether higher CSF tau levels result from dying neurons and the related release of tau from the cells, from tau increased synthesis or tau decreased clearance (Sato et al, 2018). What is known is that CSF tau is mainly composed of tau fragments (Sato et al, 2018), and there is a strong likelihood that full-length tau is not capable of reaching CSF compartment, thus being in no part present in CSF (Meredith Jr et al, 2013). Particularly, tau species in CSF seems to be mostly composed by 20-40 kDa N-terminal fragments (Meredith Jr et al, 2013). As the attention on tau oligomers as targets for therapies against tauopathies is getting higher nowadays (Polanco et al, 2018), and tau oligomers are thought to appear even before the first clinical symptoms of AD (Maeda et al, 2006), it is important to report that tau oligomeric species seem to be part of CSF-derived tau (Sengupta et al, 2017). Particularly, tau oligomers increase in AD patients' CSF and aggregate at different molecular weights (Sengupta et al, 2017). However, more precise data *in vivo* on said fragments and their structure needs to be acquired.

Altogether, CSF-derived tau likely remains the most reliable biomarkers for AD diagnosis, and strongly advocates for the importance of fragmentation related to AD pathological mechanisms. However, the establishment of its seeding properties, the knowledge on how tau reaches the CSF compartment, the specificity of tau fragments in it and the structure of CSF tau remain mostly unknown to researchers, and can constitute an important improvement in the diagnosis of AD and in the understanding of the pathological mechanisms behind the disease.

2) Material and methods

2.1) Mice

Homozygous human P301S mutant tau transgenic mice (P301S tau mice) (Allen et al, 2002) were used as host mice for tau seeding experiments with human CSF. Transgenic homozygous mice expressing human ALZ17 mutant tau (ALZ17 mice) (Frank et al, 2008) were used as host mice for tau seeding experiments with brain stem homogenates from P301SxTAU62 mice. P301SxTAU62 mice were obtained by interbreeding P301S mice and TAU62 mice (Ozcelik et al, 2016). TAU62 mice express a human tau fragment from amino acid 151-421 ($\Delta tau_{151\text{-}421}$) under doxycycline administration (Ozcelik et al, 2016). P301SxTAU62 mice were used for the investigation of potential long-term effects of neurotoxicity caused by tau oligomers along with their heterozygous littermates (P301Shet) which were never fed with doxycycline and therefore never expressed $\Delta tau_{151\text{-}421}$. Non-transgenic C57BL/6J (BL6 mice) were used as control mice for behavioural tests. The mice were housed with a 12-hours light/dark cycle and permanent access to food and water. Animal experiments were approved by the official local Committee for Animal Care and Animal Use of the Canton of Basel (Licenses Nr. BS 2364 and 2471).

2.2) CSF collection and patients' characteristics

In order to obtain large volumes of CSF from AD and control patients, allowing subsequent concentration, CSF was collected within a clinical trial set up in collaboration with the Memory Clinic, University Center for Medicine of Aging, Basel. The trial was approved by the local Ethics Commission of the Canton of Basel, and recruitment was limited to patients with a Mini Mental State (MMS) Score (Folstein et al, 1975) >19/30. All patients examined for memory disturbances and potential neurodegenerative disorders at the Memory Clinic were consecutively allowed to enter the study.

Up to 15ml of CSF per patient were collected by lumbar puncture from a total of 23 subjects after obtaining written informed consent of the patient and a care-giver. Freshly collected CSF was spun at 3000 rpm for 30 minutes to remove cell debris. The supernatants were collected and frozen at −80° C. Clinical diagnosis was based on multimodal examinations including neuropsychological assessments, brain MRI imaging, CSF tau and Aβ level measurements. Brain perfusion SPECT imaging data was available on a subset of the patients.

2.3) CSF processing

In order to obtain reasonable tau levels in small volumes that could be injected into mouse brains, CSF samples were concentrated (Skachokova et al, 2015). In brief, 10 ml CSF per patient, was lyophilized (−80° C, 0.01 mbar vacuum pressure), reconstituted in sterile H_2O and dialyzed (using Float-A-Lyzer G2, 1kD, Spectrum Labs) to reduce the salt load, re-lyophilized, and finally reconstituted in about 10 μl H_2O. The final concentration of tau was measured by ELISA.

2.4) ELISA

CSF levels of tau, phospho-tau and Aβ, were measured by commercial kits “Innotest hTAU AG”, “Innotest PHOSPHO-TAU”, and “Innotest β-Amyloid” (1-42) according to the manufacturer’s guidelines (Innogenetics/Fujirebio Europe N.V., Belgium) (Sunderland et al, 2003)

2.5) Stereotaxic surgery

For surgery, 3-months-old ALZ17 and P301S mice were anesthetized with a mixture of ketamine (10 mg/kg) and xylazine (20 mg/kg) and placed on a heating pad to maintain body temperature. Mice were stereotactically injected into the right hippocampus (A/P, −2.5 mm from bregma; L, − 2.0 mm; D/V, −1.8 mm) using a Hamilton syringe. A unilateral stereotaxic injection of 5 µl concentrated CSF was performed on P301S mice, while brainstem homogenates of 6-months-old paralyzed homozygous P301S mice and 3-weeks-old paralyzed P301SxTAU62^{on} mice were prepared for inoculation in ALZ17 mice. Brainstems were weighted and diluted 1:10 in PBS for seeding. After dilution, samples were homogenized using an Ultraturrax T8 (IKA Labortechnik, Staufen im Breisgau, Germany) and sonicated briefly (Bandelin SONOPULS, Bandelin, Berlin, Germany; 90% power, 10% cycle, 10 sec pulses). Homogenates were then centrifuged at 4000 g for 20 minutes at 4 °C, and aliquots of the supernatant were stored at -70 °C for later usage. During inoculation, each mouse received 5 µl of brainstem homogenate at a speed of 1.25 µl/min. Following injection, the needle was kept in place for additional 3 minutes before withdrawal. The surgical area was cleaned with sterile saline and the incision sutured. Mice were monitored until recovery from anaesthesia, post-interventional analgesia was administered, and animals were checked regularly following surgery. After 20 months of incubation, the seeded mice were sacrificed.

2.6) Behavioral tests

Motor behavior, including gait ataxia, tremor, and hindlimb reflexes was assessed. Quantitative motor testing was performed by the grid test in which mice were placed on a vertical mesh grid and the latency to fall off the grid was recorded for 3 min. Motor coordination and balance were assessed

using the Panlab Harvard Rotarod (Harvard Apparaturs, Holliston, USA). The rotarod starts at a speed of 4 rpm and accelerates by 1 rpm every 3 sec. In both, grid test and rotarod assay, mice were tested for 3 consecutive days with 3 trials per day, with minimum rest intervals of 5 min, and the mean latency to fall was documented. Results were obtained by averaging the daily means of 3 consecutive days.

2.7) Sacrificing of mice and tissue preparation

Mice were deeply anaesthetized with pentobarbital (150 mg/kg) and transcardially perfused with 20 ml cold PBS. The brains were dissected and post-fixed overnight in 4% paraformaldehyde (PFA). Following paraffin embedding, 4 µm coronal sections were cut from the brain tissues of seeded mice, while 4 µm sagittal sections were cut from the brain tissues of the mice used for the behavioural tests. For Western blots and seeding material, the mice were only perfused with PBS, afterwards mouse brains were dissected and frozen in liquid nitrogen or on dry ice.

2.8) Hematoxylin and Eosin staining

Paraffin tissue sections were deparaffinized in xylene for 30 min and rehydrated through a series of EtOH/water solutions from 100 %, 96 %, 80 %, and 70 %, followed by dH2O, submerging the sections for 2 min each step. The rehydrated sections were stained with Harris' hematoxylin (J.T. Baker, Biosystems Switzerland AG, Muttenz, CH), for 0.5 min, washed twice in dH2O and differentiated in 0.2 % HCl diluted in 70 % ethanol. Afterwards the Sections were stained in 1 % Erythrosine B (RAL DIAGNOSTICS, Biosystems Switzerland AG, Muttenz, CH), a stain closely resembling eosin Y, washed in dH2O and dehydrated through an ascending series of EtOH water solutions, form 70 %, 80 %, 100 %, followed by xylene. Subsequently the sections were mounted using Pertex (Histolab Products AB, Biosystems Switzerland AG, Muttenz, CH).

2.9) Gallyas staining

Tissue sections were silver-impregnated following the method of Gallyas-Braak to visualize filamentous tau pathology (Gallyas, 1971; Braak et al, 1988). Paraffin tissue sections were deparaffinized and rehydrated as described above. Afterwards the sections were incubated in 3 % periodic acid (w/v) for 30 minutes, rinsed in dH2O and incubated in alkaline silver solution (1 M NaOH, 0.6 M KI, 2 mM $AgNO_3$) for 10 minutes. Subsequently the sections were developed by mixing developer solution A (0.5 M Na_2CO3), solution B (24 mM NH_4NO_3, 12 mM AgNO3, 3.5 mM $H4[Si(W_3O10)_4]$), and solution C (24 mM NH_4NO_3, 12 mM AgNO3, 3.5 mM $H_4[Si(W_3O_{10})_4]$, 3 %

formaldehyde) in a ratio of 30:9:21 immediately before use, for ca. 25 minutes, simultaneous to monitoring of the reaction process.

Solution	Composition	Concentration	Supplier
A	Sodium Carbonate Anhydride	0.5 M	Merck, Darmstadt, Germany
B	Ammonium nitrate	24 mM	Merck, Darmstadt, Germany
	Silver Nitrate	12 mM	Merck, Darmstadt, Germany
	Tungstosilicic Acid Hydrate	3.5 mM	Sigma-Aldrich, St. Louis, USA
C	Ammonium nitrate	24 mM	Merck, Darmstadt, Germany
	Silver Nitrate	12 mM	Merck, Darmstadt, Germany
	Tungstosilicic Acid Hydrate	3.5 mM	Sigma-Aldrich, St. Louis, USA
	Formaldehyde	3 %	Merck, Darmstadt, Germany

The developing was stopped via incubation in 0.5 % acetic acid for 30 minutes and fixed via incubation in 5 % $Na_2S_2O_3$ for 3 minutes. The silver stained sections were rinsed in dH_2O, counterstained using hematoxylin and Eosin, differentiated, dehydrated, and embedded as described on the previous page.

2.10) Immunohistochemistry

For immunohistochemistry, the following anti-tau antibodies were used: AT8 (1:1000, Pierce Biotechnology, Waltham, USA), AT100 (1:1000, Pierce Biotechnology, Waltham, USA), TauC3 (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA). Secondary antibodies were from Vector Laboratories, Burlingame, USA (Vectastain ABC kit). 4X, 10X, 20X and 40X magnified pictures of the stained slides were taken with an Olympus BX43 Upright Microscope (Life Sciences Solutions, Chicago, USA).

For immunohistochemistry, paraffin tissue sections were deparaffinized and rehydrated as described for Hematoxylin and Eosin staining. For epitope retrieval the slides were placed in ProTaqs citrate buffer with pH 6 (BIOCYC, Potsdam, Germany) and microwaved for 30 minutes at 90 °C. The secondary antibodies and peroxidase (HRP) substrate were from Vector Laboratories, Burlingame, USA (Vectastain ABC kit) and used according to manufacturer's protocol.

2.11) Western blots

For the western blots comparing total and soluble tau in 16-months-old P301S heterozygous (P301Shet) and P301SxTAU62$^{on\text{-}off}$ mice, brains were homogenized in cold extraction buffer 20 % (w/v) (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 5 mM NaPyrophosphate, 10 mM B Glycerophosphate, 30 mM NaFluoride, 10 mM NaVanadate, with addition of 100 ul/10 ml PMSF of 0.1 M just before use, and 1 Pierce protease and phosphatase inhibitor mini tablet, EDTA-free, Pierce Biotechnology, Waltham, USA) using a Polytron Homogenizer (Thomas Scientific). An aliquot of the resulting homogenate was collected as total tau. Subsequently, samples were centrifuged at 80000 g for 15 min using an ultracentrifuge (Beckman Coulter, Brea, USA; OptimaTM L-70K Ultracentrifuge), and an aliquot of the supernatant was collected as soluble tau.

For the western blot comparing total tau in 3-months-old mice, one half of the mouse brain was dissected into forebrain and brainstem, and frozen in liquid nitrogen or on dry ice. Brainstems were weighted and diluted 1:10 in TBS-Complete. Subsequently, samples were homogenized using an Ultraturrax T8 (IKA Labortechnik, Staufen im Breisgau, Germany) and briefly sonicated (Bandelin SONOPULS, Bandelin, Berlin, Germany; 90% power, 10% cycle, 10 sec pulses). Homogenates were then centrifuged at 4000 g for 20 minutes at 4 °C, and aliquots of the supernatant stored at -70 °C for later usage.

Western blots were then performed under non-reducing conditions using appropriate amounts of protein, 4X NuPAGE LDS sample buffer, and deionized water. 10X NuPAGE reducing agent was used to obtain reducing conditions. Following appropriate preparation, samples were loaded onto a 7% NuPAGE® Tris-acetate gel. After the removal of gels from the cassette and activation of PVDF membrane (Amersham Biosciences, Amersham, UK) samples were transferred on the PVDF membrane using the XCell IITM Blot Module. Unspecific binding epitopes were blocked with 5% non-fat milk in PBS-Tween, followed by incubation with primary antibody over night at 4°C on a shaker. After washing with PBS-Tween, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or -rabbit secondary antibody at room temperature. Then, the membrane was washed again in PBS-Tween and detected by electrochemiluminescence (ECL) (GE Healthcare, Little Chalfont, UK). The anti-tau antibody used for the western blots was HT7 (1:1000, Pierce Biotechnology, Waltham, USA).

Antibody	Target	Dilution	Supplier
HT7	Tau aa 159-163	WB 1:1000 IHC 1:800	Thermo Scientific, Waltham USA
AT8	Tau pSer202/Thr205	WB 1:1000 IHC 1:800	Thermo Scientific, Waltham USA
AT100	Tau pThr212/Ser214	WB 1:1000 IHC 1:1000	Thermo Scientific, Waltham USA
GAPDH (6C5)	GAPDH	WB 1:1000	Santa Cruz Biotechnology, Santa Cruz, USA

2.12) Dot blots

For dot blots, serum of 3-months-old P301Shet and P301SxTAU62$^{on\text{-}off}$ mice was separated from the clot by centrifuging the samples at 1000 rpm for 15 minutes at 4 °C, with the remaining supernatant aliquoted and stored at -20 °C, for later usage. Then, a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, USA) was divided in a grid to allow later incubations with 3-months-old P301Shet and P301SxTAU62$^{on\text{-}off}$ sera and HT7 antibody as positive control at 1:1000 dilutions. 0.2 mg/ml of 2N4R wild-type tau monomers were applied on a 4x3 grid. Next, unspecific binding epitopes were blocked with 5% BSA in PBS-Tween, followed by a 30-minutes-long incubation at room temperature with previously extracted sera from the mice and HT7 antibody as positive control. After washing with PBS-Tween, the membrane was incubated with HRP-conjugated anti-mouse secondary antibody at room temperature, washed again in PBS-Tween, and detected by ECL (GE Healthcare, Little Chalfont, UK).

2.13) Statistical analysis

Quantifications were conducted on 4X, 10X, or 20X magnified images, taken with an Olympus BX43 Upright Microscope (Life Science Solutions, Chicago, USA). Bregma levels were determined by visual comparison with the Mouse Brain Atlas (Franklin & Paxinos, 2008). The quantifications and the measurements of the area of the respective anatomical regions were performed by the Cell counter plugin in ImageJ (imagej.net). Brain images that were used and modified are from the Mouse Brain Atlas (Franklin & Paxinos, 2008).

In order to estimate the effects of AD CSF tau seeds versus control seeds, mean values of counts/area ratios were calculated from the magnified images of AT8, AT100, and Gallyas positive neurons and dot-like structures, and p-values were obtained by t-tests. Three sections per animal were analyzed. P-values are interpreted exploratory and are not adjusted for multiple comparisons. A p-value < 0.05 was considered as significant.

To evaluate behavioral test results statistically, one-way analysis of variance (ANOVA) followed by post-hoc Student's t-tests and Bonferroni correction for multiple comparisons were applied. P-values < 0.05 were considered significant.

To estimate soluble tau and total tau expression from western blots of 3-months-old and 16-months-old mice, the protein bands were normalized to GAPDH protein standard, and quantified using ImageJ software; generated mean ratio values were compared by Student's t-test.

To determine the effect of early neurotoxic stress in aged mice, AT8 and Gallyas-positive neurons were semi-quantitatively assessed in brainstem regions. Three sections per animal were analyzed. The average count/area ratios were obtained. P-values calculated by Student's t-tests were interpreted exploratory and not adjusted for multiple comparisons; P-values < 0.05 were considered significant.

Box plots were generated with R software. The lower and upper hinges correspond to the first and third quartiles (25th and 75th percentiles). The upper whisker extends from the hinge to the largest value no further than 1.5 times IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles). The lower whisker extends from the hinge to the smallest value at most 1.5 times IQR of the hinge. Data beyond the end of the whiskers are called "outlying" points and are plotted individually.

2.14) Genotyping

Compound	**Concentration**	**Supplier**
Tris	100 mM (pH 8.5)	Biomol, Hamburg, Germany
EDTA	5 mM	Fluka BioChemika, St. Gallen, Switzerland
NaCl	200 mM	Merck, Darmstadt, Germany
SDS	0.2%	Bio-Rad, Hercules, USA

For sample digestion the thermomixer was set at 55 °C and 750 µl of tense buffer and 3,75 µl of Proteinase K were added to each sample. The samples were incubated overnight (or at least for 4 hours) at 55 °C and 600 rpm.

For DNA Isolation the samples were centrifuged at 4 °C and 14000 g for 5 minutes. A new tube for each sample was labelled and filled with 750 µl of isopropanol. Subsequently the supernatant was taken from each sample and added to the respective isopropanol tube. The tubes were then centrifuged at 4 °C and 14000 g for 10 minutes. Afterwards the supernatant was discharged and 200

µl of 75% ethanol were added to each pallet. Next the samples were centrifuged at 4 °C and 14000 g for 10 minutes. Afterwards the supernatant was discharged and 250 µl of sterile water were added to each tube. Subsequently the samples were incubated for 1 hour at 50 °C without shaking.

For PCR 2.5 µl of each DNA sample were added to a PCR tube containing 22.5 µl of PCR mix (see table below). The primers and PCR programs for the Δtau allele and 4R tau allele (P301S tau detection) are sown below.

For Gel electrophoresis 3 gels were prepared by mixing 300 ml of TAE-buffer with 4.5g of agarose in an Erlenmeyer Flask and heating the mixture in the microwave at maximum temp. for 4 minutes. Afterwards the mixture was cooled down for a couple minutes and 15 µl of cyber save were added. 3 combs were placed in the gel chamber and the chamber was filled with the liquid gel. After solidifying the resulting gel was cut into 3 gels. For immediate use the gels were put in an electrophoresis chamber filled with TAE-buffer, 5 µl of loading dye were added to each sample and the samples were loaded to the gel. The gel was run in TAE-buffer for 1 hour at 150V (90 minutes at 150V if the gel is run in TBE-buffer). For storage the gels were stored in TAE-buffer at 4 °C in the fridge.

PCR mix

Compounds	**Supplier**
12,5 µl Qiagen PCR Master mix	Qiagen, Germany
4 µl sterile water	Qiagen, Germany
2,5 µl forward primer	Thermo Scientific, Waltham, USA
2,5 µl reverse primer	Thermo Scientific, Waltham, USA
1 µl DMSO (add last)	Merck, Darmstadt, Germany

Primers

Primer names	**Forward primer**	**Reverse Primer**
TauF151 (for Δtau detection)	GTG GAT CTC AAG CCC TCA AG	GGC GAC TTG GGT GGA GTA
P301S (for 4R tau detection)	GGT TTT TGC TGG AAT CCT GG	GGA GTT CGA AGT GAT GGA AG

PCR programs

TauF151 program	**P301S program**
Step 1: 95°C for 2 min	Step 1: 95°C for 4 min
Step 2: 95°C for 1 min	Step 2: 95°C for 1 min
Step 3: 60°C for 1 min	Step 3: 60°C for 1 min
Step 4: 72°C for 2 min	Step 4: 72°C for 3 min
Step 5: 72°C for 10 min (final extension)	Step 5: 72°C for 10 min (final extension)
Steps 2-4 were repeated for 30 cycles	Steps 2-4 were repeated for 30 cycles

3)Results

3.1) First published manuscript

Cerebrospinal fluid from Alzheimer's disease patients promotes tau aggregation in transgenic mice

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Abstract

Tau is a microtubule stabilizing protein that forms aggregates in Alzheimer's disease (AD). Tau derived from AD patients' brains induces tau aggregation in a prion-like manner when injected into susceptible mouse models.

Here we investigated whether cerebrospinal fluid (CSF) collected from patients diagnosed with probable AD or mild cognitive impairment (MCI) likely due to AD harbors a prion-like tau seeding potential. CSF was injected intrahippocampally into young P301S tau transgenic mice. CSF obtained from AD or MCI patients increased hippocampal tau hyperphosphorylation and tau tangle formation in these mice at 4 months post-seeding. Tau pathology was also accentuated in the contralateral hippocampus, and in anterior and posterior directions, indicative of spreading.

We provide first evidence for *in vivo* prion-like properties of AD patients' CSF, accelerating tau pathology in susceptible tau transgenic mice. This demonstrates that biologically active tau seeds reach the CSF compartment in AD. Further studies may help to evaluate strain specific properties of CSF derived tau bioseeds, and to assess their diagnostic potential.

Introduction

Tau is a soluble protein that promotes microtubule assembly in neuronal cells. However, in pathological conditions, it becomes hyperphosphorylated and forms intracellular aggregates. This is characteristic of Alzheimer's disease (AD), but also of other neurodegenerative disorders known as tauopathies, including cases of frontotemporal dementia (FTD), corticobasal degeneration (CBD), and progressive supranuclear palsy (PSP) [36]. In AD, tau aggregation propagates from one brain region to another in specific patterns [6, 18, 20, 21, 23]. It has been shown that tau pathology can be seeded in a prion-like manner, as the injection of brain extracts from tau transgenic donor mice or tauopathy patients into tau transgenic host mice causes tau aggregation in the inoculated mice [1, 8, 9, 24, 26, 27].

Pathologically modified tau forms are present in the extracellular space and can transfer between cells in the brain [10, 17, 40]. Phosphorylated tau can be secreted via exosomal release, and reach the cerebrospinal fluid (CSF) [32]. CSF tau levels are currently being used for the clinical diagnosis of AD in conjunction with cognitive tests, brain imaging and CSF amyloid-β (Aβ) level measurements [34]. Increased tau levels in the CSF correlate best with cognitive decline in AD patients.

Information on the prion-like properties of Aβ and tau from CSF in AD is limited. It has been shown that CSF amyloid-β does not exhibit prion-like properties *in vivo* [16, 35]. Recently, two studies reported a seeding-like potential of CSF-derived tau *in vitro* [31, 38]. It is however not known, whether tau-comprising CSF harbors seeding potential *in vivo.*

Here, we studied the *in vivo* seeding capacity of CSF derived from AD patients upon inoculation into P301S tau transgenic mice. These animals develop tau hyperphosphorylation and neurofibrillary tangles (NFT) when ageing. We collected CSF from patients diagnosed with probable AD or mild cognitive impairment (MCI) likely due to AD, and injected it into young P301S tau mice. As a result, we observed a significant increase in neurons positive for hyperphosphorylated tau, and an accelerated formation of Gallyas-Braak positive aggregates in the hippocampus, when compared to mice inoculated with CSF derived from elderly control patients. In parallel, there were signs of spreading of tau pathology along anatomical networks, in the form of hyperphosphorylated tau-positive neurons and dot-like structures, comparable to findings when seeding with brain-derived tau in P301S tau mice [1]. These findings provide first *in vivo* evidence for the presence of bioactive tau seeds in CSF.

Materials and Methods

Mice

Homozygous human P301S mutant tau transgenic mice (P301S tau mice) [3] were used as host mice for all seeding experiments. Animal experiments were performed in compliance with protocols approved by the official local Committee for Animal Care and Animal Use of the Canton of Basel (License Nr. BS2471).

CSF collection and patient characteristics

In order to obtain large volumes of CSF from AD and control patients, allowing subsequent concentration, CSF was collected within a clinical trial set up in collaboration with the Memory Clinic, University Center for Medicine of Aging, Basel. The trial was approved by the local Ethics Commission of the Canton of Basel, and recruitment was limited to patients with a Mini Mental State (MMS) Score [14] >19/30. All patients examined for memory disturbances and potential neurodegenerative disorders at the Memory Clinic were consecutively allowed to enter the study. Up to 15ml of CSF per patient were collected by lumbar puncture from a total of 23 subjects after obtaining written informed consent of the patient and a care-giver. Freshly collected CSF was spun at 3000 rpm for 30 min to remove cell debris. The supernatants were collected and frozen at −80° C. Clinical diagnosis was based on multimodal examinations including neuropsychological assessments, brain MRI imaging, CSF tau and Aβ level measurements. Brain perfusion SPECT imaging data was available on a subset of the patients.

CSF processing

In order to obtain reasonable tau levels in small volumes that could be injected into mouse brains, CSF samples were concentrated [35]. In brief, 10 ml CSF per patient, was lyophilized (−80° C, 0.01 mbar vacuum pressure), reconstituted in sterile H_2O and dialyzed (using Float-A-Lyzer G2, 1kD, Spectrum Labs) to reduce the salt load, re-lyophilized, and finally reconstituted in about 10 µl H_2O (Suppl. Fig. 1). The final concentration of tau was measured by ELISA.

ELISA

CSF levels of tau, phospho-tau and Aβ, were measured by commercial kits "Innotest hTAU AG", "Innotest PHOSPHO-TAU", and "Innotest β-Amyloid (1-42)" according to the manufacturer's guidelines (Innogenetics/Fujirebio Europe N.V., Belgium) [4, 37].

Stereotaxic surgery

Three month-old P301S tau mice were anaesthetized with a mixture of ketamine (10 mg/kg) and xylazine (20 mg/kg) and placed on a heating pad to maintain body temperature during surgery. Mice were injected in the right hippocampus (A/P, −2.5 mm from bregma; L, − 2.0 mm; D/V, −1.8 mm) using a Hamilton syringe. Each mouse received a unilateral stereotaxic injection of 5 µl concentrated CSF, at a speed of 1.25 µl/min. Following the injection, the needle was kept in place for an additional 3 min. The surgical area was cleaned with saline and the incision sutured. Mice were monitored until recovery from anaesthesia, provided analgetic medication for up to 48 hours post-surgery, and checked regularly following surgery.

Immunohistochemical analysis

Four months post-inoculation, the mice were deeply anaesthetized with pentobarbital (150 mg/kg) and perfused with 20 ml cold PBS, followed by 20 ml 4% paraformaldehyde in PBS. The brains were dissected and post-fixed overnight. Following paraffin embedding, 5 µm thick coronal sections were prepared. Sections were silver-impregnated following the method of Gallyas-Braak to visualize filamentous tau pathology [7, 19]. Hematoxylin and eosin staining (H&E) was performed for morphological analysis. For tau immunohistochemistry AT8 (1:1000, Thermo Scientific), and AT100 (1:1000, Thermo Scientific) were used [29]. Secondary antibodies were from Vector Laboratories, Burlingame, CA (Vectastain ABC kit).

For quantification, 3 brain tissue sections were analyzed at levels comprising the injection site (from -2 to -3 µm from bregma), and the number of Gallyas-Braak silver stained inclusions, AT8 and AT100 positive neurons (entire hippocampus) and dot-like structures (dentate gyrus (DG), fimbria) was counted on 4X, 10X, or 20X magnified images, taken with an Olympus BX43 Upright Microscope (Life Science). For a few

stainings, only 2 sections next to the indicated bregma levels were well preserved and usable for quantification. Bregma levels were determined by visual comparison with the Mouse Brain Atlas (Franklin and Paxinos, 2007). The quantifications and the measurements of the area of the respective anatomical regions were performed by the Cell counter plugin in ImageJ (imagej.net). Brain images that were used and modified are from the Mouse Brain Atlas (Franklin and Paxinos, Elsevier 2007).

AT8 positive neurons were furthermore semi-quantitatively assessed at various bregma levels in CA1, CA3, and dentate gyrus (DG) regions, and an average score was obtained. For the heatmap (Fig. 2), average tau pathology per bregma level was calculated and color-graded.

Statistical analysis

In order to estimate the effects of AD CSF tau seeds versus control seeds, mean values of counts/area were calculated, and p-values were obtained by t-tests. Data was collected from 3 to 6 seeded mice per group, depending on the stainings, as indicated in Suppl. Table 1. P-values are interpreted exploratory and are not adjusted for multiple comparisons. In the results section and the figures, mean values and standard deviations are indicated. A p-value < 0.05 was considered as significant. A comprehensive overview on the statistical data is provided in Suppl. Table 1.

Data sharing

All relevant clinical data on the patients included in this exploratory translational study is provided in table 1. Additional individual de-identified patient data will be shared by the corresponding author.

Results

CSF derived from AD patients accelerates cerebral tau pathology in P301S host mice.

CSF collection: patients' characteristics

CSF was prospectively from a total of 23 patients visiting an outpatient Memory Clinic, in the setting of an explorative clinical trial. Up to 15ml of CSF were collected per patient without any relevant side effects. Clinical, imaging, and laboratory data of all patients were assessed. For experimental inoculation into transgenic mice, we selected CSF samples derived from the patients with the highest ("Alzheimer's disease patients": AD group, n=5) and the lowest ("control"-patients: CTR group, n=4) probability of AD-related cognitive decline. AD CSF samples were included from 2 patients fulfilling the actual criteria for "Probable AD dementia with high biomarker probability of AD etiology", two patients with "Probable AD dementia with intermediate biomarker probability of AD etiology" [25], as well as one patient with "MCI - core clinical data" [2], the latter with high evidence for neuronal injury in the CSF analysis [2]. The 4 patients attributed to the CTR CSF group fulfilled the criteria for "Dementia-unlikely to be due to AD" (n=2) or "MCI-unlikely due to AD" (n=2), without biomarker evidence for AD [2, 25]. Detailed patients' characteristics are provided in Table 1. Cognitive decline of control patients was clinically attributed to non-neurodegenerative causes (e.g. vascular dementia or alcohol abuse). Mean age (CTR 67,8y/AD 71,6y, p=0,52) and MMS Score (27,3/24,2, p=0.10) did not significantly differ between the two groups, although there was a trend to a higher MMS in the patients attributed to the control patients' group.

Given the physiologically low tau concentrations in CSF in the ng/ml range, the samples were concentrated as previously described (Suppl. Fig. 1) [16, 35]. After concentration, no significant difference of the total tau levels between the two groups was measured by ELISA (156 ng/ml / 204,6 ng/ml, P=0.65, Suppl. Fig. 2).

Hippocampal neuronal tau pathology provoked by AD-patients' CSF

Intrahippocampal injections of AD-patients' CSF into young P301S tau mice resulted in an increased number of AT8 positive hippocampal neurons at the injection level, as compared to CTR CSF inoculated mice (Fig. 1a-c, mean neuron count per area CTR CSF/AD CSF: 0,049/0,611, p=0,001, for comprehensive overview on the statistical data see Suppl. Table 1). Immunohistochemistry for AT100 also showed a significant increase

in positive hippocampal neurons in the AD CSF injected hippocampus when compared to CTR CSF administered mice (Fig.1d-f, CTR CSF/AD CSF: 0,101/0,729, p=0,013, for a detailed view on the accentuated AT8 and AT100 pathology in the CA1 field see Suppl. Figure 3). Moreover, a trend to increased tau phosphorylation was noticeable also in the contralateral, non-injected hippocampus, both for AT8 as well as for AT100 hyperphosphorylated tau, when comparing the CTR group to the AD group (AT8: 0,050/0,393, p=0,053, AT100: 0,164/0,626, p=0,053).

Semi-quantitative analysis revealed more AT8 positive hippocampal neurons in AD CSF seeded mice anterior (from -2 to -2.5 mm) and posterior (from -2.5 to -3mm) to the injection site, compared to CTR CSF seeded mice, both, ipsi-, and contralateral to the injection site (Fig. 2). This is compatible with CSF-induced spreading of tau pathology throughout the hippocampus of inoculated mice along anatomically connected regions, similarly to previous findings when seeding with tau containing brain homogenates [1, 8, 33].

Previous seedings with brain homogenates in P301S mice resulted in a prominent increase in dot-like structures, attributable to axonal tau aggregates [1]. Hippocampal tissue from AD CSF seeded mice showed similar wide-spread dot-like structures, when stained for AT8 or AT100. Dot-like structures were most prominent in the dentate gyrus (DG) (Fig. 3a-f, Suppl. Figure 4a-d), as well as in the pathways of the fimbria (Fig. 3g-l). In CTR CSF seeded mice, some dot-like structures were also present, most prominently in the DG (Fig. 3a, d), and accentuated in the dorsal hippocampus, where they occur regularly in untreated aged P301S mice. When comparing CTR CSF to AD CSF inoculated mice, the increase in AT8 and AT100 positive dot-like structures in the DG was significant ipsilateral to the injection site (AT8: 1,265/3,045, p=0,013 (Fig. 3a-c); AT100: 1,930/6,400, p=0,036 (Fig. 3d-f)), and even contra-lateral to the injection site (AT8: 1,012/2,574, p=0,031; AT100: 3,121/6,266, p=0,047). Compatible with an induced spreading of tau aggregation, an increase of tau hyperphosphorylation was also noted in the fimbria, both for AT8 and AT100 positive granular structures (AT8: 1,736/4,310, p=0,039 (Fig. 3g-i); AT100: 2,539/5,514, p=0,018 (Fig 3j-l)), while showing a similar trend on the contra-lateral side (AT8: 1,603/3,258, p=0,121; AT100: 2,598/4,302, p=0,053).

Furthermore, Gallyas-Braak staining showed an increase in silver positive hippocampal neuronal inclusions in AD CSF seeded host mice, both ipsilateral (Fig. 4a-c, 0,029/0,151, p=0,0007), as well as contralateral to the injection site (0,049/0,128, p=0.014). This is compatible with the induction of later stage tau

hyperphosphorylation and tau aggregation by the AD CSF tau seeds, as also visible on higher magnification views of the CA1 region (Fig. 4d, e). Only in a subset of mice, focal granular aggregates of Gallyas-Braak positive tau structures became visible. In a mouse seeded with CSF collected from patient #2, we noted a focal induction of Gallyas-Braak positive grains in the ipsilateral alveus above the CA1 field (Fig 4f), while in a mouse inoculated with CSF derived from patient #1, prominently focal granular tau structures appeared in the dorsal fornix, strictly limited to the inoculated side (Fig 4g). The latter finding was reminiscent of previous observations of focal tau aggregation in the dorsal fornix following injection of human brain extracts derived from tauopathy patients [5].

Discussion

Here we demonstrate the *in vivo* tau seeding effects of cerebrospinal fluid (CSF) derived from patients suffering from AD dementia or mild cognitive impairment (MCI) likely due to AD. CSF containing hyperphosphorylated tau species accelerated tau pathology after intrahippocampal inoculation into P301S tau transgenic host mice. These findings corroborate two recent reports of *in vitro* seeding-like activity of CSF tau [31, 38], providing now first evidence for biological seeding activity of patients' derived CSF tau in susceptible host mice.

CSF collected from patients with a high probability of AD or related MCI was used, and compared with CSF derived from age-matched patients with diagnosis other than AD. Inoculation of CSF collected from AD and MCI patients resulted in a significant increase of markers of tau pathology in the hippocampus of P301S tau host mice. Advanced tau pathology was also notable in the contralateral hippocampus, and in anterior and posterior directions. This is comparable to the spreading we and others have described after seeding with brain extracts of tau transgenic mice or tauopathy patients [1, 5, 8, 9, 22].

Besides an overall increase in tau inclusion positive hippocampal neurons, injection of concentrated AD patients' CSF into young P301S tau mice resulted in focal granular, dot-like tau accumulation particularly in the fimbria and the dentate gyrus (DG) in a subset of the inoculated mice. This granular pathology is very similar to earlier findings in tau transgenic mice seeded with brain extracts from a patient who suffered from corticobasal degeneration [5]. Punctate Gallyas-Braak silver staining positive structures indicative of axonal tau aggregates accumulated in the alveus and the dorsal fornix close to the injection site in two AD CSF inoculated mice, reminiscent of a similar pathology induced in the fornix of P301S tau mice seeded with P301S tau mouse donor brain extracts [1]. These characteristic focal Gallyas-Braak positive, axonal dot-like structures were visible in a subset of AD CSF inoculated host mice. Seeding patterns might differ due to varieties in the tau strains involved in the donor patients. It has been shown that brain samples from different tauopathy patients may accelerate tau aggregation variably in different structures or cell types, and also propagate in distinctive ways, indicating the presence of different tau strains [5]. Recently, different ultrastructural polymorphs of patients' brain-derived tau strains have been identified by cryo-EM [12, 13].

Our findings suggest that in AD patients, pathological tau species with biological seeding competence drain from the brain interstitial fluid to the CSF compartment. There, they retain their potential to evoke seed-like effects *in vivo*, similarly as previously reported for brain parenchymal tau species.

The here observed tau seeding competence of AD patients' CSF contrasts our earlier findings in seeding experiments with Aβ. There, even very long seeding times after inoculation of concentrated human or murine Aβ comprising CSF into APP transgenic host mice failed to provide evidence for the presence of bioactive Aβ seeds in the CSF compartment [16, 35]. The here described biological tau seeding activity of CSF is however not entirely surprising. In contrast to Aβ that aggregates broadly in the brain parenchyma and the perivascular space, tau is readily drained towards the CSF compartment. Opposite to Aβ, tau levels increase in the CSF with the progression of AD [28].

Furthermore, our evidence for *in vivo* seeding activity of CSF tau follows recent findings on the ability of CSF tau to induce seeding-like conformational changes *in vitro* [31, 38]. Ventricular CSF samples collected post mortem at autopsies of AD patients have been inducing tau aggregation in HEK293 cells expressing a P301S tau repeat domain [38]. CSF tau derived from Pick's disease patients has furthermore been found to induce real-time quaking conversion (RT-QuIC) of a 3 repeat tau fragment [31]. *In vitro* seeding activity in cellular systems, or prion-like substrate conversion capacity by RT-QuIC, has furthermore recently been demonstrated for CSF in the context of other neurodegenerative proteinopathies, including sporadic Creutzfeldt Jakob disease [15] and Huntington's disease [39].

The presence of bioactive tau seeds in the CSF of AD patients suggests a diagnostic use of the aggregation induction capacity of CSF in tauopathies. In contrast to brain tissue, CSF can easily be collected from patients with a low rate of side effects [30]. The simple analysis of CSF tau levels is an established part of the diagnostic criteria for AD, but it lacks yet high specificity and is unable to provide prognosis at early or even preclinical disease stages [11, 28]. The latter would however be important also for the development of novel therapies. Our detection of bioactive tau seeds in CSF of AD patients augurs well for the development of novel diagnostic tools that are based on the specific conformational properties and the bioactivity of tau.

The biological *in vivo* seeding capacity of AD patients' CSF that we describe calls for considerations on the biosafety of operators when handling CSF samples. Compared to previous seedings with brain tissues [8], the seeding effects described here after inoculation of concentrated CSF into a tau overexpressing murine host

system are relatively mild. We therefore consider the seeding potential of AD tau in CSF rather low. We however did not compare the seeding capacity of AD CSF to proportionately diluted AD brain lysates, so that a direct comparison of the seeding potential of CSF to brain-derived tau remains yet to be done. The host mice used in the present study furthermore express a human P301S mutant tau form which is particularly prone to aggregation. The present findings therefore can't be directly translated into the setting where people with normal wild-type tau would be exposed to AD patients' tau samples. Altogether, the seeding potential of CSF might be of limited relevance in a routine laboratory or clinical setting when adhering to standard safety procedures for the handling of potentially infectious bio-samples.

This study is subject to some limitations. Due to the large amounts of CSF needed, CSF had to be collected consecutively from donating patients. This resulted in a limited number of patients' samples attributable to either the AD- or the control-group, similar to the previous *in vitro* study done in HEK293 cells [38]. As tau seeding is a time and concentration dependent process [9], we concentrated the CSF samples before the intracerebral inoculations. We can't therefore exclude, that the seeding potential of tau was increased by the concentration process itself. The absence of any seeding activity in our previous experiments with concentrated CSF-Aβ [16, 35], as well as the significant differences between the CSF tau derived from AD patients versus the controls, however speak against a relevant influence of the concentrating procedure. Given the short life span of homozygous P301S tau mice, the maximum seeding time was limited to 4 months. We therefore can't exclude that a more robust seeding response would become apparent at later time points post-seeding.

The exact nature of bioactive tau seeds has yet remained unknown. The need of large amounts of CSF for seedings in the present study has precluded any detailed analysis of the bioactive seeds. In their post mortal CSF samples, Takeda et al. found high molecular weight tau (HMW) species, and considered them to be the bioactive species *in vitro* [38]. Future studies will be needed to analyze the structural nature of CSF tau bioseeds.

In conclusion, we here provide first *in vivo* evidence for the presence of bioactive tau aggregation accelerating seeds in CSF of AD patients. Further studies will be needed to elaborate whether CSF derived bioactive tau seeds might be of use as a marker of AD, as well as to study mechanistic aspects of potentially strain specific patterns of CSF bioseeds in different tauopathies.

Author contributions

D.T.W. designed the study; Z.S., A.M., A.U.M., and D.T.W. designed research; Z.S., A.M., F.S., M.F, Y.N., V.S.-M., M.S. performed research; Z.S., A.M., F.S., M.F., A.U.M., M.S., M.T., M.G., and D.T.W. analyzed data; and Z.S., A.M., M.G., and D.T.W. wrote the paper.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Table 1

patient number	group	diagnosis NIA-AA criteria	alternative clinical diagnosis	age	MMS	Brain MRI	SPECT	CSF tau (pg/ml)	CSF p-tau (pg/ml)	CSF Aβ (pg/ml)
1	CTR	Dementia unlikely to be due to AD	vascular encephalopathy	80	26	vascular encephalopathy	no signs of AD or FTD, compatible with vascular encephalopathy	500	**78**	544
2	CTR	Dementia unlikely to be due to AD	neurocognitive deficits associated with progressive pancreas carcinoma and tumorectomy	73	25	mild vascular microangiopathy	-	206	50	580
3	CTR	MCI - unlikely to be due to AD	early Wernicke–Korsakoff syndrome	61	30	compatible with Wernicke-Korsakoff syndrome	compatible with Wernicke-Korsakoff syndrome	162	43	928
4	CTR	MCI - unlikely to be due to AD	depression	57	28	normal	-	360	53	1133
1	AD	Probable AD with high BP	-	73	25	hippocampal atrophy, no vascular lesions	-	**840**	**105**	**366**
2	AD	Probable AD with high BP	-	61	24	temporal brain atrophy	Left parietal decreased glucose metabolism compatible with early AD	**500**	**92**	**318**
3	AD	MCI - core clinical data	-	79	28	mild hippocampal atrophy	-	**759**	**119**	516
4	AD	Probable AD with intermediate BP	-	71	23	left hippocampal atrophy, microangiopathy	-	308	**77**	**240**
5	AD	Probable AD with intermediate BP	-	74	21	Global and hippocampal brain atrophy, mild microangiopathy	-	238	32	**402**

Table.1: Clinical, imaging, and laboratory findings of the patients selected for the preparation of CSF seeds (CTR CSF: control patients' group, AD CSF: Alzheimer's disease group). Bold letters indicate pathological values of CSF tau, p-tau or Aβ. NIA-AA criteria: National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Criteria abbreviation: BP = "Biomarker probability of AD etiology"[26].

Figures

Figure 1

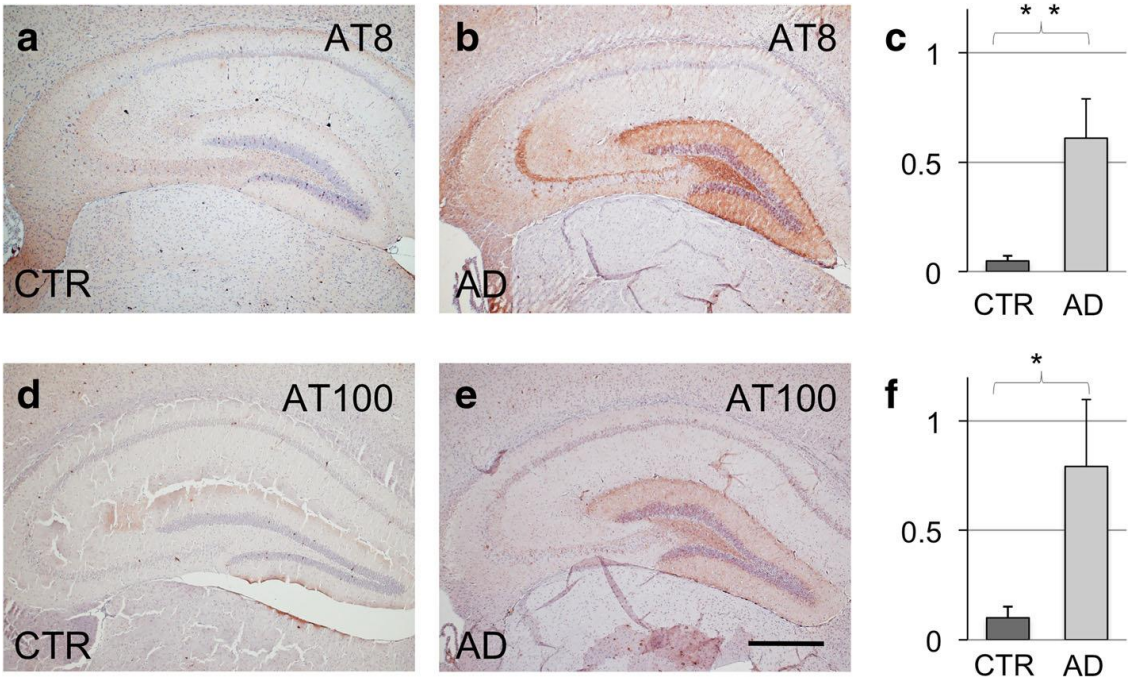


Fig.1: Immunohistochemistry for AT8 and AT100 tau phosphorylation markers of 7 months old P301S transgenic mice, sacrificed 4 months after unilateral hippocampal inoculation with CSF derived from control patients (CTR) or AD patients (AD) (a, d; b, e). AT8 positive hippocampal neurons (c, p=0,001) ipsilateral to the inoculation site; AT100 positive hippocampal neurons, ipsilateral (f, p=0,0013). Scale bar in e equals 500 μm, and applies to a, b, d, and e.

Figure 2

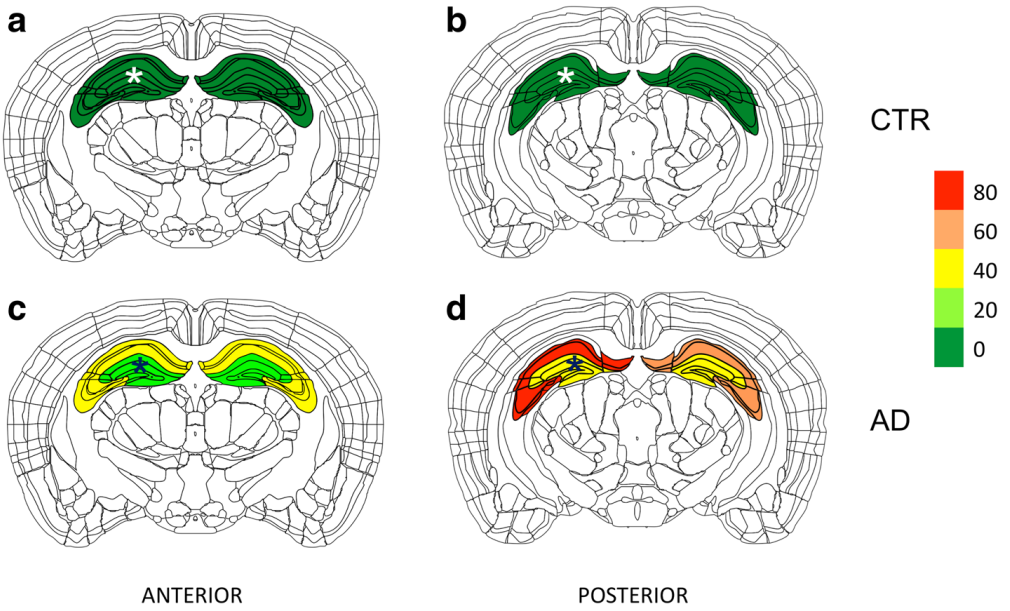


Fig.2: Heat map of a semiquantitative analysis of AT8 tau positive neurons anterior and posterior, as well as ipsilateral (asterisks) and contralateral to the injection site after CTR CSF and AD CSF seedings. Bregma levels 2-2.5/2-2.5 mm (a, c, anterior), and 2.5-3/2.5-3 mm (b, d, posterior).

Figure 3

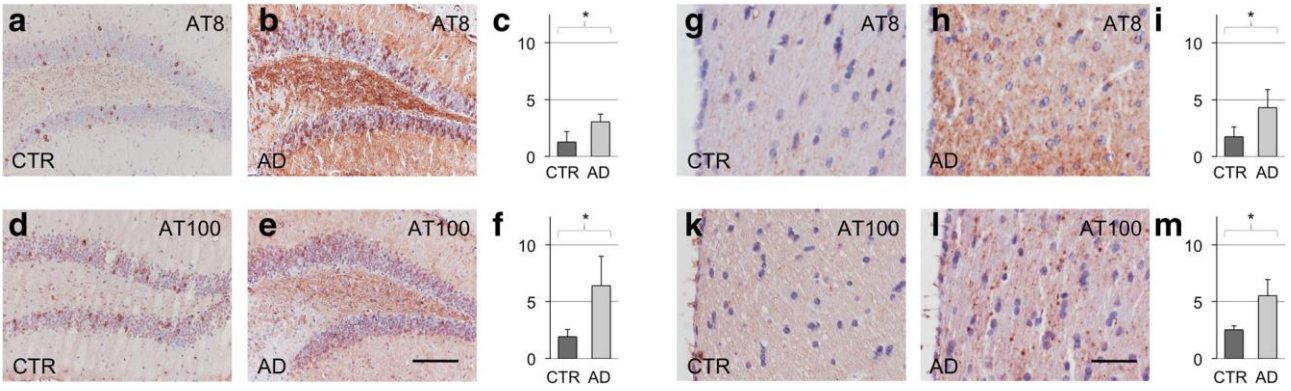


Fig.3: AT8 and AT100 immunohistochemistry after unilateral hippocampal seeding with CTR CSF (a, g ; d, j) and AD CSF (b, h; e, k) into P301S mice. Accumulation of AT8 (c, p=0.013) and AT 100 (f, p=0.036) positive dot-like structures in the DG ipsilateral to the injection site. Increase in AT8 (i, p=0.039) and AT 100 (l, p=0.018) positive dot-like structures in the fimbria. Scale bar in e equals 150 µm in a, b, d and e; scale bar in l equals 37,5 µm in g, h, j, and k.

Figure 4

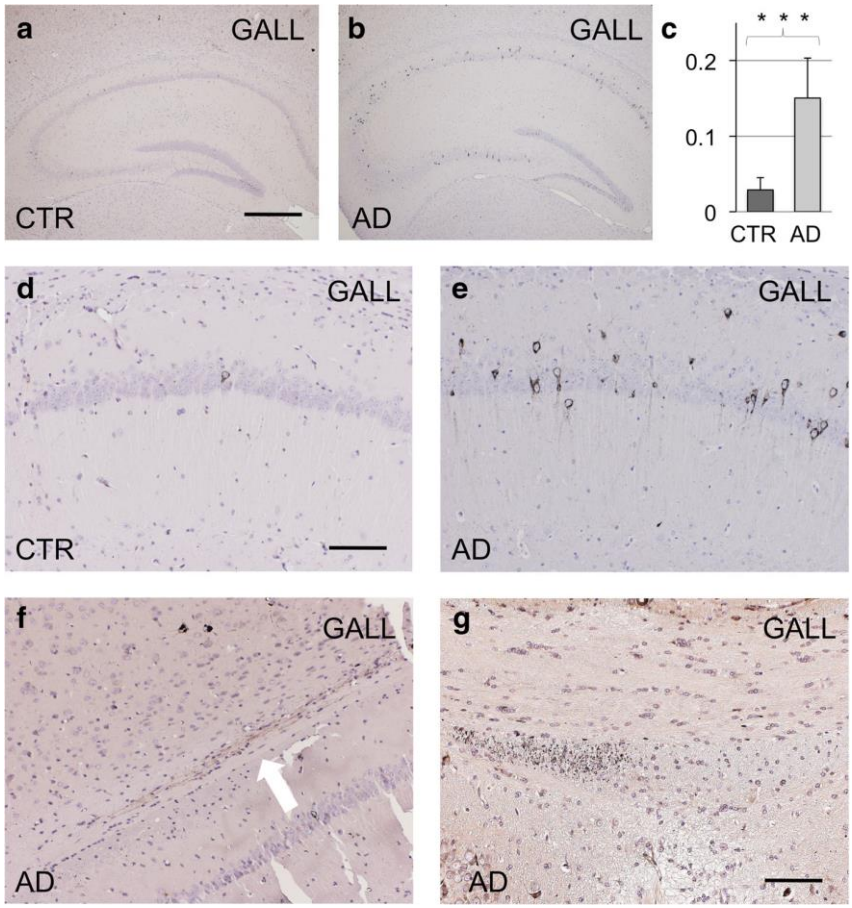
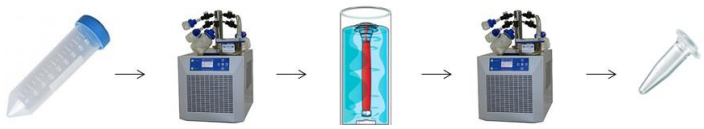


Fig.4: Gallyas-Braak stainings. Overview on the hippocampus (CTR CSF: a, AD CSF: b). Increase in Gallyas-Braak positive hippocampal neurons in AD CSF seeded mice compared to CTR CSF seedings (c, p=0.0007). Gallyas positive neurons in the CA1 subfield (CTR CSF: d, AD CSF: e). Focal dot-like Gallyas-Braak positive structures in the ipsilateral alveus above the CA1 field after inoculation of CSF from patient #2 (f, arrow), and in the ipsilateral dorsal fornix after seeding with CSF from patient #1 (g). Scale bar in a equals 500 μm in a and b; scale bar in d equals 100 μm and applies to d-f; scale bar in g equals 76 μm.

Supplemental Information

Supplemental Figures:

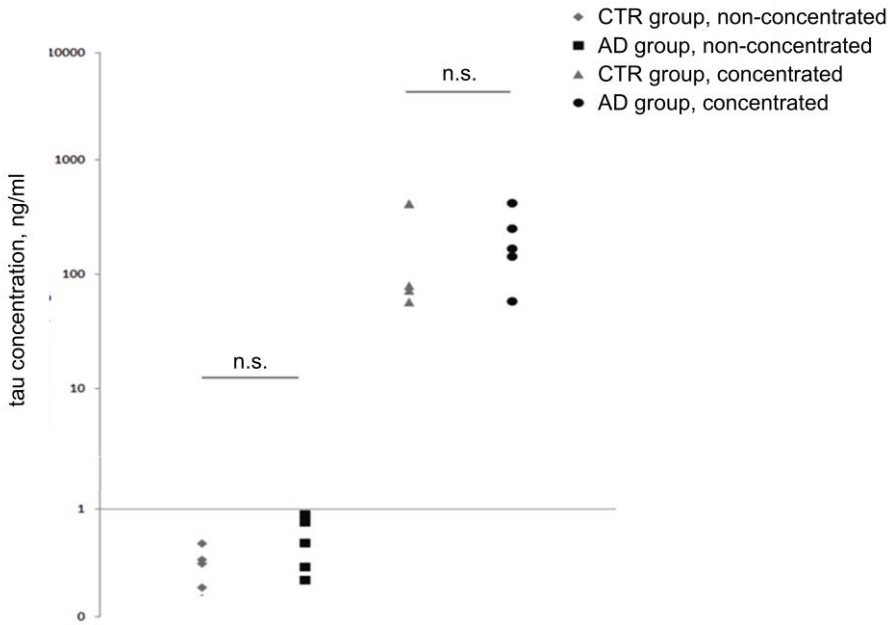
Suppl. Figure 1: CSF processing



Suppl. Figure 1:

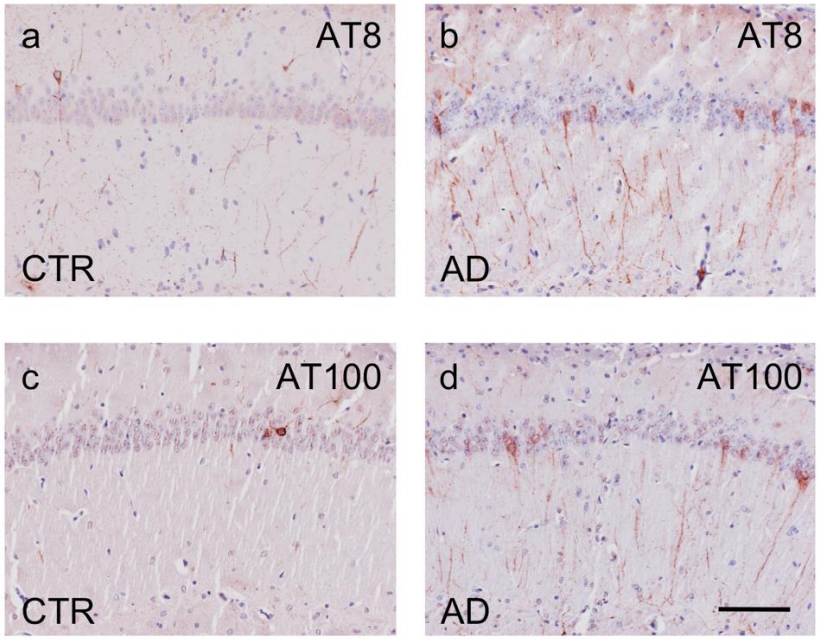
CSF was collected from patients and controls by a lumbar puncture, obtaining about 10ml on average. From those, 8ml were used for subsequent lyophilization, dialysis, and re-lyophilization, and finally dissolved in 66µl sterile water, with a theoretical concentration factor of 120x.

Suppl. Figure 2: CSF tau concentrations



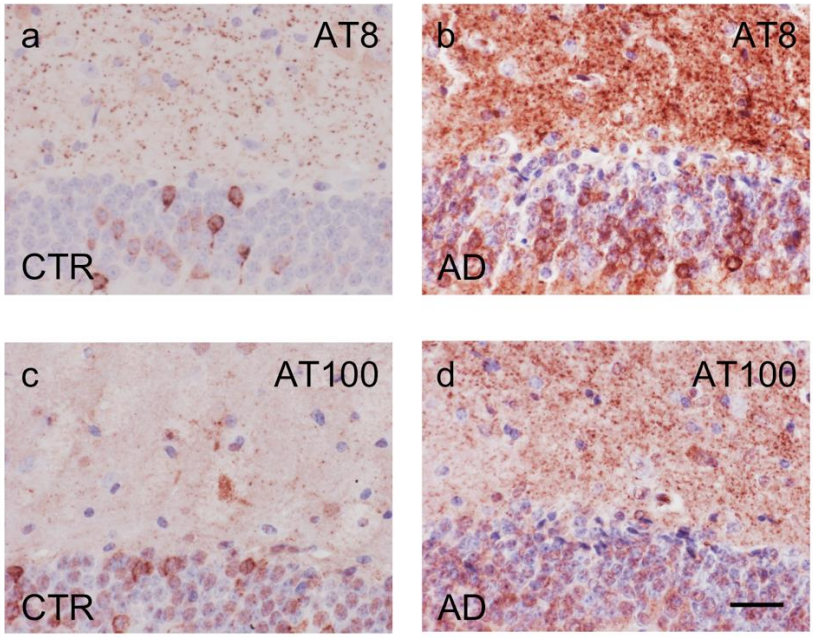
Suppl. Figure 2: Tau and phospho-tau (P-Tau, T231) concentration of the patients CSF used for seeding experiments, as measured by ELISA. There was no significant difference of the total tau levels between the AD and the CTR group, as measured by ELISA, before (0,530 ng/ml / 0,307 ng/ml, p=0,183) and after concentration (204,6 ng/ml / 156 ng/ml, p=0.646).

Suppl. Figure 3: CA1 pathology



Suppl. Figure 3: Immunohistochemistry of the hippocampal CA1 field for AT8 and AT100 tau phosphorylation markers of 7 months old P301S transgenic mice, sacrificed 4 months after unilateral hippocampal inoculation with CSF derived from control patients (CTR) or AD patients (AD) (a-d). Scale bar in d equals 100 μm and applies to a-d.

Suppl. Figure 4: DG pathology



Suppl. Figure 4: Immunohistochemistry of the dentate gyrus for AT8 and AT100 tau phosphorylation markers of 7 months old P301S transgenic mice, sacrificed 4 months after unilateral hippocampal inoculation with CSF derived from control patients (CTR) or AD patients (AD) (a-d). Scale bar in d equals 37,5 μm and applies to a-d.

Supplemental Table:

Suppl. Table 1: Statistical data CSF seedings

	AT8 HIPP neurons	AT100 HIPP neurons	Gallyas HIPP neurons	AT8 DG grains	AT100 DG grains	Gallyas DG grains	AT8 fimbria grains	AT100 fimbria grains	Gallyas fimbria grains
N per group (AD/CTR)	6/3	4/3	6/5	6/3	4/3	6/5	6/3	4/3	6/5
Mean IPSI (AD/CTR)	*0,611/ 0,049*	*0,792/ 0,101*	*0,151/ 0,029*	*3,045/ 1,265*	*6,400/ 1,930*	*1,660/ 1,616*	*4,310 /1,736*	*5,514/ 2,539*	*2,829/ 1,884*
Mean CONTRA (AD/CTR)	*0,393/ 0,050*	*0,626/ 0,164*	*0,128/ 0,049*	*2,574/ 1,012*	*6,266/ 3,121*	*2,379/ 2,232*	*3,258/ 1,603*	*4,302/ 2,598*	*2,619/ 1,744*
P value IPSI (AD vs CTR)	*0,001*	*0,013*	*0,0007*	*0,013*	*0,036*	*0,942*	*0,039*	*0,018*	*0,218*
P value CONTRA (AD vs CTR)	*0,053*	*0,053*	*0,014*	*0,031*	*0,047*	*0,884*	*0,121*	*0,053*	*0,309*

Suppl. Table 1: Overview of the statistical data of unilateral intrahippocampal seedings with CSF derived from AD patients (AD) and control patients (CTR) into P301S mice. Abbreviations: HIPP: Hippocampal, DG: dentate gyrus, IPSI: ipsilateral to the intrahippocampal inoculation of the seed, CONTRA: contralateral to the intrahippocampal inoculation of the seed.

3.2)Second manuscript in revision

Severe oligomeric tau toxicity can be reversed without long-term sequelae

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Short title: Long-term fitness after tauopathy recovery

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Abstract

Tau is a microtubule stabilizing protein that forms abnormal aggregates in many neurodegenerative disorders, including Alzheimer's disease. We have previously shown that co-expression of fragmented and full-length tau in P301SxTAU62^{on} tau transgenic mice results in the formation of oligomeric tau species and causes severe paralysis. This paralysis is fully reversible once expression of the tau fragment is halted, even though P301S tau expression is maintained. Whereas various strategies to target tau aggregation have been developed, little is known about the long-term consequences of reverted tau toxicity.

Therefore, we studied the long-term motor fitness of recovered, formerly paralyzed P301SxTAU62$^{on\text{-}off}$ mice. To further assess the seeding competence of oligomeric toxic tau species, we also inoculated ALZ17 mice with brainstem homogenates from paralyzed P301SxTAU62^{on} mice.

Counterintuitively, after recovery from paralysis due to oligomeric tau species expression, aging P301SxTAU62$^{on\text{-}off}$ mice did not develop more motor impairment or tau pathology when compared to heterozygous P301S tau transgenic littermates. Thus, toxic tau species causing extensive neuronal dysfunction can be cleared without inducing seeding effects. Moreover, these toxic tau species also lack long-term tau seeding effects upon intrahippocampal inoculation into ALZ17 mice.

In conclusion, tau species can be neurotoxic in the absence of seeding-competent tau aggregates, and mice can clear these tau forms permanently without tau seeding or spreading effects. These observations suggest that early targeting of non-fibrillar tau species may represent a therapeutically effective intervention in tauopathies. On the other hand, the absent seeding competence of early toxic tau species also warrants caution when using seeding-based tests for preclinical tauopathy diagnostics.

Key words: Alzheimer's disease; Neurodegeneration; Tau; Seeding; Oligomers

Abbreviations

AD: Alzheimer’s disease

NFT: Neurofibrillary tangles

H&E: Hematoxylin and eosin

HRP: Horseradish peroxidase

ANOVA: Analysis of variance

AgD: Argyrophilic grain disease

PSP: Progressive supranuclear palsy

CBD: Corticobasal degeneration

Introduction

Tau is a soluble protein acting as a microtubule stabilizer in neuronal cells. Under pathological conditions it becomes hyperphosphorylated and eventually forms intracellular aggregates. Tau aggregates are a hallmark of Alzheimer's disease (AD) and other neurodegenerative disorders, including forms of frontotemporal dementia (Spillantini and Goedert, 2013; Spires-Jones and Hyman, 2014). The mechanisms underlying pathological tau aggregation remain only partly understood. Tau is thought to aggregate through the formation of oligomeric species and subsequent aggregation into fibrils, culminating in the formation of tangles. Tau aggregation can spread towards anatomically connected regions in a prion-like manner (Clavaguera *et al.*, 2009; Goedert *et al.*, 2017).

We have recently shown that tau toxicity can be mediated by oligomeric tau species (Ozcelik *et al.*, 2016). Co-expression of full-length P301S mutant tau with a 3R $tau_{151-421}$ fragment ($\Delta tau_{151-421}$) in 3-weeks-old P301SxTAU62^{on} transgenic mice leads to the formation of soluble high molecular weight tau oligomers. In the P301SxTAU62^{on} mouse model, high molecular weight tau oligomers are sufficient to cause extensive nerve cell dysfunction and severe motor palsy, both of which occur in the absence of insoluble tau aggregates or neurofibrillary tangles (NFTs) (Ozcelik *et al.*, 2016). Strikingly, once the doxycycline-inducible expression of $\Delta tau_{151-421}$ is switched off in these mice, their severe phenotype reverses within 3 weeks, and animals regain their motor competence, even though heterozygous P301S mutant tau expression is maintained (Ozcelik *et al.*, 2016). This phenotype reversibility renders P301SxTAU62$^{on\text{-}off}$ mice suitable for investigating potential long-term consequences of reverted tau toxicity. The relevance of oligomeric tau toxicity is being increasingly recognized, and recent studies have shown that it can also be pharmacologically attenuated (Bittar *et al.*, 2019; Lo *et al.*, 2019; Abskharon *et al.*, 2020; Lo Cascio *et al.*, 2020; Puangmalai *et al.*, 2020). Hypothesized to depend on oligomer conformation, tau oligomeric toxicity can also be aggravated by biological interactions with chaperones (Oroz *et al.*, 2018), RNA-binding proteins (Jiang *et al.*, 2019), and nuclear complexes (Eftekharzadeh *et al.*, 2018). Due to their interaction with cellular components and their importance in tau propagation, tau oligomers have been considered crucial for tau-mediated neurodegeneration.

Long-term effects after recovery from oligomeric, non-fibrillar tau toxicity have not yet been assessed. Here, we study the late effects of reverted tau stress in aged P301SxTAU62$^{on\text{-}off}$ mice. Seeding and spreading of tau

pathology have been widely studied in human tauopathies as well as in tau transgenic mouse models (Maeda *et al.*, 2007; Clavaguera *et al.*, 2009; Sanders *et al.*, 2014; Mudher *et al.*, 2017). Based on this accumulated evidence, we hypothesized that the tau oligomers abundantly present in young paralyzed P301SxTAU62^{on} mice might act as seed for tau accumulation, as P301S full-length tau expression is maintained, while doxycycline-driven $\Delta tau_{151\text{-}421}$ expression is switched off. To our surprise, P301SxTAU62$^{on\text{-}off}$ mice that had recovered from neurotoxic stress and motor palsy did not develop worse motor function nor more pronounced tau pathology during aging when compared to their P301S tau heterozygous littermates. This demonstrates that P301SxTAU62$^{on\text{-}off}$ mice clear their toxic tau aggregates without seeding NFT formation, and hence do not show pronounced motor decline upon aging.

To study whether the toxic tau species causing palsy in young P301SxTAU62^{on} mice also lack classical seeding competence, we next stereotactically inoculated brainstem homogenates of paralyzed P301SxTAU62^{on} into ALZ17 human wild-type tau transgenic mice. In contrast to ALZ17 mice seeded with P301S brainstem homogenates containing fibrillary tau species (Clavaguera *et al.*, 2009), ALZ17 mice inoculated with P301SxTAU62^{on} homogenate did not develop fibrillar tau pathology, arguing against a seeding competence of the early toxic tau forms in P301SxTAU62^{on} mice.

Lacking seeding competence of non-fibrillar toxic tau species as well as absent long-term sequelae after halting their expression warrants the exploration of early therapeutic interventions targeting pre-fibrillar tau species. Our observations may also question the reliability of seeding-based assays for early preclinical tauopathy diagnostics.

Methods

Mice

Transgenic homozygous mice expressing human ALZ17 mutant tau (ALZ17 mice) (Probst *et al.*, 2000), transgenic homozygous mice expressing human P301S mutant tau (P301S mice) (Allen *et al.*, 2002), transgenic heterozygous mice expressing human $\Delta tau_{151-421}$ (TAU62 mice) (Ozcelik *et al.*, 2016), and non-transgenic C57BL/6J (BL6 mice) control mice were used. P301S mice and TAU62 mice were interbred to obtain double transgenic P301SxTAU62 mice (Ozcelik *et al.*, 2016). All animal experiments were performed in compliance with protocols approved by the Committee for Animal Care and Animal Use of the Canton of Basel (Licenses Nr. BS 2364 and 2471).

Behavioral tests

Motor behavior, including gait ataxia, tremor, and hindlimb reflexes was assessed. Quantitative motor testing was performed by the grid test in which mice were placed on a vertical mesh grid and the latency to fall off the grid was recorded for 3 min. Motor coordination and balance were assessed using the Panlab Harvard Rotarod (Harvard Apparatus, Holliston, USA). The rotarod starts at a speed of 4 rpm and accelerates by 1 rpm every 3 sec. In both, grid test and rotarod assay, mice were tested for 3 consecutive days with 3 trials per day, with minimum rest intervals of 5 min, and the mean latency to fall was documented. Results were obtained by averaging the daily means of 3 consecutive days.

Stereotactic surgery

For surgery, 3-months-old ALZ17 mice were anesthetized with a mixture of ketamine (10 mg/kg) and xylazine (20 mg/kg) and placed on a heating pad to maintain body temperature. Mice were stereotactically injected into the right hippocampus (A/P, −2.5 mm from bregma; L, − 2.0 mm; D/V, −1.8 mm) using a Hamilton syringe. Brainstem homogenates of 6-months-old paralyzed homozygous P301S mice and 3-weeks-old paralyzed P301SxTAU62^{on} mice were prepared for inoculation in ALZ17 mice. Brainstems were weighted and diluted 1:10 in PBS for seeding. After dilution, samples were homogenized using an Ultraturrax T8 (IKA Labortechnik, Staufen im Breisgau, Germany) and sonicated briefly (Bandelin SONOPULS, Bandelin, Berlin, Germany; 90% power, 10% cycle, 10 sec pulses). Homogenates were then centrifuged at 4000 g for 20 minutes

at 4 °C, and aliquots of the supernatant were stored at -70 °C for later usage. During inoculation, each mouse received 5 µl of brainstem homogenate at a speed of 1.25 µl/min. Following injection, the needle was kept in place for additional 3 minutes before withdrawal. The surgical area was cleaned with sterile saline and the incision sutured. Mice were monitored until recovery from anaesthesia, post-interventional analgesia was administered, and animals were checked regularly following surgery. After 20 months of incubation, the seeded mice were sacrificed.

Immunohistochemistry

Mice were deeply anesthetized with pentobarbital and transcardially perfused with 20 ml cold PBS, followed by 20 ml 4% paraformaldehyde in PBS. The brains were dissected and post-fixed overnight. Following paraffin embedding, 4 µm coronal sections were cut from the brains of seeded mice, whereas 4 µm sagittal sections were prepared from the brains of mice used for the behavioral tests. Sections were silver-impregnated following the method of Gallyas-Braak to visualize filamentous tau pathology. Hematoxylin and eosin staining (H&E) was performed for morphological analysis. For immunohistochemistry, the following anti-tau antibodies were used: AT8 (1:1000, Pierce Biotechnology, Waltham, USA), AT100 (1:1000, Pierce Biotechnology, Waltham, USA), TauC3 (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA). Secondary antibodies were from Vector Laboratories, Burlingame, USA (Vectastain ABC kit). 10X, 20X and 40X magnified pictures of the stained slides were taken with an Olympus BX43 Upright Microscope (Life Sciences Solutions, Chicago, USA).

Western blots

For the western blots comparing total and soluble tau in 16-months-old P301S heterozygous (P301Shet) and P301SxTAU62$^{on\text{-}off}$ mice, brains were homogenized in cold extraction buffer 20 % (w/v) (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 5 mM NaPyrophosphate, 10 mM B Glycerophosphate, 30 mM NaFluoride, 10 mM NaVanadate, with addition of 100 ul/10 ml PMSF of 0.1 M just before use, and 1 Pierce protease and phosphatase inhibitor mini tablet, EDTA-free, Pierce Biotechnology, Waltham, USA) using a Polytron Homogenizer (Thomas Scientific). An aliquot of the resulting homogenate was collected as total tau. Subsequently, samples were centrifuged at 80000 g for 15 min using an ultracentrifuge (Beckman Coulter, Brea, USA; OptimaTM L-70K Ultracentrifuge), and an aliquot of the supernatant was collected as soluble tau.

For the western blot comparing total tau in 3-months-old mice, one half of the mouse brain was dissected into forebrain and brainstem, and frozen in liquid nitrogen or on dry ice. Brainstems were weighted and diluted 1:10 in TBS-Complete. Subsequently, samples were homogenized using an Ultraturrax T8 (IKA Labortechnik, Staufen im Breisgau, Germany) and briefly sonicated (Bandelin SONOPULS, Bandelin, Berlin, Germany; 90% power, 10% cycle, 10 sec pulses). Homogenates were then centrifuged at 4000 g for 20 minutes at 4 °C, and aliquots of the supernatant stored at -70 °C for later usage. Western blots were then performed under non-reducing conditions using appropriate amounts of protein, 4X NuPAGE LDS sample buffer, and deionized water. 10X NuPAGE reducing agent was used to obtain reducing conditions. Following appropriate preparation, samples were loaded onto a 7% NuPAGE® Tris-acetate gel. After the removal of gels from the cassette and activation of PVDF membrane (Amersham Biosciences, Amersham, UK) samples were transferred on the PVDF membrane using the XCell IITM Blot Module. Unspecific binding epitopes were blocked with 5% non-fat milk in PBS-Tween, followed by incubation with primary antibody over night at 4 °C on a shaker. After washing with PBS-Tween, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or -rabbit secondary antibody at room temperature. Then, the membrane was washed again in PBS-Tween and detected by electrochemiluminescence (ECL) (GE Healthcare, Little Chalfont, UK). The anti-tau antibody used for the western blots was HT7 (1:1000, Pierce Biotechnology, Waltham, USA).

Sarkosyl extraction and immunoelectron microscopy

Following PBS perfusion, mouse brainstem tissue was dissected and frozen in liquid nitrogen. Sarkosyl extraction was performed as described (Delobel *et al.*, 2008). Briefly, the brainstem tissue was homogenized in A68 buffer (0.5 ml of 800 mM NaCl, 10% sucrose, 10 mM Tris-HCl pH 7.4, 1mM EGTA) using a Kinetica polytron. Samples were centrifuged at 5,000g for 15 min. The collected supernatant was analysed as total tau samples. Following sarkosyl addition to 1% and shaking for 1 h, samples were centrifuged at 80,000g for 30 min. The resulting pellet was resuspended in 50mM Tris-HCl pH 7.4.

For immunoelectron microscopy, aliquots were placed on carbon-coated 400 mesh grids and allowed to dry partially. Grids were blocked in droplets of 0.1% gelatin (Sigma G7041, Sigma-Aldrich, St. Louis, MO, USA) and stained with HT7 primary antibody (1:50; Pierce, Rockford, IL, USA). Grids were then washed briefly

with blocking buffer, stained with anti-mouse IgG-Gold secondary antibody (Sigma G7652), washed with water, and stained with 2% uranyl acetate. Electron microscopy was performed using a FEI Tecnai Spirit TEM at a magnification of 21,000x and images recorded using a Gatan Orius SC200B CCD camera (Gatan, Pleasanton, CA, USA).

Dot blots

For dot blots, serum of 3-months-old P301Shet and P301SxTAU62$^{on\text{-}off}$ mice was separated from the clot by centrifuging the samples at 1000 rpm for 15 minutes at 4 °C, with the remaining supernatant aliquoted and stored at -20 °C, for later usage. Then, a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, USA) was divided in a grid to allow later incubations with 3-months-old P301Shet and P301SxTAU62$^{on\text{-}off}$ sera and HT7 antibody as positive control at 1:1000 dilutions. 0.2 mg/ml of 2N4R wild-type tau monomers were applied on a 4x3 grid. Next, unspecific binding epitopes were blocked with 5% BSA in PBS-Tween, followed by a 30-minutes-long incubation at room temperature with previously extracted sera from the mice and HT7 antibody as positive control. After washing with PBS-Tween, the membrane was incubated with HRP-conjugated anti-mouse secondary antibody at room temperature, washed again in PBS-Tween, and detected by ECL (GE Healthcare, Little Chalfont, UK).

Statistical analysis

To evaluate behavioral test results statistically, one-way analysis of variance (ANOVA) followed by post-hoc Student's t-tests and Bonferroni correction for multiple comparisons were applied. P-values < 0.05 were considered significant. To estimate soluble tau and total tau expression from western blots of 3-months-old and 16-months-old mice, the protein bands were normalized to GAPDH protein standard, and quantified using ImageJ software; generated mean ratio values were compared by Student's t-test. To determine the effect of early neurotoxic stress in aged mice, AT8 and Gallyas-positive neurons were semi-quantitatively assessed in brainstem regions. Three sections per animal were analysed. The total area analysed per animal was comparable between animals (AT8: P-value = 0,34, and Gallyas: P-value = 0,91). P-values calculated by Student's t-tests were interpreted exploratory and not adjusted for multiple comparisons; P-values < 0.05 were considered significant. Box plots for figures 2 to 5 were generated with R software. The lower and upper hinges correspond to the first and third quartiles (25th and 75th percentiles). The upper whisker extends from the

hinge to the largest value no further than 1.5 times IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles). The lower whisker extends from the hinge to the smallest value at most 1.5 times IQR of the hinge. Data beyond the end of the whiskers are called "outlying" points and are plotted individually.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Co-expression of tau fragment and full-length tau induces severe reversible neurotoxicity in P301SxTAU62^{on} mice in the absence of tau fibrils

P301SxTAU62^{on} transgenic mice co-expressing human $\Delta tau_{151-421}$ with full-length P301S mutant tau were obtained by interbreeding P301S tau transgenic mice with TAU62 transgenic mice, where $\Delta tau_{151-421}$ expression is regulated by a doxycycline-responsive promoter element (Ozcelik *et al.*, 2016). 3-weeks-old heterozygous P301S transgenic littermates (P301Shet) did not exhibit signs of motor dysfunction, as also illustrated by their normal tail suspension test (Fig. 1A). In contrast, P301SxTAU62^{on} mice of the same age showed a severe hindlimb palsy, only being able to move by the use of their forelimbs (Fig. 1B). Upon halting doxycycline administration to stop $\Delta tau_{151-421}$ expression, this phenotype was fully reversible within three weeks, even though P301S tau expression continued (Fig. 1C).

Histological examination did not reveal any tau pathology in young P301Shet mice (Fig. 1A). In contrast, extensive AT8-positive pre-tangle stage tau pathology was found in paralyzed, 3-weeks-old P301SxTAU62^{on} mice (Fig. 1B). Remarkably, three weeks after $\Delta tau_{151-421}$ expression had been stopped, AT8-positive tau pathology was no longer detectable, and by the age of six weeks, P301SxTAU62^{on-off} mice had regained normal walking capability (Fig. 1C). Gallyas-Braak silver stain positive tau pathology was absent in 3-weeks-old P301Shet mice (Fig. 1A), their paralyzed P301SxTAU62^{on} littermates (Fig. 1B), as well as in recovered P301SxTAU62^{on-off} mice (Fig. 1C, for high magnification pictures see Supplementary Fig. 1).

Immunoelectron microscopy confirmed the absence of tau filaments in brainstem samples of paralyzed P301SxTAU62^{on} mice (Fig. 2). Total tau as well as sarkosyl extracts of brainstem homogenates collected from 6-months-old homozygous P301S tau transgenic mice showed HT7-positive tau filaments (Fig. 2A). In contrast, only oligomeric, non-fibrillar tau structures were detectable in total tau extracts of 3-weeks-old paralyzed P301SxTAU62^{on} mice, and no aggregated tau structures could be found in sarkosyl extracts from paralyzed P301SxTAU62^{on} mice (Fig. 2B). Extracts from non-transgenic 3-weeks-old BL6 mice were used as negative controls (Fig. 2C). These observations demonstrate that the severe, but reversible motor palsy in P301SxTAU62^{on-off} mice is mediated by non-fibrillar tau species (Ozcelik *et al.*, 2016). A graphical representation of these findings is given in Supplementary Fig. 2.

Absence of excessive motor impairment in aged P301SXTAU62$^{on\text{-}off}$ mice after recovery from early severe neurotoxicity

To study the long-term effects of the severe non-fibrillar tau stress in P301SxTAU62^{on} mice, we followed their motor capabilities after their initial recovery. As only $\Delta tau_{151\text{-}421}$ expression is under the control of a doxycycline-responsive promoter element, recovered P301SxTAU62$^{on\text{-}off}$ mice maintain expression of P301S mutant full-length tau.

We compared formerly paralyzed, recovered and aged P301SxTAU62$^{on\text{-}off}$ mice to their P301Shet littermates and to non-transgenic BL6 mice by tail suspension, rotarod, and grid tests at the age of 16 months. P301Shet littermates showed signs of hindlimb clasping, while recovered P301SxTAU62$^{on\text{-}off}$ mice were still able to spread their hindlimbs, as were non-transgenic BL6 mice (Fig. 3A).

As expected, at 16 months of age, motor balance (rotarod) was significantly reduced in both tau expressing mouse models, compared to non-transgenic BL6 mice. Somewhat surprisingly, however, we did not detect a worse motor performance of P301SxTAU62$^{on\text{-}off}$ mice in comparison to their P301Shet littermates. In contrast, formerly paralyzed P301SxTAU62$^{on\text{-}off}$ mice performed even slightly better in the rotarod test when compared to P301Shet littermates, although this did not reach statistical significance (Fig. 3B). Similar observations were made for the grid test, revealing a significantly reduced motor strength in P301Shet transgenic mice in comparison to non-transgenic BL6 mice. Again, at the age of 16 months, P301SxTAU62$^{on\text{-}off}$ mice showed a slightly better grid test performance than their P301Shet littermates, although again without statistical significance (Fig. 3C, see Supplementary Table 1 for complete values). Thus, P301SxTAU62$^{on\text{-}off}$ mice recovered from early neurotoxic tau stress and did not show increased motor impairment with aging when compared to P301Shet littermates. These findings are graphically summarized in Supplementary Fig. 2.

Absence of excessive tau pathology in aged P301SxTAU62$^{on\text{-}off}$ mice after recovery from severe neurotoxicity

We next aimed at studying whether the absence of a pronounced motor phenotype in P301SxTAU62$^{on\text{-}off}$ mice would also be mirrored by the extent of tau pathology upon aging. To this end, we comparatively analysed brains of 16-months-old P301SxTAU62$^{on\text{-}off}$ and P301Shet mice by histology. In all mice, tau pathology was most extensive in the brainstem. AT8 immunohistochemistry revealed extensive tau hyperphosphorylation in P301Shet mice whereas significantly less tau hyperphosphorylation was present in P301SxTAU62$^{on\text{-}off}$ animals (Fig. 4A, B, for high magnification pictures see Supplementary Fig. 3). Additional immunohistochemistry with the AT100 antibody revealed similar results for 16-months-old mice, confirming the significantly more abundant hyperphosphorylation in the heterozygous mice which did not experience the early neurotoxic stress as the recovered mice did (see Supplementary Fig. 4).

Gallyas-Braak silver staining revealed widespread, robust tau tangle formation in the brainstem of P301Shet mice, whereas formerly paralyzed P301SxTAU62$^{on\text{-}off}$ mice remained almost devoid of fibrillar tau pathology (Fig. 4C, D, for high magnification pictures see Supplementary Fig. 3). Semiquantitative assessment confirmed the lower extent of tau pathology in aged P301SxTAU62$^{on\text{-}off}$ compared to P301Shet mice (see Supplementary Table 2 for complete values). Immunohistochemistry with TauC3 antibody did not reveal leakage of $\Delta tau_{151\text{-}421}$ expression in 16-months-old P301SxTAU62$^{on\text{-}off}$ mice (Supplementary Fig. 4). A graphical representation of these findings can be found in Supplementary Fig. 2.

We confirmed our histological findings by western blotting, using the human tau targeting HT7 antibody. Significantly higher levels of total (Fig. 5A, B) and soluble tau (Fig. 5C, D) were detected in P301Shet mice compared to P301SxTAU62$^{on\text{-}off}$ mice at 16 months of age (see Supplementary Table 2 for complete values). Next, we wanted to rule out that the mild motor phenotype as well as the comparatively mild tau pathology in aged P301SxTAU62$^{on\text{-}off}$ mice were caused by loss of human mutant P301S tau expression. To this end, we analysed tau protein levels in these mice and their heterozygous littermates at the age of 3 months. Western blot analysis revealed comparable total tau levels in P301Shet and P301SxTAU62$^{on\text{-}off}$ mice (Fig. 6A, B; see Supplementary Table 2 for complete values). It is therefore unlikely, that the maintained motor competence of aged P301SxTAU62$^{on\text{-}off}$ mice occurs despite a potential previous loss of tau-expressing neurons in young

paralyzed P301SxTAU62^{on} mice. Nevertheless, based on western blotting, discretely reduced tau expression levels in P301SxTAU62$^{on\text{-}off}$ compared to P301Shet mice cannot be excluded. Therefore, a mildly lowered tau expression might have been contributed to the slightly better motor performance and the less extensive tau pathology in aged P301SxTAU62$^{on\text{-}off}$ mice. In light of the extensive early tau toxicity occurring in P301SxTAU62^{on} mice, an immunological reaction could also have been triggered by the toxic high molecular weight tau, and may have caused the lower tau levels in aged P301SxTAU62$^{on\text{-}off}$ mice. However, dot blot analysis did not detect any anti-tau antibodies in sera of 3-months-old P301Shet and P301SxTAU62$^{on\text{-}off}$ mice (Supplementary Fig. 5).

Brainstem homogenates from paralyzed P301SxTAU62^{on} mice do not induce NFT formation in ALZ 17 mice

Our observations made so far argued against a long-term seeding effect of the early, non-fibrillar neurotoxic tau species which caused the severe motor palsy in P301SxTAU62^{on} mice. To assess the seeding capacity of these non-fibrillar toxic tau species also in a classical tau seeding setting (Clavaguera *et al.*, 2009), we next prepared brainstem homogenates from paralyzed 3-weeks-old P301SxTAU62^{on} mice, and inoculated them intrahippocampally into 3-months-old ALZ17 tau transgenic mice. Brain homogenates derived from aged tangle-bearing homozygous P301S tau transgenic mice were used as positive controls, as these had previously been proven to provoke the formation of Gallyas-Braak silver stain-positive tangles when inoculated into ALZ17 mice (Clavaguera *et al.*, 2009). As expected, ALZ17 mice seeded with P301S brainstem homogenates developed granular focal tau pathology, primarily in CA1 (Fig. 7A) and the ipsilateral dorsal fornix above CA1 (Fig. 7B). In contrast, ALZ17 mice seeded with P301SxTAU62^{on} brainstem homogenates remained devoid of Gallyas-Braak silver stain positive structures up to the age of 20 months. This observation indicates that the non-fibrillar toxic tau species associated with the severe paralysis of P301SxTAU62^{on} mice are not seeding-competent. A graphical representation of these findings is given in the Supplementary Fig. 6.

Discussion

We here report that early neurotoxic tau stress causing severe motor paralysis in P301SxTAU62^{on} mice is fully reversible and does not cause late seeding or adverse effects in recovered P301SxTAU62$^{on\text{-}off}$ mice during aging. Furthermore, we find that the inoculation of brainstem homogenates from paralyzed P301SxTAU62^{on} mice into ALZ17 mice does not result in tau seeding in the host mice, demonstrating that non-fibrillar neurotoxic tau species can lack classical seeding competence.

Co-expression of a $\Delta tau_{151\text{-}421}$ with full-length P301S mutant tau in P301SxTAU62^{on} mice causes severe paralysis after three weeks, as we have previously reported (Ozcelik *et al.*, 2016). This motor impairment is paralleled by an increase in hyperphosphorylated tau, but occurs in the absence of fibrillar tau forms or tau tangles. Upon switching off $\Delta tau_{151\text{-}421}$ expression, hyperphosphorylated tau species are no longer detectable and P301SxTAU62$^{on\text{-}off}$ mice regain their motor function. This confirms that hyperphosphorylated non-fibrillar tau oligomers can mediate early neurotoxicity, and that clearance of these tau species rescues the paralyzed mice from their motor impairment (Ozcelik *et al.*, 2016).

While over-expression of non-aggregating tau deletion mutants lacks relevant neurotoxicity (Macdonald *et al.*, 2019), we here confirm that soluble oligomeric, yet non-fibrillar tau aggregates can exert extensive tau toxicity. This is also in line with reported clinical data: tau oligomers appear early in the brains of patients developing AD (Patterson *et al.*, 2011; Lasagna-Reeves *et al.*, 2012; Koss *et al.*, 2016) and progressive supranuclear palsy (Gerson *et al.*, 2014), possibly even before first clinical symptoms become apparent (Maeda *et al.*, 2006). Tau oligomers have also been shown to correlate well with neurodegeneration in tau transgenic mice (Gomez-Isla *et al.*, 1997; Lasagna-Reeves *et al.*, 2011; Jiang *et al.*, 2019). Based on this, oligomeric tau species are now considered to play a central role in the pathogenesis of neuronal dysfunction in tauopathies (Ghag *et al.*, 2018; Polanco *et al.*, 2018; Sengupta *et al.*, 2018; Bittar *et al.*, 2019), comparably to oligomeric structures in other neurodegenerative disorders (Hong *et al.*, 2018; Sekiya *et al.*, 2019). The severe early neurotoxic stress provoked by non-fibrillar tau forms in our P301SxTAU62$^{on\text{-}off}$ mice strengthens this view.

Counterintuitively, we here find that P301SxTAU62$^{on\text{-}off}$ mice, which have been exposed to non-fibrillar neurotoxic tau, do not show an accelerated course of their tauopathy during aging. In contrast, aged P301SxTAU62$^{on\text{-}off}$ mice show rather better motor performance and less tau pathology in comparison to their

heterozygous P301S littermates, even though P301S tau expression remains unaffected upon switching off $\Delta tau_{151\text{-}421}$ expression. To our knowledge, this is the first analysis of long-term consequences of early tau stress in a model maintaining the expression of an aggregation-prone tau form. Previous studies were conducted in mouse models conditionally expressing a single mutant human tau form, and the recovery of these mice was achieved by its suppression (Van der Jeugd *et al.*, 2012; Polydoro *et al.*, 2013; Wang *et al.*, 2018). Thus, upon switch-off, aggregation-prone human tau forms were no longer expressed in these mice – a scenario, which does not reflect the situation of potential interventional tau clearance in humans. In addition, previous studies in rTgTauEC mice have shown that NFT-associated toxicity can be reversed by suppressing tau overexpression (Polydoro *et al.*, 2013). In pro-aggregant hTau40 transgenic mice, motor fitness improved after stopping mutant tau expression (Van der Jeugd *et al.*, 2012). Similar results were obtained in PS19 mice by antisense oligonucleotide-mediated downregulation of mutant tau expression (DeVos *et al.*, 2013; DeVos *et al.*, 2017). Whereas our previous studies showed that tau oligomeric stress can result in severe motor impairment in the absence of tau fibrils (Ozcelik et al, 2016), our present findings now further separate tau toxicity from tangle formation by providing first evidence that early tau toxicity can be reverted even without subsequent seeding of fibrillar tau tangles. This indicates that halting tau oligomer-related toxicity prior to the appearance of filamentous tau can not only reverse clinical symptoms (as reflected by the motor phenotype recovery of our mice), but may also efficiently prevent long-term tau seeding and spreading. Strikingly, interventions with compounds targeting tau oligomers have already shown beneficial effects, including the reversal of brain dysfunction (Castillo-Carranza *et al.*, 2014; Soeda *et al.*, 2015; Gerson *et al.*, 2018; Lo Cascio *et al.*, 2019; Lo *et al.*, 2019). Our new findings suggest that early therapeutic tau oligomer removal might efficiently prevent the progression of tauopathies.

We cannot exclude that the lack of a late tau seeding effect by early toxic tau species in aging P301SxTAU62$^{on\text{-}off}$ mice is caused by immunological reactions targeting tau in these mice. The slower decline of motor function of recovered, aging P301SxTAU62$^{on\text{-}off}$ mice could also be associated with a protective mechanism triggered by the early neurotoxic tau stress. While a dot blot analysis of sera of P301SxTAU62$^{on\text{-}off}$ mice did not reveal any antibodies targeting recombinant tau, this does not fully rule out alternative immunological mechanisms (e.g. T-cell mediated; antibodies against specific epitopes of oligomeric tau assemblies). Furthermore, the slightly lowered tau levels in aged P301SxTAU62$^{on\text{-}off}$ mice could also be a consequence of autophagy

induction by the early oligomeric tau stress that these mice experienced; in support of the latter notion, it was previously found that pharmacological autophagy activation can defer tau pathology progression in P301S mice (Schaeffer *et al.*, 2012; Ozcelik *et al.*, 2013).

As we cannot exclude that the lack of a late tau seeding effect by early toxic tau species in P301SxTAU62$^{on\text{-}off}$ mice is attributable to immunological reactions or the induction of the autophagy pathway targeting oligomeric tau in these mice, we next investigated whether the early toxic tau species exert classical prion-like seeding competence upon intracerebral inoculation. Fibrillar tau induces the aggregation of soluble tau species in a prion-like manner upon inoculation into tau transgenic host mice (Clavaguera *et al.*, 2009; Goedert *et al.*, 2017). We inoculated ALZ17 mice with brainstem homogenates from paralyzed P301SxTAU62^{on} mice. These seeded ALZ17 mice remained completely free of fibrillar tau pathology up to high ages. In contrast, seedings with P301S brainstem homogenates induced focal granular Gallyas-Braak silver stain-positive tau aggregates, as these homogenates also contain short tau filaments, known to be the most seeding-competent tau species (Jackson *et al.*, 2016). The absence of fibril induction in ALZ17 mice inoculated with brainstem homogenates from paralyzed P301SxTAU62^{on} mice confirms that early toxic tau oligomers can lack fibrillar tau seeding competence.

At late disease stages, seeding-competent tau structures are present in most tauopathies, and capable of seeding fibrillar tau aggregates when injected into ALZ17 mice (Clavaguera *et al.*, 2013). Interestingly, the only homogenates not provoking the formation of Gallyas-positive fibrils in ALZ17 mice, comparable to our P301SxTAU62^{on} brainstem homogenates, were extracts collected from argyrophilic grain disease (AgD) patients. This has been linked to the histological predominance of pre-tangles over fully formed tangles in AgD (Ferrer *et al.*, 2008), similar to the pre-tangle stage pathology present in our paralyzed P301SxTAU62^{on} mice. A low seeding activity of AgD extracts has recently also been found in an RT-QuIC *in vitro* seeding model (Kraus *et al.*, 2019). In that study, also brain extracts from other tauopathies exerted only low seeding effects, demonstrating the presence of non-, or not-yet seeding-competent tau forms in human tauopathies including progressive supranuclear palsy (PSP) and corticobasal degeneration (CDB) (Kraus *et al.*, 2019). A comparably low seeding efficacy of PSP and CDB homogenates was found in a cell-based aggregation assay, while seeds of AD patients resulted in high tau aggregation (Chung *et al.*, 2019). In this light, our present findings warrant

caution when developing aggregation and seeding-based diagnostic assays. Very early tau forms could be toxic, but undetectable by seeding-based diagnostic tools.

In conclusion, we here demonstrate that high molecular weight tau oligomers can provoke a severe, but reversible neurotoxicity. Rescued mice do not develop long-term sequelae, which argues for early therapeutic interventions targeting oligomeric tau forms. Furthermore, we confirm that these early toxic tau species lack classical seeding competence. Therefore, caution should be exercised when using seeding-based assays for the detection of very early preclinical tauopathy manifestations. Our findings also warrant a deepened analysis of early, non-fibrillar toxic tau forms in the future. While the knowledge on filamentous tau strains is rapidly growing thanks to Cryo-EM studies in particular (Fitzpatrick *et al.*, 2017; Falcon *et al.*, 2018a; Falcon *et al.*, 2018b; Falcon *et al.*, 2019), the puzzling characteristics of non-, or not-yet fibrillar tau forms remain only incompletely understood.

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Competing interests

The authors declare no competing interests.

Author notes

DTW designed the study; AM, MF, FS and DTW designed research; AM, MF and FS performed research; AM, MF, MT, and DTW analysed data; and AM, SF and DTW wrote the paper. AM, MF and FS contributed equally to the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1: Early neurotoxic stress depends on full length and Δtau co-expression and is reversible.

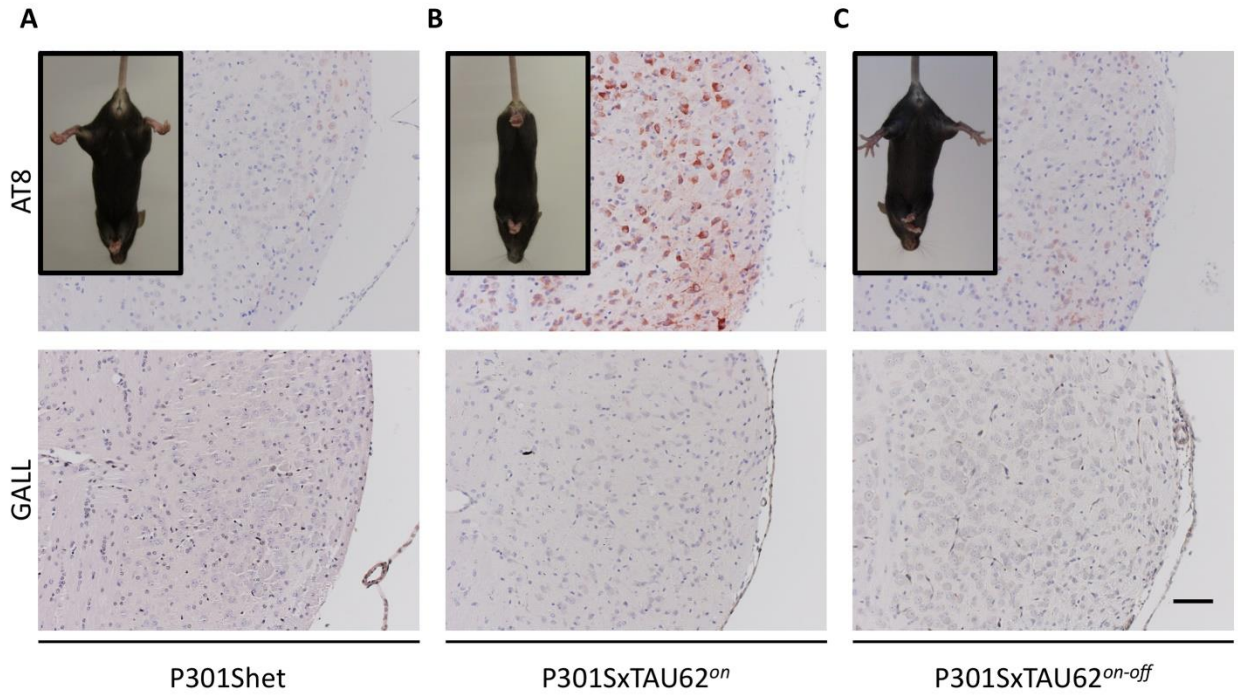


Fig.1: P301S heterozygous mice (P301Shet; n=5) at 21 days of age show no signs of paralysis in the tail suspension test, and histological tests with AT8 antibody did not detect hyperphosphorylated tau or Gallyas-positive tau fibrils in the tegmental reticular nucleus (A). Hindlimbs of P301SxTAU62^{on} (n=5) mice the same age as their heterozygous littermates are paralyzed; upon histological characterization, paralyzed P301SxTAU62^{on} mice show hyperphosphorylated tau but no Gallyas-positive tau fibrils in the same region (B). After suspending doxycycline administration for 3 weeks, P301SxTAU62$^{on\text{-}off}$ (n=5) mice recover their motor functions and do not show hyperphosphorylated or fibrillar tau (C). Altogether, the findings confirm that the severe neurotoxicity in P301SxTAU62^{on} mice does not depend on filamentous tau pathology, but solely on hyperphosphorylated tau oligomers. Scale bar equals 75 µm, and applies to A, B and C.

Figure 2: Absence of tau filaments in paralyzed P301SxTAU62^{on} mice

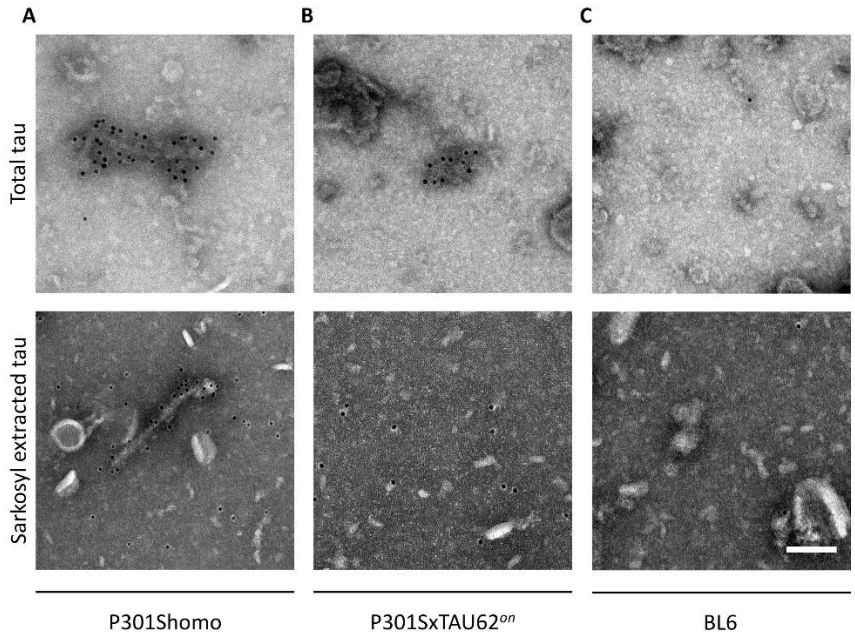


Fig.2: Immunoelectron microscopy shows HT7-positive tau filaments in total tau and sarkosyl extracts of brainstem tissue from a 6-months-old homozygous P301S tau transgenic mouse (A). Total tau extract collected from a 3-weeks-old paralyzed P301SxTAU62^{on} mouse reveals non-filamentous, oligomeric HT7 antibody positive tau structures while tau filaments are absent in its sarkosyl extract (B). Absence of tau aggregates in a 3-weeks-old BL6 control mouse (C). The findings confirm that the severe neurotoxicity in P301SxTAU62^{on} mice does not depend on filamentous tau pathology, but is linked to hyperphosphorylated tau oligomers. Scale bar equals 100 nm, and applies to A, B and C.

Figure 3: P301SxTAU62$^{on\text{-}off}$ mice do not develop an impaired motor phenotype upon aging.

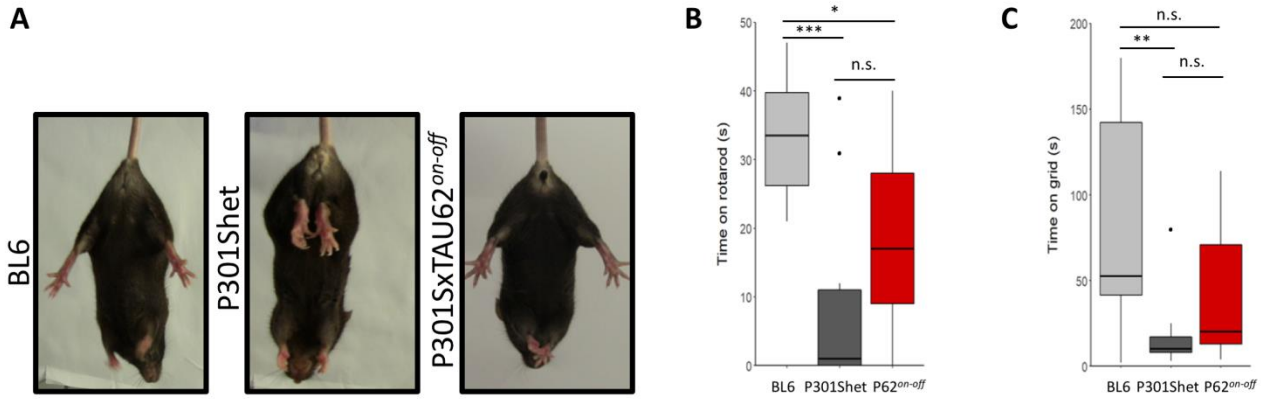


Fig.3: Tail suspension tests revealed normal hindlimb spreading for BL6 mice, and pathological hindlimb spreading predominantly in heterozygous P301S transgenic littermates at 16 months of age, while this was less pronounced in age-matched P301SxTAU62$^{on\text{-}off}$ (abbreviated P62$^{on\text{-}off}$) mice (A). Rotarod test comparing BL6 (n=10), P301Shet (n=15), and P301SxTAU62$^{on\text{-}off}$ mice (n=13) at 16 months of age revealed that P301SxTAU62$^{on\text{-}off}$ animals were not significantly more impaired than P301Shet mice which did not undergo early neurotoxic stress (B, P-value = 0,14). Grid tests of BL6 (n=10), P301Shet (n=17), and P301SxTAU62$^{on\text{-}off}$ mice (n=11) at 16 months also showed that P301SxTAU62$^{on\text{-}off}$ mice were not experiencing heavier motor impairment when compared to their heterozygous littermates (C, P-value= 0,09). n.s. = P-value > 0,05; * = P-value < 0,05; ** = P-value < 0,01; *** = P-value < 0,001. See also Supplementary Table 1.

Figure 4: Aged P301SxTAU62$^{on\text{-}off}$ mice show decreased hyperphosphorylated and fibrillar tau pathology compared to their heterozygous littermates.

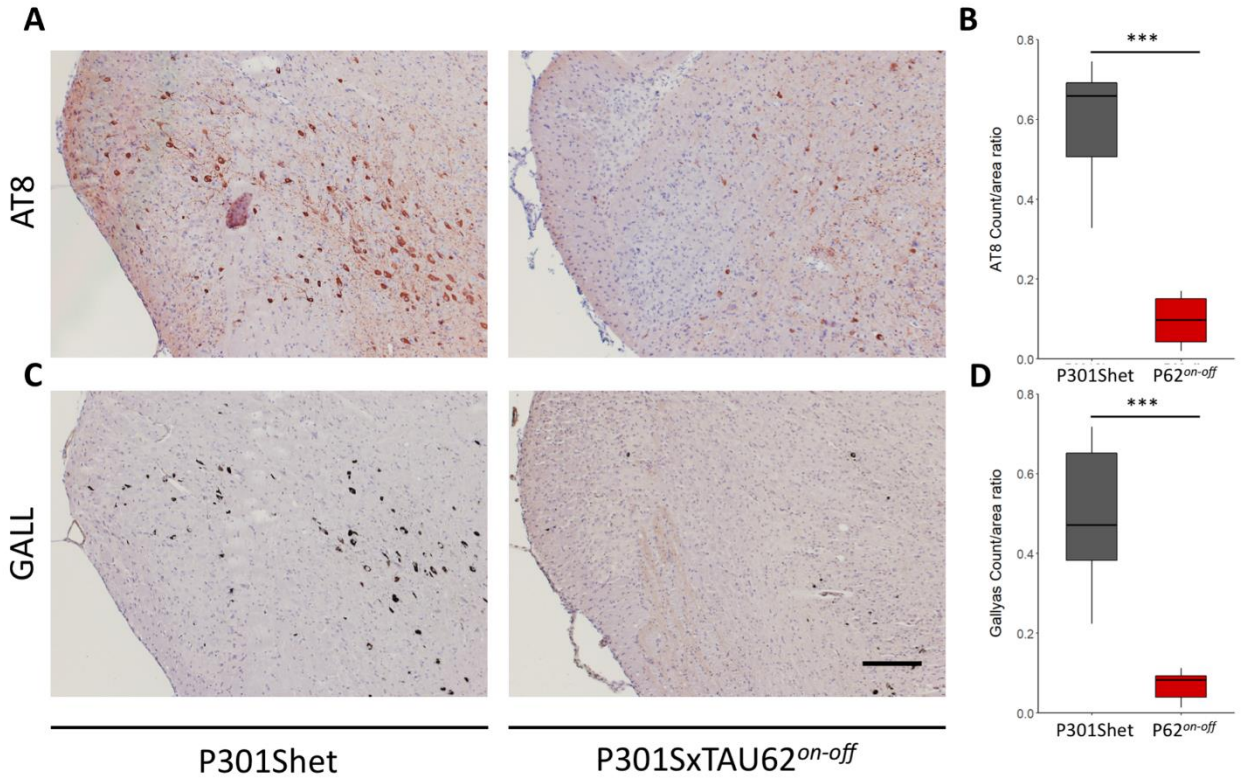


Fig.4: Histological tests comparing AT8 stained brainstem sections (A) of 16-months-old P301Shet (n=7) and P301SxTAU62$^{on\text{-}off}$ (abbreviated P62$^{on\text{-}off}$) mice (n=7) revealed that the different performance in the behavioral tests was paralleled by different degrees of hyperphoshorylated tau pathology (B, P-value = 3,39E-06). These results were also mirrored by Gallyas-stained brainstem sections (C) of 16-months-old P301Shet (n=7), and P301SxTAU62$^{on\text{-}off}$ mice (n=7), which revealed different degrees of fibrillar tau pathology between the two groups (D, P-value = 7,77E-05). *** = P-value < 0,001. Scale bar equals 200 µm, and applies to A and C. See also Supplementary Table 2.

Figure 5: Aged P301SxTAU62^{off} mice show lower total and soluble tau levels than their heterozygous littermates.

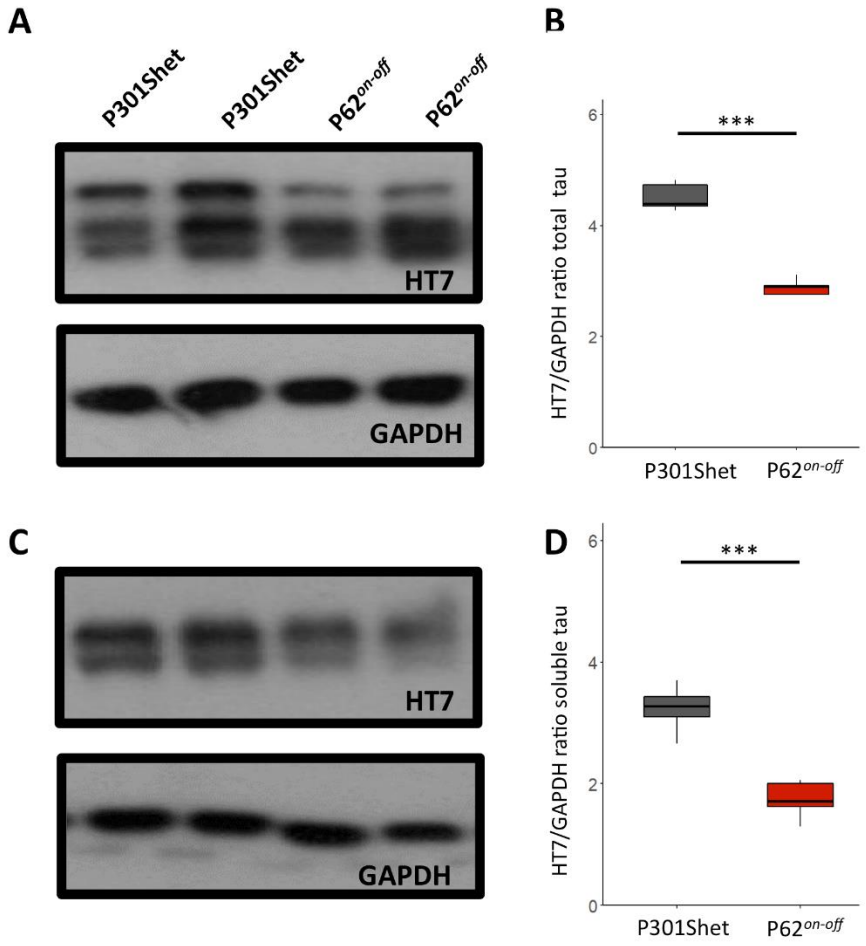


Fig.5: Western blot analysis of total tau (HT7 antibody) revealed that P301Shet mice (n=5) have a significantly higher level of total tau when compared to P301SxTAU62$^{on\text{-}off}$ (abbreviated P62$^{on\text{-}off}$) mice (n=5) at 16 months of age (A), which was confirmed by HT7/GAPDH ratio quantification (B, P-value = 1,32E-06). Soluble tau levels (C) in P301Shet mice (n=5) were also significantly higher than in P301SxTAU62$^{on\text{-}off}$ mice (n=5), as reflected by HT7/GAPDH ratio quantification (D, P-value = 0,0001). *** = P-value < 0,001. See also Supplementary Table 2.

Figure 6: Total tau levels in young P301SxTAU62$^{on\text{-}off}$ mice do not decrease compared to their heterozygous littermates.

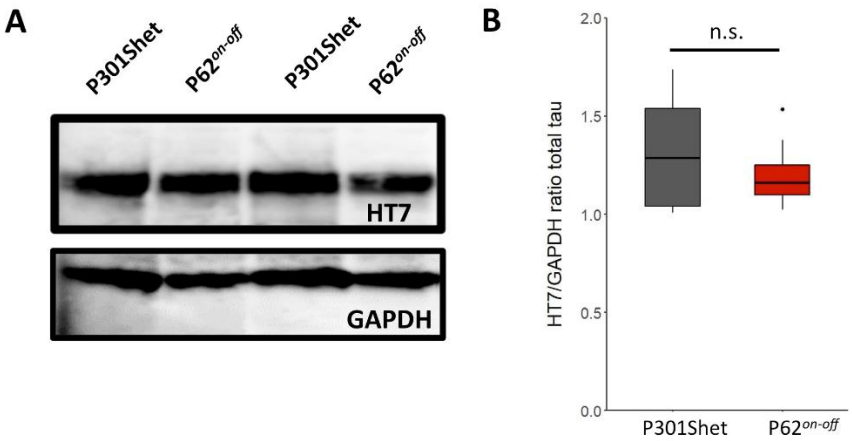


Fig.6: Western blot analysis (HT7; A) and HT7/GAPDH ratio (B) for both P301Shet (n=6) and P301SxTAU62$^{on\text{-}off}$ (abbreviated P62$^{on\text{-}off}$) mice (n=6). Tau levels were not significantly different between the two groups (P-value = 0,35). n.s. = P-value > 0,05. Box plots with hinges, whiskers and outliers. See also Supplementary Table 2.

Figure 7: Seeding with high molecular weight tau results in complete absence of fibrils in ALZ17 mice.

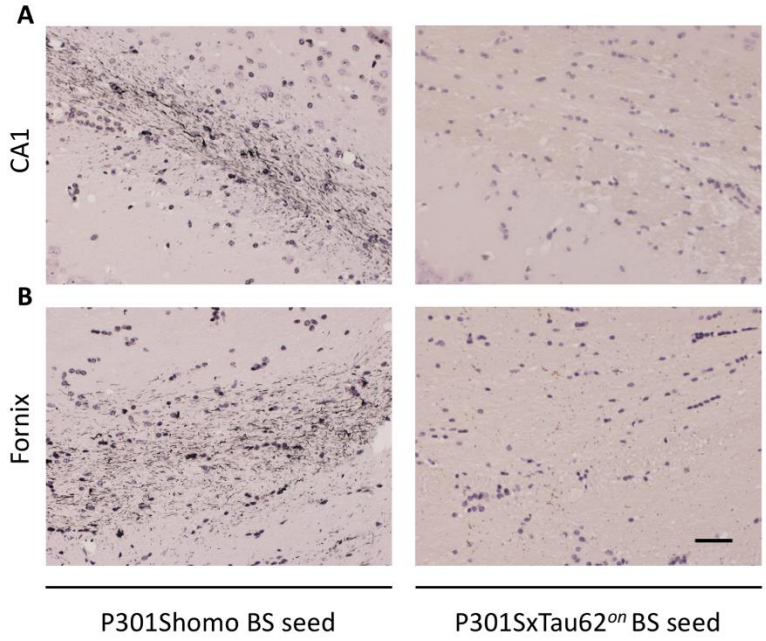
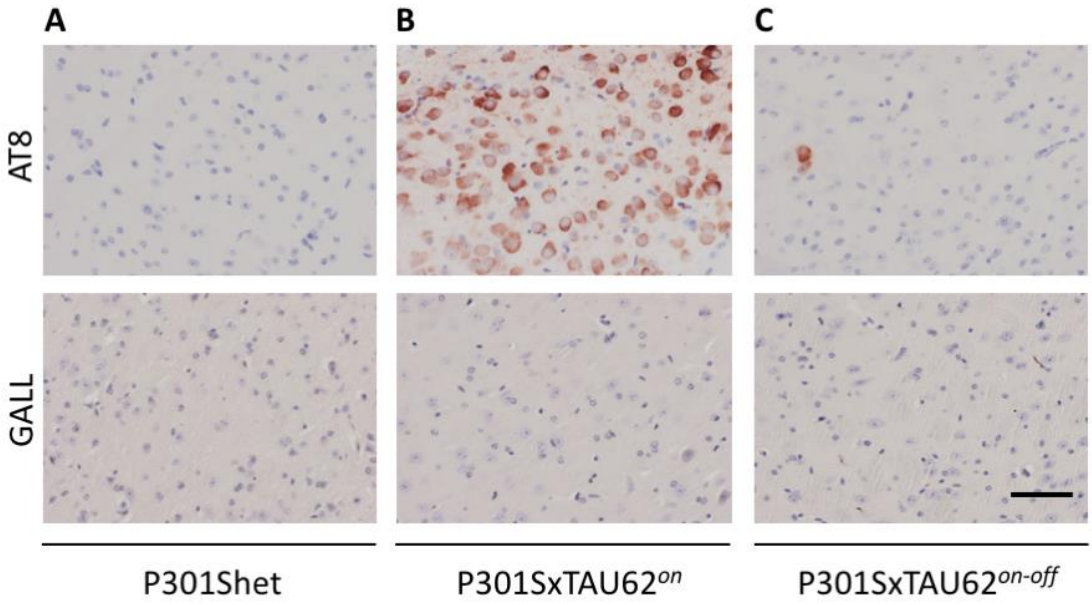


Fig.6: ALZ17 mice seeded with brainstem homogenates from paralyzed P301S homozygous (abbreviated P301Shomo) mice (n=5) revealed granular focal tau pathology in CA1 (A), which was not detected in ALZ17 mice seeded with P301SxTAU62^{on} brainstem homogenate (n=4). Granular focal tau pathology was also found in the fornix (B), and Gallyas-positive structures were detected in ALZ17 mice seeded with brainstem homogenates of paralyzed P301Shomo mice, but not in ALZ17 mice seeded with P301SxTAU62^{on} brainstem homogenate. Scale bar equals 37,5 µm, and applies to A and B.

Supplementary Material

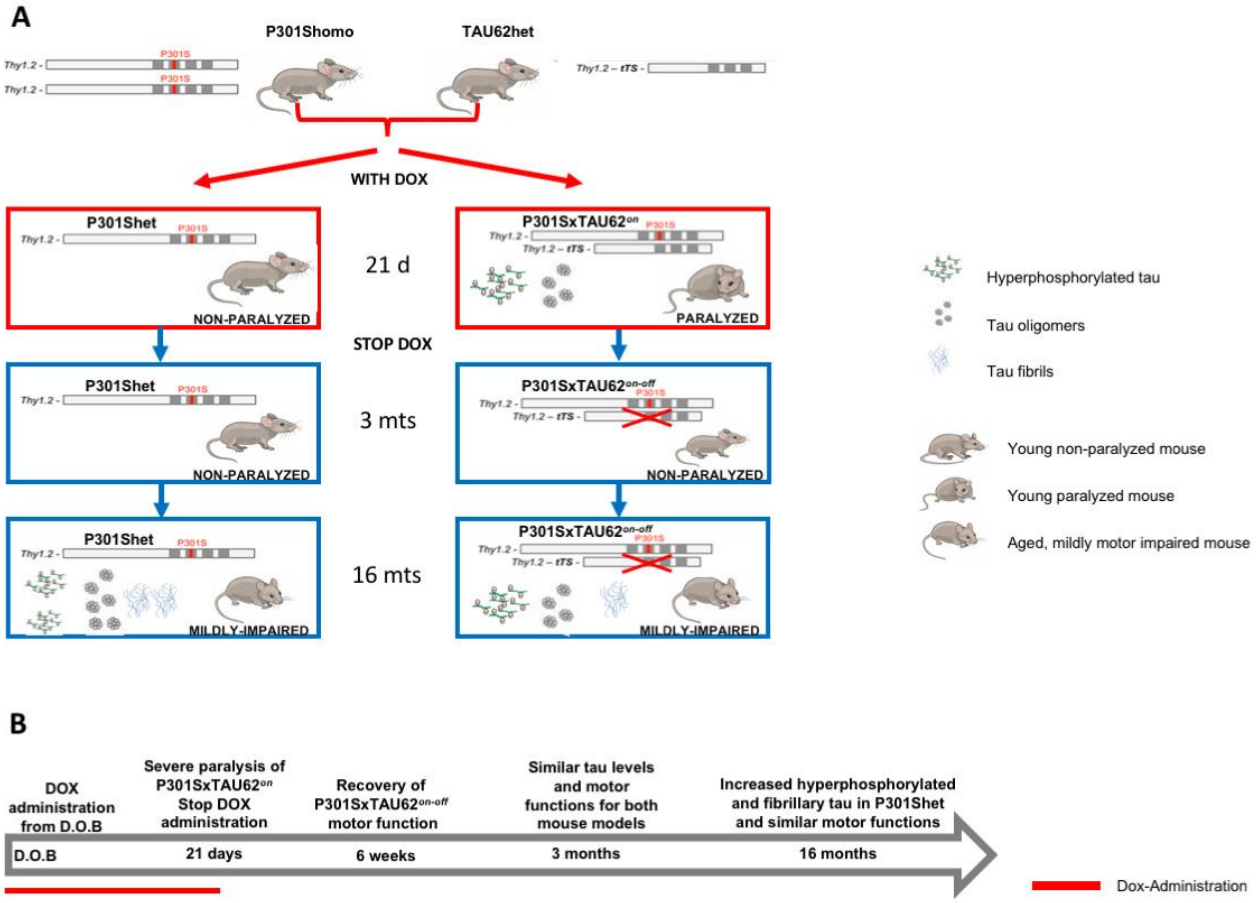
Supplementary Figure 1:



Suppl. Fig. 1: High magnification images extending the findings shown in Figure 1.

P301S heterozygous mice (P301Shet; n=5) at 21 days of age show no signs of hyperphosphorylated tau or Gallyas-positive tau fibrils in the tegmental reticular nucleus (A). Paralyzed P301SxTAU62^{on} mice (n=5) show hyperphosphorylated tau but no Gallyas-positive tau fibrils in the same region (B). After suspending doxycycline administration for 3 weeks, P301SxTAU62$^{on\text{-}off}$ mice (n=5) do not show hyperphosphorylated or fibrillar tau (C). Scale bar equals 50 µm, and applies to A, B and C.

Supplementary Figure 2:

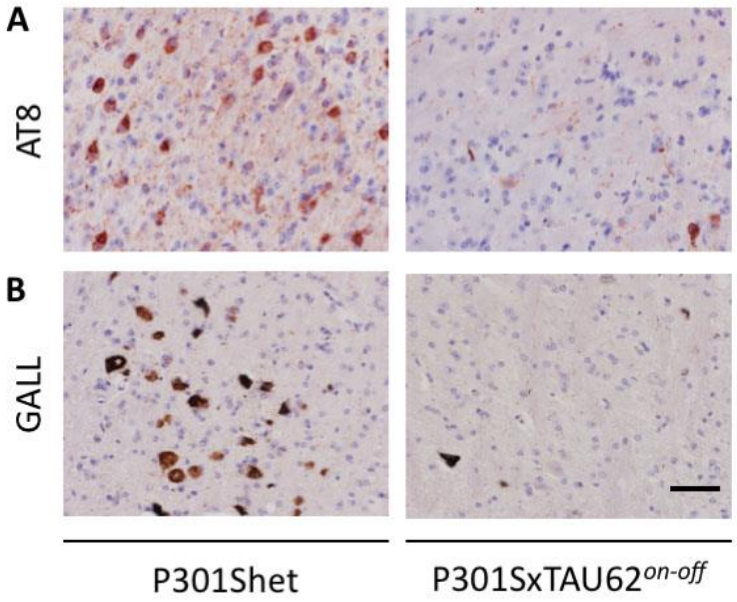


Suppl. Fig. 2: Schematic of background and main conclusions showing no long-term sequelae of reverted tau toxicity.

P301SxTAU62^{on} mice co-express human P301S mutant tau and, only under oral doxycycline (DOX) administration, $\Delta tau_{151\text{-}421}$ fragment. This results in hyperphosphorylated tau oligomers that cause a severe paralysis after 21 days. If these mice are fed normally, they only express heterozygous mutant P301S tau but not the tau fragment, and are thus comparable to P301Shet mice which remain without oligomeric tau formation or severe paralysis after 21 days. After expression of the doxycycline-responsive $\Delta tau_{151\text{-}421}$ fragment is switched off (P301SxTAU62$^{on\text{-}off}$), hyperphosphorylated tau oligomers are cleared efficiently and mice recover their motor function in the following 3 weeks, even though mutant P301S tau expression is being maintained. Therefore, P301SxTAU62$^{on\text{-}off}$ mice exhibit a similar phenotype as P301Shet mice at three months of age. At 16 months of age, P301SxTAU62$^{on\text{-}off}$ mouse motor functions do not differ significantly from P301Shet mice, while tangles and hyperphosphorylated tau oligomers are significantly less abundant in

P301SxTAU62$^{on\text{-}off}$ compared to P301Shet mice. This proves that P301SxTAU62$^{on\text{-}off}$ mice do not experience sequelae of the early neurotoxic stress, when compared to their heterozygous P301S tau transgenic littermates.

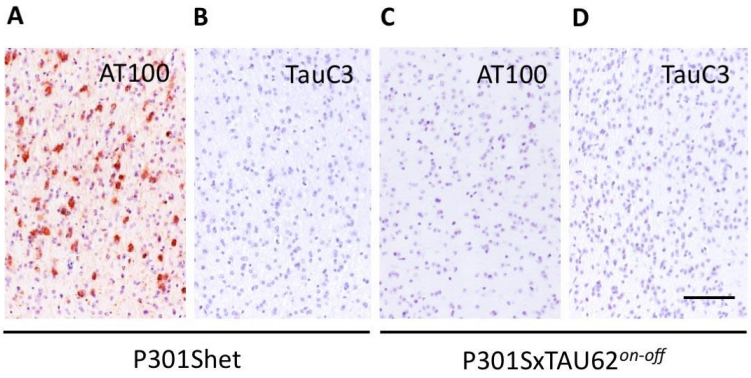
Supplementary Figure 3:



Suppl. Fig. 3: High magnification images extending the findings shown in Figure 3.

Aged P301SxTAU62$^{on\text{-}off}$ mice exhibit less AT8 and Gallyas-positive neurons than their heterozygous littermates. AT8 (A) and Gallyas silver (B) stained brainstem sections of 16-months-old P301Shet mice (n=7) show more hyperphosphorylated tau and silver stain positive pathology than P301SxTAU62$^{on\text{-}off}$ mice (n=7). Scale bar equals 50 µm, and applies to A and B.

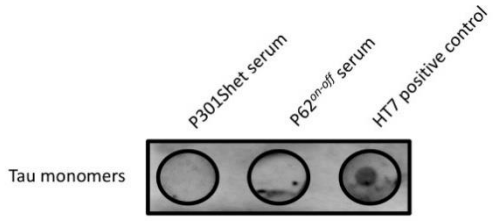
Supplementary Figure 4:



Suppl. Fig. 4: Immunohistochemistry targeting the AT100 phospho-epitopes at Thr 212 and Ser 214, and Δtau (TauC3 antibody) in 16-months-old mice.

In parallel to Gallyas silver stain positivity (Suppl. Fig. 3), extensive hyperphosphorylation of AT100-positive tau was seen in the brainstem of heterozygous P301S littermates (n=7) (A), but was almost absent in P301SxTAU62$^{on\text{-}off}$ mice (n=7) (C). Immunohistochemistry with TauC3 antibody did not find any tau fragment in non-fragment expressing P301Shet mice (n=7) (B), nor were signs of fragment expression leakage detectable in P301SxTAU62$^{on\text{-}off}$ mice (n=7) (D). The scale bar corresponds to 100 µm, and applies from A to D.

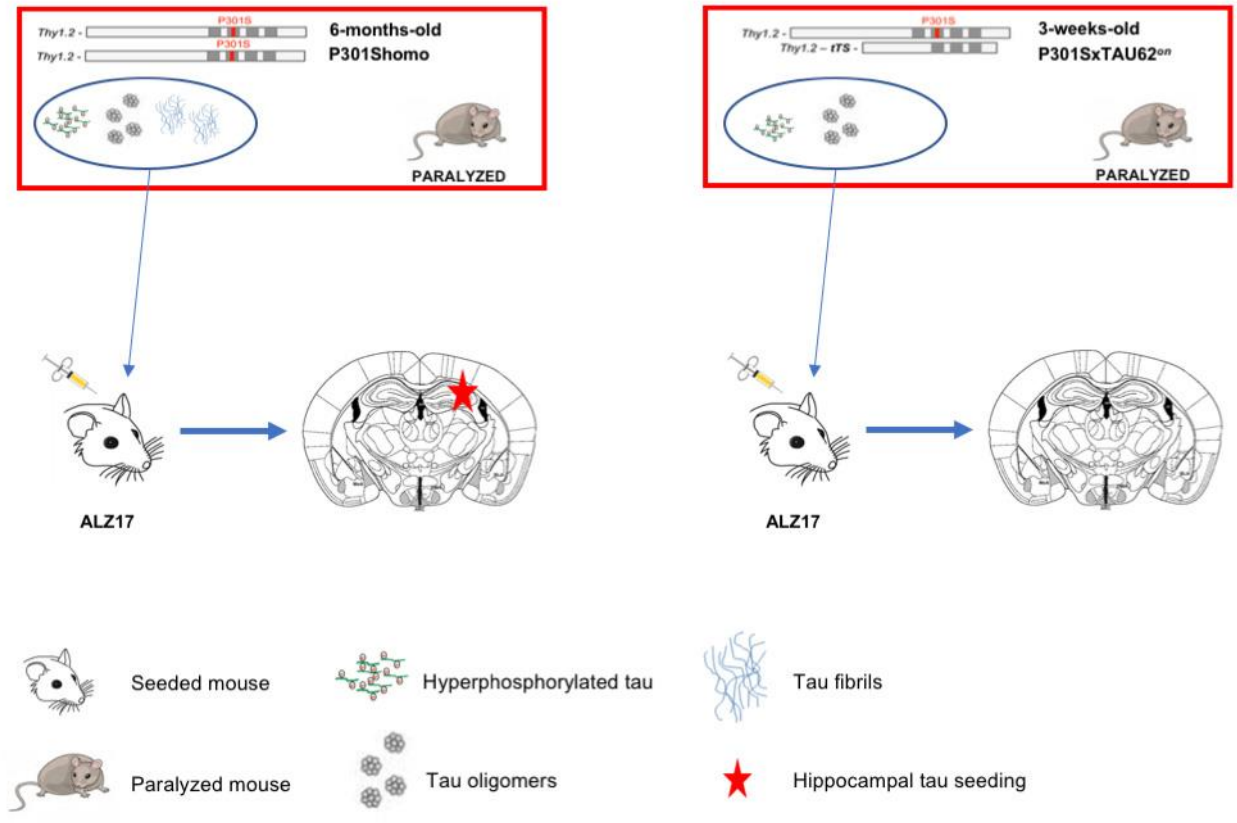
Supplementary Figure 5:



Suppl. Fig. 5: 3-months-old P301Shet and P301SxTAU62$^{on\text{-}off}$ mice do not show anti-tau antibodies in their sera.

Dot blot with the sera of 3-months-old P301Shet (n=4) and P301SxTAU62$^{on\text{-}off}$ (labelled P62 $^{on\text{-}off}$; n=3) mice was conducted, to test for the presence of anti-tau antibodies, using HT7 antibody as a positive control. 2N4R wild-type tau monomers were applied on the membrane and both sera did not show any immunological activity.

Supplementary Figure 6:



Suppl. Fig. 6: Schematic of background and main conclusions showing that oligomeric toxic tau species can lack seeding competence.

As positive control, ALZ17 mice were injected with tau fibril-containing brainstem homogenates of paralyzed, 6-months-old P301S mice (left box). These ALZ17 control mice developed granular focal tau pathology in the injected hippocampus. Next, we analyzed the seeding capacity of oligomers resulting from co-expression of P301S tau and $\Delta tau_{151\text{-}421}$. To this end, we inoculated ALZ17 transgenic mice with brainstem homogenates collected from paralyzed, 3-weeks-old P301SxTAU62^{on} mice, which do not show fibrillary tau pathology (right box). ALZ17 mice seeded with P301SxTAU62^{on} mice brainstem homogenates did not develop focal tau aggregates in the inoculated hippocampus. This proves that brainstem tissue of paralyzed P301SxTAU62^{on} mice lacks *in vivo* tau seeding competence.

Supplementary Table 1:

TEST	***Mean time ± STD BL6***	***Mean time ± STD P62^{on-off}***	***Mean time ± STD P301Shet***	***P-value ANOVA***	***P-value BL6 vs P301Shet***	***P-value BL6 vs P62^{on-off}***	***P-value P301Shet vs P62^{on-off}***
Rotarod 16 months	33 s ± 8,77 s	17,46 s ± 12,78 s	7,73 s ± 12,01 s	2,8E-05 (***)	2,5E-05 (***)	0,01 (*)	0,14 (n.s)
Grid 16 months	81,9 s ± 65,14 s	40,27 s ± 38,84 s	15,35 s ± 17,58 s	0.001 (**)	0,001 (**)	0,4 (n.s)	0.09 (n.s)

Suppl. Table 1: Overview on behavioral tests.

The table shows the overview of the statistical values of the behavioral tests executed on P301S heterozygous mice (P301Shet), P301SxTAU62^{on-off} mice (P62^{on-off}) and BL6 mice; n.s = P-value > 0,05; * = P-value < 0,05; ** = P-value < 0,01; *** = P-value < 0,001.

Supplementary Table 2:

TEST	***Mean ± STD P301Shet***	***Mean ± STD P62^{on-off}***	***P-value P301Shet vs P62^{on-off}***
AT8 Count/area 16 months	0,59 ± 0,15	0,10 ± 0,06	3,39E-06 (***)
Gallyas Count/area 16 months	0,50 ± 0,19	0,07 ± 0,04	7,77E-05 (***)
HT7/GAPDH signal total tau WB 16 months	4,51 ± 0,24	2,88 ± 0,15	1,32E-06 (***)
HT7/GAPDH signal soluble tau WB 16 months	3,23 ± 0,39	1,71 ± 0,31	0,0001 (***)
HT7/GAPDH signal WB 3 months	1,32 ± 0,3	1,20 ± 0,17	0,35 (n.s)

Suppl. Table 2: Overview on immunohistochemistry and western blots.

The table shows the overview of the statistical values of the quantified stained sections and western blots on 3 and 16 months old P301S heterozygous (P301Shet) mice and P301SxTAU62^{on-off} (P62^{on-off}) mice; n.s = P-value > 0,05; * = P-value < 0,05; ** = P-value < 0,01; *** = P-value < 0,001.

4) Discussion

Alzheimer's disease (AD) is the most prevalent form of senile dementia with a global prevalence estimated to be above 30 millions (WHO, 2015). Its death toll is constantly rising, as it remains among the most frequent causes of death in the world, and the researchers even suspect that the official number are an underestimation of the real number of deaths associated to AD (James et al, 2014). Nowadays, the actual lack of therapeutic strategies resulting into useful treatments limits pharmacological therapies to symptomatic interventions, and with millions of people continuously diagnosed worldwide, AD is considered an enormous source of social, health and economic problems (Scheltens et al, 2016).

Therefore, the research to understand the basic mechanisms behind the origin and development of the disease is fundamental, in order to establish successful therapeutic strategies to diminish its incidence. In this regard, AD has been particularly challenging. The pathological hallmarks of the disease have been established more than a century ago, since the observation of Prof. Alzheimer on fibrillary tangles and amyloid plaques. In time, the proteins constituting these neurological lesions have been identified, respectively, as the microtubule stabilizer tau and the resulting product from the processing of amyloid precursor protein (APP), amyloid β (Aβ).

However, it has been more than 15 years since the last newly marketed drug against memory loss and AD (McShane et al, 2019), and during the years, therapies targeting Aβ have all failed soundly. Therefore, research has switched towards an increased attention on tau role on AD development. In this regard, tau recently-proven ability to seed pathological fibrils through anatomically connected regions (Clavaguera et al, 2009; Clavaguera et al, 2013) has opened new doors for the understanding of the disease and for hypothetical new therapeutic strategies stopping tau spreading. However, new aspects of tau pathology in AD have risen, particularly regarding the conformation of pathological tau, which is diversifying from the simple involvement of fibrils: tau toxicity has indeed been related also to oligomeric forms, which precede tau tangles and seem to be toxic by themselves. Furthermore, tau fragmentation has been found to participate to the neurotoxicity of both tau fibrils and oligomers, pointing the researchers' attention also on cleavage patterns of the microtubule stabilizer.

It is also difficult to diagnose AD, as the certainty of it comes only from post-mortem analysis, even though the quality of the diagnostic tests and instruments is constantly increasing. Apart from the imaging techniques and cognitive tests, biochemical biomarkers are becoming more reliable and suitable to track to understand the progression of the disease. On behalf of this, cerebrospinal fluid (CSF) tau is one of the most utilized and studied biomarkers nowadays, and the presence of

fragmented forms of tau also puts tau cleavage patterns into the spotlight for the understanding of the disease.

4.1) Tau fragmentation and the role in tau oligomer-related toxicity

The first presented results confirmed that high molecular weight tau oligomers cause a severe motor phenotype in tau transgenic mouse models, which is reversible and does not result neither in later motor adverse effects, nor in seeding effects. To conduct our study, we built a double transgenic mouse line, coming from previous papers published in our group, interbreeding P301S mice, carrying the mutant P301S mutation on MAPT gene (Allen et al, 2002), and TAU62 mice, which express Δtau151-421 fragment under a conditional $Thy_{1,2}$ promoter, activated by doxycycline administration (Ozcelik et al, 2016). The resulting line, P301SxTAU62^{on} mice, undergoes through a sever motor palsy, after three weeks, that can be reversed after stopping the expression of the $\Delta tau_{151\text{-}421}$ fragment, while the expression of human mutant P301S tau is ongoing. This is obtained in three weeks after halting the administration of doxycycline. This early neurotoxic stress does not cause later adverse effects in the recovered P301SxTAU62$^{on\text{-}off}$ mice, neither in the form of motor impairments, nor in the form of tau tangles. Injecting P301SxTAU62^{on} brainstem homogenates in ALZ17 mice did not result in tangles either, thus proving that our high molecular weight tau is not seeding competent.

Tau oligomers have been proven to appear in the brain of AD patients (Lasagna-Reeves et al, 2012b) and progressive supranuclear palsy (Gerson et al, 2014). These oligomers have been shown to correlate well with neuronal death in aged mice (Lasagna-Reeves et al, 2011) and to correlate very well with clinical symptoms in AD patients, even more than tangles (Dujardin et al, 2020). Moreover, oligomers are generally thought to initiate disease development in tauopathies (Lasagna-Reeves et al, 2012) and even to appear before the first clinical symptoms (Maeda et al, 2006). Tau oligomeric toxicity has been hypothesized to depend on the conformation of the oligomers (Lo et al, 2019) and can also be aggravated by biological interactions with other complexes, such as chaperones (Oroz et al, 2018), RNA-binding proteins (Jiang et al, 2019), and nuclear complexes (Eftekharzadeh et al. 2018); furthermore, immunological intervention against oligomers has shown to stop tau seeding *in vitro* (Abskharon et al, 2020). Based on this, oligomeric tau species are now considered to play a central role in the pathogenesis of neuronal dysfunction in tauopathies (Ghag et al, 2018; Polanco et al, 2018; Sengupta et al, 2017; Bittar et al, 2019), comparably to oligomeric structures in other neurodegenerative disorders (Hong et al, 2018; Sekiya et al, 2019).

The severe early neurotoxic stress experienced by P301SxTAU62$^{on\text{-}off}$ mice strengthens this hypothesis as it relies solely on tau oligomers and can be reversed by cleaning said tau oligomers, with no formation of tangles during neurotoxicity. After exposing our P301SxTAU62 mice to this early

neurotoxic stress, we found that these mice exhibit a less pronounced motor phenotype at 14 and 16 months of age compared to their heterozygous P301S littermates that did not experience previous compelling neurotoxicity. Aged P301SxTAU62off also show an absence of increased tau pathology, no formation of tangles, and less total and soluble tau protein levels compared to P301Shet mice. Our P301SxTAU62$^{on\text{-}off}$ model therefore suggests that there is no later effect after clearing the mice of high molecular weight tau forms consisting of toxic oligomers, even while still expressing a mutant human tau form, such as P301S tau.

To our knowledge, this is a surprising result when compared to previous studies in mouse models with conditional expression of mutant human tau, as recovery of mice was obtained only by suppressing the expression of said mutant tau forms (Polydoro et al, 2013; Wang. X et al, 2018; Van der Jeugd et al, 2012). Previous studies in rTgTauEC mice have shown that NFT-associated toxicity can be reversed by suppressing tau overexpression (Polydoro et al 2013), and that early intervention in rTg4510 mice (Wang. X et al, 2018) and in pro-aggregant hTau40 mice (Van der Jeugd et al, 2012) can improve the motor fitness of these models when suppressing mutant human tau forms. In our study, tau neurotoxicity is unrelated to tangles and it is reversed by halting Δtau151-421 fragment expression alone. Our P301SxTAU62$^{on\text{-}off}$ mice do not need to suppress mutant human P301S tau heterozygous expression to recover from early neurotoxic stress and maintain their motor function in later stages of life, thus showing that mutant tau expression can be tolerated even in adult age if high molecular weight toxic combinations of tau have been previously cleared from the mouse brain.

It is important to remark that clearance of tau oligomers in P301SxTAU62$^{on\text{-}off}$ mice surprisingly does not result into any later development of pathological tau forms. Previous studies in rTg4510 mice have shown tau tangles could be dissociated from neuronal dysfunction (SantaCruz et al, 2005); particularly, cognitive functions are recovered once expression of transgenic P301L tau is halted, but tau tangles continue to accumulate (SantaCruz et al, 2005). Our findings confirm that early tau neurotoxicity can occur in the absence of tangles and is potentially reversible, but more importantly, we here show for a first time that reversible soluble neurotoxic tau species do not necessarily lead to formation of tangles as long term sequelae after the neurotoxic stress. This adds further knowledge on mutant human tau progression in mouse models and may bring to new therapeutic strategies in human patients. It is known in humans that tau oligomers appear in prefrontal cortex at Braak stage 1, preceding the formation of tangles (Maeda et al, 2006) which are confined in trans-entorhinal cortex at that stage (Braak & Braak, 1991). Oligomers are thought to act as intermediates for assembly of tangles in later Braak stages (Maeda et al, 2007). Stopping their conversion to NFT in mice by acting with small molecule compounds on tau oligomers reduced neuronal loss and reversed brain dysfunction (Soeda et al, 2015), thus giving hope that halting oligomer-related toxicity before appearance of NFT could indeed yield therapeutic applications.

To test whether or not our toxic tau forms were seeding competent, we injected brainstem homogenates containing high molecular weight tau species of P301SxTAU62^{on} mice in ALZ17 mice, devoid of fibrillary pathology. This resulted in ALZ17 mice being completely free of fibrillary tau pathology, particularly when compared to the same mice seeded with P301S brainstem homogenates, which show focal granular pathology in CA1 and fornix. Our experiments therefore prove that toxic tau oligomers do not necessarily have to be seeding competent and this early co-expression might halt seeding competence of tau oligomers and spreading of tau pathology through anatomical networks in the brain.

Tau spreading mechanisms are not fully understood yet (Mudher et al, 2017). Tau propagation has been shown to occur in different ways comparing various tauopathies (Sanders et al, 2014). Seeding experiments with P301SxTAU62^{on} brainstem homogenates follow previous similar experiments with brainstem homogenates of various tauopathies being injected into the same fibrils-free ALZ17 mice (Clavaguera et al 2013). Our particular combination of $\Delta tau_{151\text{-}421}$ and mutant P301S tau was previously proven as highly neurotoxic but not fibrillogenic, and here we add further results showing that it does not have any seeding potential when injected in ALZ17 mice, thus proving that high molecular weight toxic tau species can also be not-seeding competent.

Many fibrillogenic brainstem homogenates proved capable of spreading tau fibrils when injected into ALZ17 mice (Clavaguera et al, 2013); interestingly, the only ones not yielding Gallyas-positive fibrils, just like our injected P301SxTAU62^{on} brainstem homogenates, were argyrophilic grain disease (AGD) extracts. This could have been linked to AGD histological features, consisting mainly of pre-tangles rather than fully formed tangles (Ferrer et al, 2008). It may be that Gallyas-negative brain extracts, or extracts containing very few tangles, are not able to cause formation of fibrils and their subsequent spreading in mice. Previously, tau oligomers prepared *in vitro* have been proven to propagate when injected in BL6 mice, and cause neurodegeneration and β-sheet-rich neurotoxic aggregates (Lasagna-Reeves et al, 2011). β-sheet structures were also proven to be necessary for mutant human P301S tau aggregation in transgenic mice (Macdonald et al, 2019). Our P301SxTAU62^{on} brainstem homogenates may not seed due to the inability of our high molecular weight tau oligomers to form enough β-structures and induce tau aggregation in ALZ17 mice. Another explanation could be related to cellular mechanism of propagation, as tau is mainly thought to spread thanks to exosomal secretion (Wang. J et al, 2017). This same process can lead tau to cerebrospinal fluid (Saman et al, 2012), without losing its seeding potential (Skachokova et al, 2019). This shows that exosomes maintain seeding capacity through different anatomical networks and brain compartments, even though tau is differentially phosphorylated in said exosomes compared to pathological brain cells (Polanco et al, 2016). It is possible that our specific high molecular weight tau oligomers cannot be transmitted via exosomes, thus explaining lack of seeding competence.

Oligomer-related toxicity, particularly related to fragmentation is not unprecedented in neurodegenerative diseases. The second most common neurodegenerative disorder in the world, behind AD, is Parkinson's disease (PD) (Blauwendraat et al, 2020), which is characterized by a massive cell death of dopaminergic neurons in the substantia nigra caused by pathogenic inclusions known as Lewy bodies (Deuschl & deBie, 2019). However, it has also been hypothesized that oligomers could initiate toxic pathways in PD as well (Chen et al, 2015). β-sheets oligomers cause a significant loss of dopaminergic neurons (Meade et al, 2019), and fragments of α-synuclein can contribute decisively to fibrillogenic aggregation in PD (Guerrero-Ferreira et al, 2018). The link between fragmentation and oligomerization was recently established more in detail, when it was proven that fragments of α-synuclein can recruit monomers to constitute pathogenic oligomers and even worsen the pathological condition in mouse models of PD (Froula et al, 2019). The intervention on said fragments was shown to halt the aggregation of fibrils and to have a therapeutic potential against PD (Froula et al, 2019).

Another similar case of pathogenic assembly linked to fragmentation, particularly complexing full-length proteins and fragments, occurs in TDP-43 proteinopathies, such as amyotrophic lateral sclerosis (ALS) (Wang.X et al, 2015; Li.Q et al, 2015). Although the pathogenic mechanism of ALS has yet to be fully understood, TDP-43 accumulates into cytoplasmic inclusions which are highly cytotoxic; particularly, the aggregates are formed mainly by the full-length protein and a 25 kDa C-terminal fragment (CTF-25), which appears to be highly neurotoxic (Li.Q et al, 2015). This CTF-25 fragment is thought to enhance endoplasmic reticulum (ER) stress-mediated apoptosis in the neurons, thus increasing neuronal cell death (Wang.X et al, 2015). Recently, toxic oligomers formed by TDP-43 have also been linked to aggregation initiation in ALS (French et al, 2019), and it is coherent with previous observations of C-terminal fragments of the same protein guiding pathological aggregation (Shimonaka et al, 2016), thus also bringing to light a possible link between fragmentation, oligomerization and initiation of neurotoxic pathways. Altogether, the results presented in the thesis follow therefore nascent lines of research where oligomers are questioning the importance of established hallmarks in neurodegenerative diseases, and oligomerization might be indeed linked to fragmentation of protein at the center of neurological proteinopathies.

4,2) CSF tau and its seeding properties

The second presented results followed previous studies on the seeding properties of CSF-derived amyloid aggregates of our group, and goes further in the analysis by testing CSF-derived tau and its seeding capabilities. Our quest for the evidence for *in vivo* seeding activity of CSF tau follows recent findings on the ability of CSF tau to induce seeding-like conformational changes *in vitro* (Saijo et al, 2017; Takeda et al. 2016). Ventricular CSF samples collected post-mortem at autopsies of AD

patients have been inducing tau aggregation in HEK293 cells expressing a P301S tau repeat domain (Takeda et al, 2016). CSF tau derived from Pick's disease patients has furthermore been found to induce real-time quacking conversion of a 3 repeat tau fragment (Saijo et al 2017). In vitro seeding activity in cellular systems, or prion-like substrate conversion capacity by real-time quacking induced conversion (RT-QuIC), has furthermore recently been demonstrated for CSF in the context of other neurodegenerative proteinopathies, including sporadic Creutzfeld Jakob Disease (CJD) (Foutz et al, 2017), and Huntington's Disease (HD) (Tan et al, 2015).

We therefore investigated *in vivo* seeding properties of CSF tau. Particularly, we tested whether human CSF from AD patients exhibits signs of *in vivo* seeding competence when inoculated into P301S mice. In the setting of an explorative clinical trial, high volume samples of 15ml CSF were collected from 23 consecutive patients of an outpatient memory clinic. Out of this prospective cohort, we identified 5 patients fulfilling the criteria for "Probable AD dementia" or MCI – core clinical data", constituting the AD group, and 4 age-matched patients with a diagnosis "Dementia-unlikely to be due to AD" or "MCI-unlikely due to AD" (McKhann et al, 2011), attributable to the control group.

AD patients' derived CSF provoked a significant increase in hippocampal tau hyperphosphorylated neurons, as well as a significant increase in Gallyas positive hippocampal neurons. Furthermore, injection of concentrated human AD CSF samples into pre-tangle stage P301S mice resulted in focal granular, dot-like tau accumulation particularly in the fimbria and the dentate gyrus in a subset of the inoculated mice, very similar to earlier findings in PS19 mice seeded with brain extracts from a human CBD patient (Boluda et al, 2015). Punctate Gallyas silver staining positive dot-like structures indicative of axonal tau aggregates accumulated in the dorsal fornix ipsilateral to the injection site in two AD-CSF inoculated mice, reminiscent of a similar pathology induced in the fornix of P301S mice seeded with P301S donor brain extracts (Ahmed et al, 2014).

The reported seeding competence of CSF tau contrasts our earlier aforementioned findings in seeding experiments with Aβ. There, extensive long-term seedings, inoculating concentrated human or murine Aβ comprising CSF into APP transgenic host mice, failed to provide evidence for the presence of bioactive Aβ seeds in the CSF compartment (Fritschi et al, 2014; Skachokova et al, 2015). The here found biological seeding activity of CSF tau is however not entirely surprising. In contrast to Aβ that aggregates broadly in the brain parenchyma and the perivascular space, tau is readily drained towards the CSF compartment. Opposite to Aβ, tau levels increase in the CSF with progressive Alzheimer's disease (Olsson et al, 2016). Tau is also known to be present in the CSF compartment in its post-translationally modified, hyperphosphorylated form, as a first evidence of an efflux of pathologically altered tau species from the brain towards the CSF.

The presence of bioactive tau seeds in the CSF of AD patients suggests a diagnostic use of the aggregation induction capacity of CSF tau in tauopathies. In contrast to brain tissue, CSF can easily be collected from patients with a low rate of side effects through standardized procedures (Disanto

et al, 2017). The simple analysis of CSF tau levels is an established part of the diagnostic criteria for AD, but it lacks yet high specificity and is unable to provide prognosis at early or even pre-symptomatic disease stages (Olsson et al, 2016). The latter would however be important also for the development of novel therapies. Our detection of bioactive tau seeds in human CSF of AD patients augurs well for the development of novel diagnostic tools that are based on the specific conformational properties and the bioactivity of tau.

The biological *in vivo* seeding capacity of AD patients' CSF we here describe asks for considerations on the biosafety of operators when handling human CSF samples. Taking into account that, compared to previous seedings with brain tissues (Clavaguera et al 2013), relatively mild seeding effects occurred after inoculation of concentrated CSF into an artificially tau overexpressing murine host system, we would still consider the seeding potential of human AD tau in CSF rather low, and of limited relevance in a routine laboratory or clinical setting when adhering to standard safety procedures for the handling of potentially infectious human biosamples.

Our results come however from a study subject to some limitations. Due to the high amounts of human CSF needed to allow its concentration, CSF had to be collected from consecutively donating patients. This resulted in a small number of patients' samples attributable to either the AD or the group, similar to the previous *in vitro* study done in HEK293 cells (Takeda et al 2016). As all patients were similarly aged, we furthermore can't exclude that patients attributed to the control group were subjected to pre-symptomatic, yet possibly seeding competent tau pathology, and thereby might have lessened the differences between the AD and the control group. As tau seeding is a time and concentration dependent process (Clavaguera et al 2009), we concentrated the CSF samples before the intracerebral inoculations. It might be, therefore, that the seeding potential of tau was increased by the concentration process itself. The absence of any seeding activity in our previous experiments with concentrated CSF-Aβ (Fritschi et al 2014, Skachokova et al 2015), as well as the significant differences between the CSF tau derived from P301S mice and AD patients versus the controls, however speak against a relevant influence of the concentrating step. Given the life span of homozygous P301S host mice, the maximum seeding time is limited to 4 months. We therefore can not exclude that a more robust seeding response would become apparent at later times points post-seeing.

The exact nature of bioactive tau seeds has yet remained unknown. The need of high amounts of CSF seeding in the present study has precluded any detailed analysis of the bioactive seeds. In their post mortal CSF samples, Takeda et al. found high molecular weight tau (HMW) species and considered them to be the bioactive species (Takeda et al 2016). HMW tau species were however also detectable in one of the control patients, what might be due to post mortal tau aggregation given their CSF sampling at autopsies. The next steps should therefore analyze the biochemical and structural nature of CSF tau bioseeds.

Recent studies correlated tau truncated at residue 368 in the CSF compartment with tau PET analysis (Blennow et al, 2020), stating that the aforementioned fragment correlates well with tau pathology in AD patients (Blennow et al, 2020). Other studies focused on the relationship between tau truncation at residue 224 in the CSF compartment and AD, suggesting a strong association (Cicognola et al, 2019). Both these fragments seem to be part of tau tangles (Blennow et al, 2020; Cicognola et al, 2019), thus suggesting that CSF tau forms can be seeding competent, possess an aggregating nature and, again, are mainly present and relevant in cleaved forms. CSF-derived tau has been also recently linked to mnemonic worsening by itself, as higher levels of CSF tau were associated with a worse memory performance in old adults (Berron et al, 2019). With other studies previously hinting at a correlation between levels of specific forms of tau in CSF, such as tau phosphorylated at Thr231, and cognitive decline (Ashton et al, 2020), CSF-derived tau could therefore clarify many doubts regarding diagnostic procedures for AD patients, once its seeding properties, and its correlation to cognitive features is further elucidated.

As said before, studies on the diagnostic value of CSF tau in AD mirror other approaches in the various neurodegenerative diseases, particularly CJD and HD (Foutz et al, 2017; Tan et al, 2015). Adding to the aforementioned RT-QuIC studies, reducing prion levels in the CSF has been recently shown to be a good option to test the efficacy of prion-reducing drugs in the CNS (Vallabh et al, 2019), thus suggesting that prion protein levels in CSF could be used as a pharmacodynamic biomarker (Vallabh et al, 2019). CSF mutant huntingtin levels were instead related to HD stages and the presence, in the central nervous system, of mutant huntingtin (Southwell et al, 2015), with CSF mutant huntingtin increasing along with the stage of the disease (Southwell et al, 2015). Pharmaceuticals companies, developing pioneering antisense oligonucleotides targeting mutant huntingtin, used CSF levels of that same mutant protein to test the efficacy of the treatment in the various phases of the trials (Mullard, 2019b), thus speaking highly of the reliability of huntingtin measurements in CSF related to HD stages.

Altogether, these findings advocate for an increasing importance of CSF biomarkers for many neurodegenerative diseases, thus underlying the need for further research on the mechanisms bringing aggregation-prone proteins, and most likely fragments of them, to the CSF compartment, and the consequent relevance for the diagnostic tools available to clinicians. The results presented into the thesis follow this research direction and provide a little, but significant advancement in the understanding of CSF-derived tau properties, establishing its seeding competence and its possible relevance for AD diagnostics.

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6) Abbreviations

ABri = Amyloid-Bri

AD = Alzheimer’s disease

ADan = Amyloid-Dan

AGD = Argyrophilic grain disease

ALS = Amyotrophic lateral sclerosis

ApoE4 = Apopoliprotein E4

APP = Amyloid precursor protein

Aβ = Amyloid beta

BACE = Beta-site APP cleaving enzyme

BBB = Blood brain barrier

CaMKII = Calcium or calmodulin-dependent protein kinase II

CBD = corticobasal degeneration

Cdk5 = Cyclin-dependent kinase-5

CDR = Clinical Dementia Rating

CJD = Creutzfeld Jakob Disease

CK1 = Casein kinase 1

CNS = Central nervous system

CSF = Cerebrospinal fluid

CTE = Chronic traumatic encephalopathy

EEG = Electroencephalography

EOAD = Early onset Alzheimer’s disease

ER = Endoplasmic reticulum

FBD = Familial British dementia

FDD = Familial Danish dementia

FTDP-17 = Frontotemporal dementia and parkinsonism linked to chromosome 17

FTLD = Frontotemporal lobar degeneration

GGT = Globular glial tauopathy

GSK-3 = Glycogen synthase kinase-3

HD = Huntington’s disease

HDAC6 = Histone deacetylase 6

HMW = High molecular weight

LOAD = Late onset Alzheimer's disease

MAPK = Mitogen-activated protein kinase

MARK = Microtubule affinity-regulating kinase

MCI = Mild cognitive impairment

MMS = Mini Mental State

MMSE = Mini-Mental State Examination

MOCA = Montreal Cognitive Assessment

MRI = Magnetic resonance imaging

NFL = Neurofilament light protein

NFT = Neurofibrillary tangles

OGT = O-GlcNAc transferase

PART = Primary age-related tauopathy

PD = Parkinson's disease

PDPK = Proline-directed protein kinases

PET = positron emission tomography

PFA = Paraformaldehyde

PHF = Paired helical filaments

PKA = Cyclic AMP-dependent protein kinase

PSEN 1 = Presenilin-1

PSEN 2 = Presenilin-2

PSP = Progressive supranuclear palsy

PTM = Post-translational modification

RT-QuIC = Real-time quacking induced conversion

sAPPβ = Soluble amyloid precursor protein beta

SF = Straight filament

$\Delta tau_{151\text{-}421}$ = human tau fragment from amino acid 151-421

7) Curriculum Vitae

PERSONAL INFORMATION Alfonso Martinisi

Via Francesco Borromini 7, 6900 Lugano

0041796802392

alfonso.martinisi@hotmail.it

Sex Male | Date of birth 02/06/1992 | Nationality Swiss and Italian

JOB APPLIED FOR, PREFERRED POSITION

Biologist/Researcher/Pharmaceutical Manager

WORK EXPERIENCE

February 2017-Present

Ph.D Internship at University of Basel in Neurobiology under the supervision of Prof. Dr. David Winkler, February 2017-Present

Full time internship in a Neurobiology Lab in order to understand biochemical and structural roles of tau protein for Alzheimer's disease development. My task was particularly to study long-lasting effects of tau oligomeric structures in murine models of Alzheimer's disease, and to study the propagation potential of tau protein contained in the cerebrospinal fluid of the patients. My work was published in a peer-reviewed journal as an article, and a second one is following soon.

Business or sector Research

November 2016

Invited speaker for Italian-speaking students at the preparatory week for the Swiss Olympics of Biology 2017, November 2016; topics treated: Neurobiology and Biochemistry

Six hours of teaching to high-school level students for the Swiss Olympics of Biology 2017, four in Biochemistry and two in Neurobiology. My task was to provide the students with basic but thorough notions on both topics, and prepare them for the international competition. I was invited by a selected group of ETHZ students.

Business or sector Teaching

March-November 2016

Substitute teacher at the Scuola Media Massagno, four times:
March 2016, one week, Science teacher, 17 hours
April 2016, one week, Mathematician teacher (first week), 15 hours
April 2016, one week, Science and Mathematician teacher (last week), 25 hours
November 2016, one week, Science and Mathematician teacher, 23 hours

Experience as a substitute teacher in Science and Mathematics, teaching lessons left by the titular teacher, but also preparing lessons on my own.

Business or sector Teaching

January 2014-May 2015

Graduate student researcher at the University of Pavia (Italy), thesis internship

Thesis internship in Structural Biology in the Structural Biology lab led by Prof. Federico Forneris, resulting in my Master thesis "Expression, purification and characterization of two laminin G domains of EGFLAM/Pikachurin, a novel proteoglycan involved in synapse formation". The work aimed at expressing, purifying and characterizing this retinal protein, starting from bioinformatical analysis and construct design, cloning, expressing, and purifying it enough to crystallize the protein and obtaining its 3D structure through X-ray crystallography. I focused particularly on the LG1 and 2 domains of the protein, the polymerization and dystroglycan-binding domains, purifying the second enough to carry on with stability assays at different pHs. The thesis was awarded the maximum score (110/110)

Business or sector Research

EDUCATION AND TRAINING

October 2013-December 2015

Master Degree in Molecular Biology and Genetics, University of Pavia (Italy), 110/110

- Excellent theoretical skills on Molecular biology and genetics, on many fields and topics
- Particular focus on oncology, immunology, neurology
- Practical knowledge in molecular biology, biochemistry, and structural biology acquired in Prof. Federico Forneris' Structural biology lab, during a 18-month thesis internship
  Advisor: Prof. Federico Forneris

October 2010-July 2013

Bachelor Degree in Biotechnology, University of Pavia (Italy), 106/110

- Basic theoretical skills in biology and biologic related technology
- Basic practical molecular biology skills (comet assay) acquired during a three-month internship under the supervision of Dr. Daniela Necchi, Pharmacology lab

PERSONAL SKILLS

Mother tongue(s) Italian

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C1	C1	C1	C1	C1
	Certificate of Advanced Knowledge in English				
German	B2	B2	B2	B2	B2

Levels: A1/A2: Basic user - B1/B2: Independent user - C1/C2 Proficient user
Common European Framework of Reference for Languages

Communication skills
- Good teamwork skills, good communication skills, great ability to adapt to multicultural places

Organisational / managerial skills
- Good resistance to stress, ability to organise daily work

Job-related skills
- Good teaching skills with secondary school and high school level students

- Knowledge of bioinformatics, molecular biology and biochemistry techniques: construct design, cloning procedure in yeast and bacterial expression system (PCR, transformation, ligation, digestion with restriction enzymes), protein expression procedure in yeast and bacterial expression system (small and large scale), protein purification procedure (small and large scale), SDS-PAGE, Western blot, dot blot, immunohistochemistry essays (antibody stainings), Nickel affinity chromatography (both with manual Nickel beads and AKTA Purifier automated system), HPLC (using AKTA Purifier), crystallization trials (manual and automated trials), protein stability assays (using AKTA Purifier)
- Animal handling: FELASA Certificate B, obtained in 2017; injection and handling of mice, behavioural tests

Digital competence

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem solving
Independent user	Independent user	Independent User	Independent User	Independent User

Levels: Basic user - Independent user - Proficient user
Digital competences - Self-assessment grid

Replace with name of ICT-certificate(s)

Good command of Microsoft Office suite (Word, Excel, Power Point); Adobe (Adobe Reader)

ADDITIONAL INFORMATION

References

- Prof. Federico Forneris, head of Structural Biology Armenise-Harvard lab at the University of Pavia Master Thesis and Internship supervisor, federico.forneris@unipv.it
- Dr. Daniela Necchi,former researcher in the Pharmacology at the University of Pavia, Bachelor Thesis supervisor, necchid@unipv.it
- Prof. Dr. David Winkler, neurologist at the University Hospital Basel, Ph.D supervisor, david.winkler@usb.ch

Projects

Expression, purification and characterization of two laminin G domains of EGFLAM/Pikachurin (Prof.Federico Forneris)
Biochemical and structural characterization of tau protein in tau transgenic mouse models (Prof. Dr. David Winkler)

Publications

First Co-Authorship (unofficially First Authorship, however we granted Drs. Skachokova her requests on the paper) on scientific paper describing the propagation potential of Cerebrospinal Fluid derived from Alzheimer's disease patients, focusing on its ability to propagate tau pathology on tau transgenic mice

Certifications

Certificate of Advanced Knowledge in English, June 2013, Pavia
Certificate FELASA B, for animal handling, April 2017, Basel