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Biochemical characterization of extracellular Tau from *in vivo* models of Tau aggregation

Dissertação de Mestrado em Biologia Celular e Molecular, orientada pelo Doutor Kristof Van Kolen (Janssen Pharmaceutica) e pelo Professor Doutor Carlos Duarte (Universidade de Coimbra) e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

Junho de 2016

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UNIVERSIDADE DE COIMBRA

The work presented in this thesis resulted from a partnership between the University of Coimbra and Janssen Pharmaceutica NV, Beerse I. All experimental activities were performed at Janssen Pharmaceutica NV, Beerse I, a Johnson & Johnson pharmaceutical research and development facility in Beerse, Belgium.

All data presented in this thesis is strictly confidential

Beerse, 2016

Acknowledgments

First I would like to thank Dr. Kristof van Kolen for accepting me in this project and for his guidance during this year. Thanks for all the confidence, support and advices, for everything that I learned with you and for the good moments that we spent in the lab. It was a true pleasure doing this thesis with you and your scientific knowledge and enthusiasm will, certainly, be a role model for my career. Dank u wel!!

I would like to thank Dr. Marc Mercken for welcoming me in his research group, for the pleasant scientific discussions in the lab meetings and all the help with the development of this project. To the microdialysis lab, particularly Christ Nolten and Pim Drinkenburg, I would also like to thank for their helpful work and feedback throughout the year.

I would like to acknowledge all the members of the Neuroscience Department at Janssen for welcoming me in a foreign country and for the great atmosphere in the company. A special thanks to Marc Vandermeeren, Guy Daneels and Clara Theunis, for the helpful scientific discussions, for all the support and company in the lab.

To the "Portuguese community" at Janssen, André Marreiro, Alberto Soares, Sara Calafate and Miguel Matias, I would like to express my deepest gratitude for all the great moments we lived this year, for the scientific and academic support and, especially, for all the laughs and beers that we shared. Thanks to you I felt completely at home in Belgium.

To professors Emília Duarte and Carlos Duarte, I would like to thank for giving me the opportunity to enroll in the Master and in this internship at Janssen, for sharing your knowledge and for the amazing first year that I spent in Coimbra.

I would like to thank all my friends in Portugal. Thanks for your support and trust, and for your visits to Belgium. For your affection and for helping me feel closer to home, thank you! Thank you to all the students at Janssen for the great nights and weekends that we spent. It was a real pleasure to meet you!

A special thanks to my housemates, Rafaela and Francisco, for being with me in this adventure. Thanks for the friendship, for all the amazing moments that we had together, for everything! You were a big support during this year and I couldn't do this without you.

Por fim, gostaria de agradecer à minha família, principalmente aos meus pais, por todo o sacrifício e dedicação ao longo destes anos. Não tenho palavras para vos agradecer tudo o que fizeram por mim e sem vocês nao conseguia ter chegado até aqui. Obrigado pelas longas conversas e pelas visitas, obrigado por me terem feito sentir perto de casa. Por sempre me terem apoiado e acreditado em mim, um grande obrigado do fundo do meu coração.

Abstract

Alzheimer's disease (AD) is the most common neurodegenerative disorder, which is characterized by two major hallmarks: deposition of Aβ plaques and incorporation of neurofibrillary tangles composed by hyperphosphorylated Tau (NFTs). Recently, progress has been made in the comprehension of AD mechanisms, however neither disease modifying treatments nor biomarkers capable of detecting the disease at early stages have been discovered.

Several studies supported the hypothesis that Tau pathology spreads throughout the brain, particularly by a mechanism involving extracellular Tau release and subsequent uptake by synaptically connected neurons. Thus, it is supposed that extracellular Tau species are responsible for Tau seeding and spreading of Tau pathology. Although several studies have investigated extracellular Tau, it not yet clear which Tau species is responsible for transmission of Tau pathology, with some authors considering Tau oligomers, while others consider larger aggregated filamentous Tau as the pathogenic Tau species.

Particularly, a study performed in P301S mice using an *in vivo* microdialysis technique demonstrated Tau protein in interstitial fluid. However, little is known about the biochemical properties of the extracellular Tau found. Thus, in this project, the objective was to characterize ISF Tau perfused from P301S mice by a 1 MDa microdialysis probe, using different biochemical assays. Furthermore, the focus was to uncover which Tau species are present in the extracellular space at very early stages of Tau pathology.

Our results suggest that the vast majority of Tau present in ISF of 3-month old P301S mice is fragmented, particularly in two abundant fragments, one from N-terminal to PRD and other in MTBD/C-terminal region. To a minor extent, also full-length and most C-terminal Tau was detected. Importantly, mouse endogenous Tau was found in ISF samples from WT mice, however at much lower concentration. Although these two cleavage processes seem evident, it is hypothesized that multiple Tau fragments are present in ISF, but may only be detected depending on the specificity for a particular region of the antibody combination used.

Furthermore, the *in vivo* microdialysis technique was confirmed as a manner to investigate ISF Tau levels, which may also be used to monitor Tau or antibody levels during a passive immunization treatment.

In conclusion, this project provided insight about the nature of extracellular Tau, emphasizing the relevance of the study of these fragments in the discovery of a new Tau pathology biomarker, as well as, for the understanding of Tau pathology mechanisms.

Keywords: Alzheimer's disease; Tau pathology; microdialysis; ISF; Tau fragments

Resumo

Alzheimer é a doença neurodegenerativa mais comum a nível mundial, sendo caracterizada pela deposição de placas de Aβ e pela incorporção de aggregados de Tau, sob a forma de tranças neurofibrilares. Recentemente, grandes avanços têm ocorrido na compreensão dos mecanismos que causam a doença de Alzheimer. No entanto, até à data, ainda não foram encontrados tratamentos que travem a progressão da doença nem biomarcadores que permitam fazer um diagnostico precoce da doença.

Inúmeros estudos têm apoiado a hipótese de que a patologia da Tau se propaga pelo cérebro, nomeadamente por um mecanismo envolvendo a libertação de Tau para o meio extracelular e, consequentemente, a sua incorporação por neurónios conectados através de sinapses. Assim, é sugerido que espécies extracelulares de Tau são responsáveis pela propagação da patologia da Tau. Apesar de alguns estudos terem investigado a Tau extracelular, ainda nao é evidente que forma da Tau é responsável pela transmissão da patologia. Enquanto alguns autores consideram os oligómeros da Tau como patogénicos, outros consideram que são formas maiores e agregadas em filamentos as causadoras da patologia da Tau.

Nomeadamente, um estudo em ratinhos transgénicos (P301S) usando uma técnica de microdiálise *in vivo*, demonstrou a presença de Tau no fluído intersticial cerebral (ISF). No entanto, ainda pouco se sabe acerca das propriedades bioquímicas da Tau extracelular encontrada. Assim, neste projecto, o objectivo foi caracterizar a Tau de ISF extraido de ratinhos transgénicos (P301S), por uma sonda de microdiálise com 1 MDa, usando diferentes ensaios bioquímicos. Para além disso, outro objectivo foi descobrir que espécies da Tau estão presentes no meio extracelular numa fase inicial da patologia da Tau.

Os resultados deste projecto sugerem que a Tau no ISF de ratinhos transgénicos (P301S) com 3 meses de idade, está, maioritariamente, fragmentada. Nomeadamente, dois fragmentos abundantes foram encontrados, um do N-terminal até ao PRD, e outro na região do MTBD/C-terminal. Em menor concentração foi também encontrada Tau “full-length” e um fragmento composto pela parte final do C-terminal. Adicionalmente, “murine” Tau endógena foi detectada nas amostras de ISF de ratinhos WT, apesar de em muito menor concentração. Para além dos dois pontos de fragmentação evidentes encontrados, os resultados sugerem que múltiplos fragmentos da Tau estarão presentes no ISF. No entanto, determinados fragmentos so poderão ser detectados dependendo da combinação de anticorpos usada, dado que combinações distintas têm diferentes especificidades para regiões particulares da Tau.

Ainda para mais, a técnica de microdiálise *in vivo* foi confirmada como uma forma de estudar os níves de Tau no ISF, o que poderá ser usado para monitorizar os níveis de Tau ou de anticorpo durante um tratamento de imunização passiva.

De uma forma geral, os resultados aqui descritos contribuíram para aumentar o conhecimento sobre as formas extracelulares da Tau, realçando a importância do estudo destes fragmentos na descoberta de um novo biomarcador da patologia da Tau, assim como na compreensão dos mecanismos patogénicos da Tau.

Palavras-chave: Doença de Alzheimer; patologia da Tau; microdiálise; ISF; fragmentos da Tau

Abbreviations

aCSF – Artificial cerebrospinal fluid

AD – Alzheimer's disease

APOE – Apoliprotein E

APP – Amyloid-precursor protein

ASOs – Antisense oligonucleotides

Aβ – amyloid β

CSF – Cerebrospinal fluid

EC – entorhinal cortex

ECM – Extracellular matrix components

FAD – Familial Alzheimer's disease

FTD – Frontotemporal dementia

FTDP-17 – Frontotemporal dementia with Parkinsonism linked to chromosome 17

GWAS – Genomic-wide association studies

HEZ – Heterozygote

HPLC – High-performance liquid chromatography

ISF – Interstitial fluid

LTP – Long-term potentiation

MAPs – Microtubule associated proteins

MAPT – Microtubule associated protein

MTBD – Microtubule-binding domain

MTs – Microtubules

NFTs – Neurofibrillary tangles

NMDA – N-methyl-D-aspartate

NMDAR - N-methyl-D-aspartate receptor

PHFs – Paired helical filaments

PLC – Phospholipase C

PRD – Proline-rich domain

PSEN1 – Presenilin 1

PSEN2 – Presenilin 2

RNA – ribonucleic acid

SAD – Sporadic Alzheimer's disease

TG – Transgenic

WT – Wild-type

Table of Contents

1. Introduction

1.1 ALZHEIMER'S DISEASE – GENERAL REMARKS

Alzheimer's disease (AD) is the most common neurodegenerative disorder accounting for about 80% of dementia cases[1]. This disease affects nearly 2% of the population and is one of the leading causes of death in the elderly. Moreover, it is predicted that the incidence of dementia, including Alzheimer's, will almost double every 20 years, with an expected worldwide prevalence of 115.4 million people in 2050. Particularly in low and middle income countries, prevalence of dementia will highly increase, reaching the proportion of 71% of worldwide people with dementia in about 35 years[2]. At present, there are no disease modifying treatments, thus, it is essential to understand the molecular mechanisms causing the onset and progression of this disease in order to identify new therapeutic strategies.

AD is characterized by a widespread loss of neurons, namely in the cortex and hippocampus, causing early memory loss, gradual erosion of cognitive functions and behavior alterations[3]. The major histopathological hallmarks are neuronal loss, extracellular β-amyloid (Aβ) plaques and intracellular neurofibrillary tangles (NFTs). Senile plaques are formed by aggregating Aβ peptides that are generated by amyloid-precursor protein (APP) processing by a series of proteases. This can proceed through two pathways; one called the prevalent non-amyloidogenic pathway, triggered by the cleavage of APP by α-secretase[4,5,6,7]. The other mechanism of APP processing is called the amyloidogenic pathway, which will lead to the formation of Aβ. The initial cleavage is mediated by β-secretase at a position located 99 amino acids from the C terminus. This results in the release of soluble APP β into the extracellular space, and the 99-amino-acid C-terminal stub (known as C99) within the membrane. The newly generated N terminus corresponds to the first amino acid of Aβ peptide which is further cleaved by γ-secretase between residue 38 and 43 to release an intact Aβ peptide. Thus, β-secretase generates the amino-terminus and γ-secretase cleaves in the carboxy-terminus, determining Aβ length. In a pathological state, γ-secretase is responsible for the formation of $A\beta_{42,}$ which is a more fibrillogenic and neurotoxic species that can aggregate and deposit as senile plaques[8].

In turn, NFTs are composed of hyperphosphorylated Tau aggregates. Tau is rich in serine and threonine residues, being a substrate of several kinases under physiological conditions, essential for the regulation of microtubules dynamics. However, under pathological conditions, Tau may become hyperphosphorylated, dissociating from microtubules and aggregating in different conformations such as NFTs[8]. NFTs are also a feature of several additional Tauopathies

such as progressive supranuclear palsy, corticobasal degeneration, Pick's disease, and some forms of frontotemporal dementia, without formation of Aβ plaques[9,10].

Although aging is the main risk factor for AD, both early-onset familial (FAD) and late-onset sporadic (SAD) can be distinguished. FAD is rare, accounting for less than 1% of total AD cases, and is inherited in an autosomal dominant manner. Mutations in three different genes have been identified: APP, presenilin 1 (PSEN1) and presenilin 2 (PSEN2), which are components of the complex that generates Aβ[8]. Even more important, a protective mutation against AD was also found in the APP gene[11]. This A673T coding mutation exhibited reduction in the formation of amyloidogenic peptides *in vitro* and protected against cognitive decline in the elderly without AD[11]. Nevertheless, the vast majority (95%) of Alzheimer's cases are SAD, which does not have a direct genetic cause, but some susceptibility genes have been identified, such as apoliprotein E (APOE)[12]. Particularly, the ε4-allele of APOE has been consistently indicated as a risk factor for AD, especially by reducing age-at-onset. However, APOE-ε4 is neither necessary nor sufficient to cause AD, but only a genetic risk factor. Additionally, some other studies reported that the ε2-allele can have a weak, but significant, protective effect[12]. Genetic studies of SAD revealed additional loci contributing to risk of disease, including PICALM, CLU, CR1, BIN1, CD2AP, EPHA1, MS4A4A, CD33, and ABCA7[13,14]. Nevertheless, a genomic-wide association study (GWAS) performed recently, only confirmed APOE, CR1, BIN1 and PICALM association with SAD cases[15]. Although sporadic cases are not yet fully understood, it is believed that lifestyle and environmental factors, combined with genetic susceptibility may play a role in AD appearance[16,17].

1.1.1 Protein oligomerization – a key event for AD pathology

Since AD is defined by the presence of Aβ plaques and Tau NFTs, it is believed that protein oligomerization may be the essential event causing AD pathology. Consistently, several other neurodegenerative diseases are related to aggregation and accumulation of misfolded proteins (proteinopathies), such as α-synuclein in Parkinson's disease or huntingtin in Huntington's disease[18]. Moreover, both Aβ and Tau aggregates have been found 15 years before the onset of symptoms[19]. This is further supported by the observation that mutations found in FAD (APP, PSEN1 and PSEN2 genes) result in increased production of $A\beta_{42}$, causing presenile dementia with early plaque formation[20]. Thus, the "amyloid cascade hypothesis" for AD emerged[21]. This hypothesis postulates that the increased production and aggregation of $A\beta_{42}$ leads to Aβ oligomerization and deposition in plaques which, in turn, will accelerate Tau

hyperphosphorylation and aggregation, synaptic dysfunction and widespread neuronal death[21]. Although it can explain AD-causing mutations, it may not apply to the late-onset sporadic cases (the vast majority), for several reasons: 1) in SAD, mutations or certain susceptibility genes cannot directly explain the formation of Aβ plaques, however these genes may also influence indirectly the pathology by interfering with different processes, such as inflammation and protein trafficking; 2) Aβ aggregates are commonly found in aged normal subjects (non-dementia)[22]; 3) biomarkers of neurodegeneration appeared in normal elderly individuals independently of Aβ deposition[23].

Therefore, in the last decade, the paradigm has changed and several groups focused in the study of Tau in order to understand the mechanisms involved in Tau alterations in AD, namely hyperphosphorylation, aggregation, pathology and propagation throughout the brain. Accumulating evidence suggests that Tau may play a crucial role in mediating neurodegeneration. First, Tau pathology, in the absence of Aβ aggregates, occurs in several Tauopathies. Particularly, most cases of FTDP-17 are associated with mutations in the Tau-encoding microtubule association protein (MAPT) and is characterized by a progression of NFTs throughout the brain similar to the one of Alzheimer's disease[24], establishing a role for Tau in neurodegenerative diseases[25]. Second, studies developed by Braak et al[26,27], suggested the appearance of Tau inclusions earlier than Aβ plaques. Third, *in vitro* and *in vivo* studies evidenced interplay between Aβ and Tau in causing toxicity in AD. Although the molecular mechanism beyond this link is unknown, different modes of interaction may occur: Aβ can mediate Tau pathology; Tau is required for Aβ toxicity; synergistic effects of Aβ and Tau, targeting cellular processes or organelles, which may be due to cross seeding between proteins, combined aggregated stress and/or independent neurotoxic pathways that result in increased neuronal vulnerability and, thus, increased susceptibility for pathology spreading [28,29].

Initially, Aβ-Tau interaction was revealed when injection of synthetic Aβ into mutant Tau transgenic mice induced a 5-fold increase in the number of Tau NFTs[9]. Later, it was observed that Aβ formation in APP transgenic mice caused hyperphosphorylation of Tau and that the offspring of hybrid mice (APP transgenic mice Vs Tau transgenic mice) only increased NFTs pathology, but not Aβ pathology[30]. Thus, Aβ-mediated Tau pathology was suggested as a hierarchical mode of Aβ-Tau interplay. Nevertheless, evidences emerged that Tau pathology is not only a consequence of Aβ pathology, but also an important mediator of Aβ toxicity. One *in vitro* study reported that wild type neurons when exposed to Aβ displayed neurite degeneration and neuronal death, but in neurons derived from Tau-knock out mice, this effect was not observed[31]. Furthermore, a study

with Tau knockout mice crossed with mice that overexpress human APP (hAPPJ20) showed that Tau was required for Aβ toxicity since the absence of Tau genes protected hybrids against memory and learning deficits[32]. Regarding the possible synergistic effects of Aβ and Tau, a good example of this interaction mode is the mitochondrial dysfunction that occurs in AD, with both Aβ and Tau being responsible for mitochondria respiration impairment[33].

One possible protein that functionally connects Aβ and Tau is the tyrosine protein kinase FYN, which binds directly to Tau and positively regulates NMDA receptor activity[30]. FYN-Tau interaction targets FYN to dendrites in wild-type mice, but in Tau knockout mice FYN accumulates in soma. Under physiological conditions, Tau is essentially found in the axon (very low levels in dendrites), however, in AD, Tau is redistributed to the somatodendritic compartment[34]. FYN phosphorylates NMDAR, mediating their interaction with PSD95, which is required for the Aβ toxicity[34]. Therefore, under pathological conditions, increased levels of dendritic Tau will target increased levels of FYN to the post-synaptic region, enhancing NMDAR-PSD95 interaction and, thus, resulting in excitotoxicity, memory deficits and premature mortality. Other possible mechanism of Aβ-Tau interaction is the cross-seeding between these two misfolded proteins, since studies with animal models of AD revealed that Aβ is able to enhance Tau aggregation, but Tau aggregates did not have the same effect on Aβ. Accordingly, a recent study demonstrated both *in vitro* (well-characterized cellular assay of Tau aggregation) and *in vivo* (P301S Tau transgenic mice) that pre-aggregated Aβ-seeds induce and accelerate Tau aggregation, particularly by a heterotypic seeding mechanism of filamentous Tau by Aβ[35]. This process may also occur indirectly, that is, Aβ aggregates may activate certain kinases that phosphorylate Tau, inducing higher misfolding and aggregation of Tau[9,36,37]. Another hypothesis may be the combined aggregate stress that Aβ and Tau induce to the cells. Both protein aggregates could overload cell clearance mechanisms, saturating the system and inducing neurotoxicity. These findings exemplify possible mechanisms of Aβ-Tau interplay and strongly support the paradigm that both Aβ and Tau can mediate each other functions and act together to cause toxicity in AD (Fig.1).

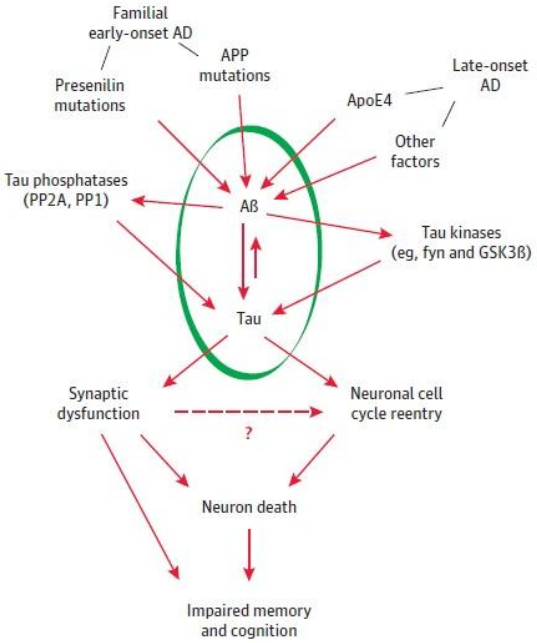

Fig.1: Interplay between Aβ and Tau driving AD progression. From: Bloom., 2014[38]

Taking into account these findings, a combined hypothesis for AD has appeared, in which the interaction between Aβ and Tau is responsible for the main toxic events in Alzheimer's disease. Thus, in 2011, Ittner and Gotz[28] proposed the "Tau axis hypothesis" for AD, which postulates that progressively increasing levels of dendritic Tau turn neurons more vulnerable to Aβ toxicity. First, although Aβ is already formed, the low levels of Tau in soma and dendrites limit neurons vulnerability to Aβ toxicity. With AD progression, increased levels of Tau become hyperphosphorylated and Tau accumulates in the somatodendritic compartment, increasing dendritic Tau levels. In a more advanced state of AD, these high levels of Tau are associated with more sensitized synapses to Aβ toxicity (probably through Tau-FYN interaction). Since, Aβ toxicity, in turn, increases dendritic Tau accumulation, this could be a positive feedback cycle, increasing even more Aβ effects in the post-synaptic compartment[39]. Recently, Musiek and Holtzman[40], updated the framework of the amyloid hypothesis, incorporating several factors such as: genetic mutations, synaptic activity and sleep cycles dysfunction, which may facilitate Aβ production and release; proteostatic failure (promoted by aging, oxidative stress, etc), function or genotype of certain proteins (APOE, Lrp1 and SorL1) and inflammation, which may promote Aβ aggregation. Once Aβ homeostasis is lost, Aβ through poorly understood mechanisms triggers Tau aggregation and, subsequently, Tau pathology and neurodegeneration (Fig.2)[40].

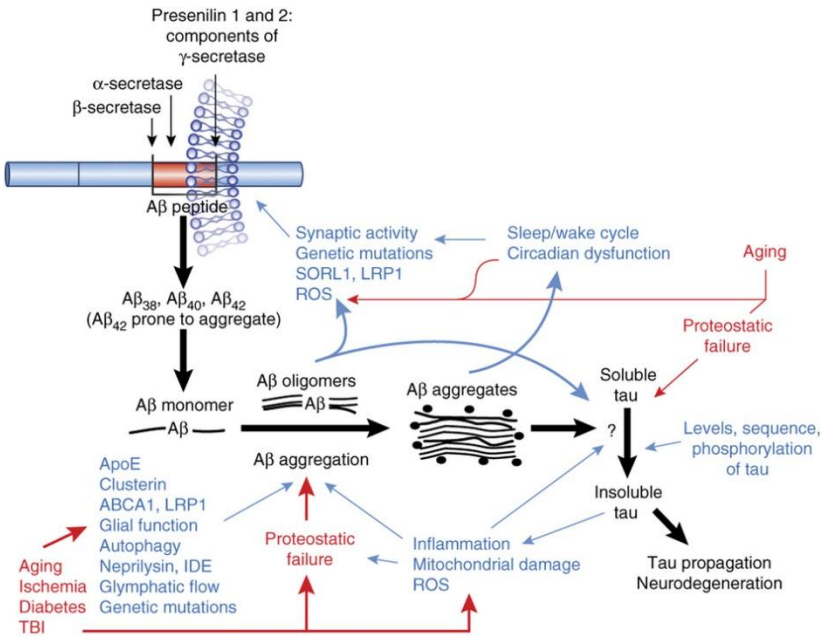

Fig.2: An updated framework of the amyloid hypothesis. Blue text and arrows illustrate proposed modifiers of the Aβ cascade, and red text and arrows show the influence of aging and comorbid pathologies. From: Musiek & Holtzman, 2015[40]

1.2 BIOCHEMISTRY AND CELL BIOLOGY OF TAU

Tau is a native unfolded microtubule-associated protein identified in 1975[41]. It is mainly expressed in neuronal axons, but can also be found in somatodendritic compartment and in oligodendrocytes[42,43]. The Tau protein can be subdivided into four regions: N-terminal region, proline-rich domain (PRD), microtubule-binding domain (MTBD) and C-terminal region[44]. The proline-rich domain includes several phosphorylation sites, which are important to regulate Tau binding to microtubules (MTs), and is able to bind SH3 domains of other proteins as FYN[45]. Six Tau isoforms can be produced by alternative splicing of the MAPT gene present on chromosome 17q21.31[46]. These isoforms differ by the presence or absence of a 29-aminoacid or 58-aminoacid insert in the N-terminal region (exon 2 and 3) and by the inclusion or exclusion of exon 10 (31 amino acid) in the MTBD.

Tau together with MAP2 and MAP4 forms a family of protein with identical domain structure, whose main function is binding to microtubules, promoting microtubule assembly and stabilization[47]. Indirectly, Tau-MTs interaction could regulate axonal transport, since absence of Tau may increase MTs disorganization and Tau can interfere with the binding of motor proteins to

MTs[48]. However, knock-down of Tau expression did not impair axonal transport, suggesting that Tau may not have an essential role in this mechanism[49]. Nevertheless, since Tau has numerous binding partners, additional functions can be supposed, namely as regulator of signaling pathways or as a scaffold protein. Some functions attributed to Tau are: Tau regulates FYN levels in post-synaptic regions, affecting NMDAR-PSD95 interaction; Tau binds to phospholipase C (PLC), particularly activating it in the presence of arachidonic acid[50]; Tau acts as a direct enzyme inhibitor in histone deacetylase-6, regulating MTs stability by a mechanism independent of tubulin binding[51]; Tau affects adult neurogenesis, since in Tau knockout mice, adult neurogenesis was greatly reduced[52].

1.2.3 Tau aggregation

Since Tau is hydrophilic and highly dynamic, its aggregation into well-organized fibers in AD was not expected[47]. On the other hand, two factors may contribute to Tau aggregation: Tau high propensity to acquire a β-structure characteristic of amyloid filaments[53]; non-phosphorylated full-length recombinant Tau interaction with negatively charged compounds, such as glycosaminoglycans (heparin binds to Tau tandem repeats and induces dimerization), RNA or free-fatty acids[54,55].

In AD and other Tauopathies, Tau assembly involves the transition from a natively unfolded monomer to large aggregate structures such as NFTs and it is thought to be a multi-step process in which phosphorylation and other post-translational modifications may play a crucial role. First, the detachment of Tau from MTs occurs, triggered by Tau hyperphosphorylation, namely by an imbalance between Tau kinases and/or phosphatases (Fig.3)[25]. Some candidate protein kinases and phosphatases involved in this process are proline-directed kinases, proteins kinases that act on Lys-X-Gly-Ser motifs, tyrosine kinases and protein phosphatases (PP1, PP2A, PP2B and PP2C)[56,57]. Other post-translational modifications, such as Tau acetylation, glycation or O-GlcNAcylation may also be an important trigger of Tau detachment and aggregation of Tau. Acetylation seems to impair Tau ability to bind MTs, increasing its propensity to self-assemble. On the other hand, O-GlcNAcylation appears to be an inverse process, causing a reduction in Tau phosphorylation, and, consequently, enhancing Tau-MTs binding[25]. Subsequently, cytosolic concentration of unbound/free Tau increases, becoming more likely to misfold and more prone to aggregation. Next, early deposits of Tau called pretangles are formed in a structure that not

exhibits β-sheets. Thus, a structural rearrangement occurs, resulting in the formation of paired helical filaments (PHFs) containing β-sheet structure. Finally, PHFs self-assemble to form NFTs (Fig.3).

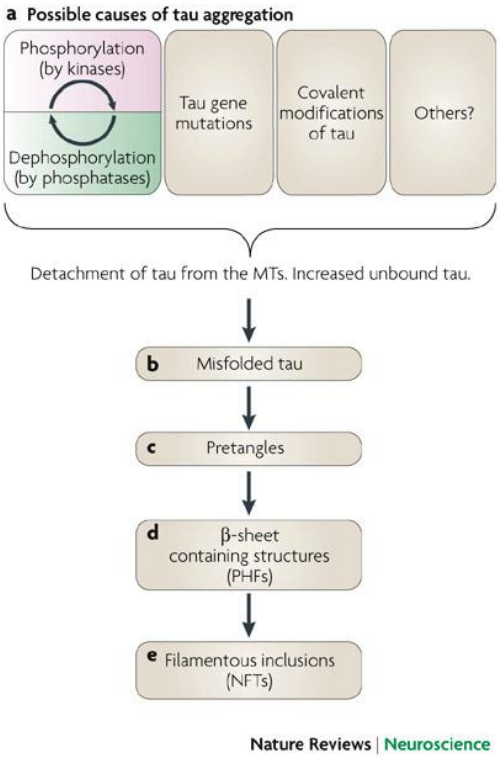

Fig.3: Pathological aggregation of Tau. From: Ballatore et al., 2007[25]

1.2.3 Tau pathology

Nowadays, it is known that Tau contributes to neuropathogenesis, but the mechanisms by which Tau causes AD pathology are not yet fully understood. It has been suggested that both loss and gain of Tau function may be included in this process. Loss of function can be due to Tau hyperphosphorylation and sequestration of soluble free Tau[58]. However, evidences revealed that increased phosphorylation of Tau may not be detrimental, since it occurs during normal physiological processes, such as hibernation and fetal development[59,60]. Furthermore, in other studies, it was verified that complete Tau ablation does not cause premature mortality or neurodegeneration and that Tau knockout lines mice have normal cognition and behavior[34]. Interestingly, downregulation of Tau might even protect against Aβ toxicity, since reduced excitotoxicity was observed in Tau$^{-/-}$ mice when compared to wild-type[34]. Although it was hypothesized that other MAPs might compensate for Tau loss, no changes in MAP1A, MAP1B or

MAP2 protein levels were observed in a study developed by Dawson and colleagues[61]. Thus, it is unlikely that loss of Tau function is the essential cause contributing to neuropathogenesis in AD and other forms of dementia. On the other hand, Tau phosphorylation and aggregation, as abnormal gain of Tau function, have been considered as possible causes of Tau pathology. Tau phosphorylation in a manner that hyperphosphorylated Tau has a tighter and more folded conformation with more propensity to aggregate[62]. Regarding Tau aggregation, studies with cell lines and mouse models with two different inducible constructs have been performed. One expressed the 4R microtubule repeat domain of human Tau with a deletion of lysine 280, which is highly prone to aggregate, and another construct with two mutations (I277P/I308P) preventing Tau aggregation. The pro-aggregation mice formed Tau neurofibrillary tangles and developed cognitive deficits, synaptic loss and impaired LTP. In contrast, the anti-aggregation mice did not develop these abnormalities. Importantly, switching of the pro-aggregation transgene rescued behavioral and electrophysiological deficits, without eliminating soluble Tau aggregates, highlighting that Tau aggregation causes toxicity[63,64]. Furthermore, a recent study assessing seeding ability of different Tau species in cellular and mouse models, concluded that large (>10 monomers) aggregated and hyperphosphorylated Tau was able to initiate the formation and spreading of Tau pathology, while no detectable seeding was observed with smaller oligomeric fractions[65]. Consistently, only immunodepletion of the large aggregated Tau with a phospho specific antibody (AT8), strongly reduced seeding[65]. Despite this, others suggested that Tau inclusions (NFTs) may not be responsible for the toxicity of Tau aggregates, since decreasing the levels of soluble Tau in inclusion-positive neurons, reduced caspase activation without affecting the number and size of Tau inclusions[66]. Thus, soluble Tau oligomers may be responsible for the activation of proapoptotic pathways. Additional studies in flies also supported that toxicity may be due to soluble Tau species, possibly dimers[27]. Moreover, Tau oligomers have been identified in AD brains and *in vitro* and *in vivo* models of AD[67,68,69]. A recent study with a mouse model of Tau pathology has also supported an important role of Tau oligomers, since the extracellular injection of oligomeric Tau impaired LTP and memory formation[70]. Consistently, other study with neurons derived from induced pluripotent cells demonstrated that only Tau oligomers, but not monomers, were able to enhance aggregation and phosphorylation of Tau, being responsible for Tau seeding and spreading of Tau pathology, which was associated with increased neurodegeneration[71]. Concluding, these findings suggest that, indeed, gain of Tau function, namely Tau aggregates/oligomers, may contribute to neuropathogenesis.

In AD, NFTs propagate in a hierarchical and predictable pattern through selective brain regions. Tau first accumulates in brainstem nerve cells, namely in noradrenergic projection

neurons of the locus coeruleus, from where the pathology spreads to the entorhinal cortex and other brain regions. To describe the pathology the so-called “Braak stages” of Tau pathology were established (Fig.4)[27].

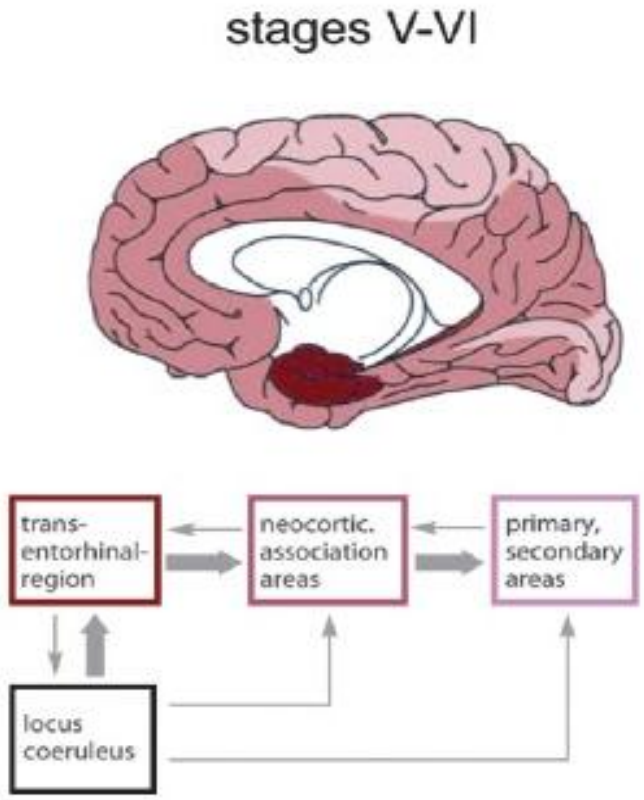

Fig.4: Braak stages of Tau pathology: Pretangle stage a, abnormally phosphorylated Tau appears abruptly in locus coeruleus (in the proximal regions of the axon) detected by AT8-immunoreactivity, which is an antibody that recognizes a phosphate-dependent epitope at serine 202 and threonine 205[69]. During stage b, pretangle material fills the somatodendritic compartment of a few coeruleus neurons. In stage c, the pathology occurs in nerve cells of other nonthalamic brainstem nuclei. Then, in stage 1a, portions of neurites with pretangle material appear in the transentorhinal region. Consequently, in stage 1b, all transentorhinal neurons become filled with the material. In these first phases, neurons do not undergo cell death despite large amounts of AT8-ir pretangle material is being produced. Next, stage I and II, which are related to a prodromal AD, show silver-stain positive aggregation confined to the upper layers of the transentorhinal cortex. In stages III and IV, associated with mild cognitive impairment, the pathology spreads to the entorhinal cortex. Finally, during stages V and VI, which characterize Alzheimer’s patients, a widespread formation of argyrophilic Tau aggregates in neocortical association areas and an increase in the pathology severity in the brain regions previously affected[15,23]. Adapted from: Braak et al., 2011[27]

1.2.4 Tau propagation

Although the pattern of propagation and the brain regions affected by Tau pathology are identified, it is not completely understood how this mechanism of Tau spreading occurs. Initially, the spread of Tau pathology in AD was attributed to the passive release of Tau from neurons due to cell death. However, recently, several research groups demonstrated active release and interneuronal transfer or Tau, suggesting a trans-neuronal spread of misfolded Tau. This idea was tested using transgenic mouse lines with localized expression of human Tau. Neuropsin promoter-driven tTa (transcriptional activator specific from medial entorhinal cortex (EC)) mice were crossed with a transgenic mouse line carrying the P301L mutation, resulting in mice expressing transgenic mutant Tau almost exclusively in the medial EC[72]. Analyzing the pathology development in these mice, a strong line of evidence emerged supporting a model of trans-synaptic spreading. First, Tau mislocalized from axons to cell bodies and dendrites in the EC. Then, Tau-positive NFT-like aggregates formed first in the EC and, later, propagated to downstream projection targets of EC neurons[73,74,75]. Furthermore, observation of human Tau protein in cells that did not expressed human Tau transgene suggested that Tau can be transferred across synapses[75].

Thus, it is evident that Tau pathology spreads across neurons, but by which mechanism is still unclear. Accumulating evidence suggests that it could occur by a prion-like mechanism similar to what was reported in prions, α-synuclein and Aβ. Prion-like spreading mechanism states that an intrinsic pathogenic protein misfolds, evades cellular clearance and induces other molecules to become similarly misfolded. In AD, it is supposed that Tau misfolds, acting as a seed that induces aggregation by recruiting additional unfolded or oligomeric Tau[76]. The term “prion-like” is only used to refer to the molecular processes of “conformational templating”, since there is no evidence that Tau aggregates can transmit between individuals as prions do[76]. The concept of “Tau seeds” refers to specific forms of Tau, ranging from free soluble Tau to NFTs, which can propagate Tau pathology. Nevertheless, this mechanism comprises different stages, since intracellular misfolded Tau, first, needs to be released in order to seed additional Tau molecules, and, then, extracellular Tau needs to be internalized by neighboring neurons in order to spread the pathology.

There is strong evidence that Tau secretion occurs, since extracellular Tau has been found in brain fluids, such as cerebrospinal fluid (CSF) and interstitial fluid (ISF)[77]. Moreover, Tau is present in CSF and ISF of healthy individuals, suggesting that Tau is also released by neurons, as a

physiological process, independently of neuronal death[78,79]. However, it is still controversial how neurons secrete Tau. Recent findings suggest that Tau may be released by two different mechanisms: packaged in exosomes or in a free soluble form. Consistent with secretion of Tau in exosomes, Tau was detected in exosomes isolated of both healthy age-matched controls and AD patients[78]. Moreover, neuroblastoma M1C cells, with inducible expression of wild type human Tau, secreted both exosomal and free soluble Tau[78]. Other study involving Tau overexpression in non-neuronal cell lines (COS-7 and HEK cells) revealed that Tau is released in microvesicles, in the absence of neurodegeneration[79]. Taking into account these results, it can be suggested that Tau may be packaged and secreted within neuron-derived vesicles, but it is unclear if exosomal Tau release is a regulated physiologic process or results from overexpression of Tau in these cell models. On the other hand, several studies suggest that Tau may be released in a free soluble form. First, studies with HEK cells with inducible expression of human Tau, revealed soluble extracellular Tau, but no Tau in the exosomal fraction[80]. Furthermore, a proteomic analysis of neuronal exosomes derived from cortical neurons did not show detectable levels of Tau in this fraction. Thus, it seems that Tau should be released in a free soluble form, instead of exosomal. However, there is a great discrepancy between the results, so is still unclear which mechanism really occurs. An explanation for these results could be due to the use of different model systems, since studies showing exosomal secretion of Tau involved overexpression of Tau (in certain cases it is not physiological), whereas studies revealing soluble Tau secretion used physiological Tau expression. Other possibility could be that both mechanisms occur in an activity-regulated process[81]. That is, with normal levels of Tau, it is secreted by a free soluble mechanism, as a constitutive/physiological release, while with Tau overexpression or toxic levels it is secreted by an exosomal/vesicular mechanism. Supporting this, studies using an inducible Tau expression cell model, evidenced that Tau release not associated with vesicles occurred in the absence of cytotoxicity[80,82]. Accordingly, the amount of Tau secreted was greatly increased after stimulation of neuronal activity[83,84].

After secretion, extracellular Tau may be internalized by neighboring cells to facilitate Tau spreading in AD and other Tauopathies. Although knowledge about neuronal Tau uptake is still scarce, recent studies have exploited the mechanism and the nature of the Tau that is internalized. Wu and colleagues[86] proposed that specific Tau species are internalized through an endocytic mechanism, since Tau aggregates co-localized with proteins involved in the endosomal pathway[86]. Internalization of extracellular Tau has been exploited *in vivo* by brain inoculation of Tau aggregates and monitoring their uptake. Injection of Tau filaments from P301S transgenic mice into young wild-type mice caused filamentous Tau inclusions of wild-type Tau and spreading

to neighboring brain regions[87]. Thus, in this model, the presence of Tau filaments expressing mutant Tau was sufficient to induce propagation of the pathology, that is, these Tau filaments can be considered as "Tau seeds". Another focus of intense research has been the determination of which forms of Tau are more prone to be internalized. For that, several studies have directly tested the uptake of specific species of Tau. In a study with either low-molecular weight aggregates, short fibrils or long fibrils of recombinant full-length Tau, only aggregates and short fibrils were internalized[86]. Furthermore, HEK cells overexpressing a monomeric or oligomeric recombinant Tau microtubule-binding domain (amino acids 243 to 375) formed aggregates which were released and internalized by other HEK cells, suggesting that these fragments can be transferred between cells *in vitro*[88]. *In vivo* studies showed that both full-length Tau and a truncated form of Tau (only with the microtubule-binding domain) were internalized by cells and propagated across the brain[89]. Intriguingly, a different study suggested that cells might discriminate the types of Tau internalized *in vivo*, since both oligomeric and PHFs Tau were internalized. However, only oligomeric Tau was able to propagate to other brain regions, suggesting that, although PHFs are internalized, they may not be transported and released by neurons[90]. In contrast, Takeda et al.[91] claim that a phosphorylated high molecular weight Tau species is internalized and synaptically propagated between neurons. In this study, different Tau species derived from postmortem human cortical extracts and ISF of Tau transgenic mice were analyzed. Surprisingly, only this rare high molecular form was considered as the endogenous form of Tau responsible for propagation of pathology, while more abundant lower molecular weight species (monomers and oligomers) were not able of spreading Tau pathology. Since contradictory results have been described, further studies are needed to clarify which Tau species are secreted and internalized both *in vitro* and *in vivo,* facilitating the transmission of Tau pathology between cells.

1.2.5 *In vivo* study models of Tau spreading

In the past years, several transgenic mouse models have been developed in order to understand Tau pathology in AD, namely to study the mechanisms of Tau spreading. The vast majority of models were achieved through the use of known Tau mutations in other Tauopathies (forms of frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17)).

One of the first models produced was a Tau transgenic mouse for the 383 amino acid isoform of human Tau with the P301S mutation[92]. This mutation occurs in exon 10 of Tau and causes early onset clinical signs and has strong functional effects. Several studies suggest a reduced ability of P301S mutant Tau protein to promote microtubule assembly and, in turn, a strong stimulatory effect to Tau self-assemble in filaments[93,94]. This transgenic mice line was expressed under the control of the murine thy1 promoter. Successfully, the transgenic mouse line described, represented the essential features of a human Tauopathy, including the formation of filaments of hyperphosphorylated Tau and nerve cell degeneration[92]. Later, several models were produced based on this mutation. In a study by Yoshiyama et al.[93], a cDNA construct of the WT human T34 isoform Tau (1N4R), harboring the P301S mutation, was cloned with an expression vector containing the mouse prion (MoPrP) promoter (PS19 line). This PS19 transgenic mice line was used in order to detect early manifestations of human Tauopathies. Indeed, PS19 mice developed synaptic pathology and microgliosis in hippocampus at 3 month-old and synaptic dysfunction at 6 months, before neuron loss and NFTs formation[92]. Furthermore, a double mutation transgenic Tau line was produced in order to obtain high pathogenicity and, thus, generate an improved model for NFTs formation in Tauopathies/AD[95]. To the P301S mutation, a K257T mutation, associated with severe phenotypes of FTD, memory loss and high Tau-immunoreactivity, was added[95]. Moreover, these two mutations are associated with insertion of potential phosphorylation sites, turning Tau more prone to form NFTs. A natural rat Tau promoter reported to direct positive control of neuronal-specific expression was used[95]. Therefore, these animals displayed several features characteristic of the disease, without non-cognitive features, such as motor deficits, which were a disadvantage of some previously reported NFT-related tg mice[92]. Also a great improvement in Tau transgenic mice was the production of the THY-Tau22 double mutation model[96]. In this case, Tau mutations G272V (mutation in the first microtubule binding repeat motif in all Tau isoforms[96]) and P301S were generated into the human 4-repeat Tau and cloned with the Thy1.2 expression vector, which specifically drives expression in neurons without affecting embryonic development. As in the previous model, the THY-Tau22 model exhibit common features of Tau pathology in the absence of any motor dysfunction. Moreover, this model showed a high resolution of different steps of Tau pathology, namely Tau phosphorylation, ranging from the start of Tau phosphorylation (3 months) to NFT-like inclusions occurrence, spreading to other brain regions (6 months) and neurodegeneration (10-14 months)[96]. Models combining Aβ pathology and Tauopathy have also been developed in order to study Aβ-tau interplay, namely the model in which APP/PS1 mice were crossed with PS19 mice[97].

Other common mutation used in models of Tau pathology is the P301L, which is also coupled to familial forms of FTD and FTDP-17. In patients with dementia and P301L mutation in Tau, widespread neuronal and glial Tau inclusions were observed[98]. Therefore, Gotz and colleagues hypothesized that P301L could be used to simulate Tau pathology[99]. They created a P301L transgenic mice model with the longest human brain Tau isoform, using the promoter Thy1.2. In this study, was demonstrated that human Tau can form filaments in mouse brains and is able to reproduce aspects of the human Tau pathology such as NFTs formation. Next, several improved P301L models emerged. One example is the Tg4510r line, which is able to express mutant Tau that could be suppressed with doxycyline[100]. This model was also generated with a TRE tet-off promoter, which restricted the expression only to forebrain structures. In this study, progressive age-related NFTs were formed and neuronal loss and behavioral impairments occurred. However, after transgenic Tau was suppressed with doxycycline, memory function recovered and neuronal death stopped, but NFTs continued to accumulate[97]. Therefore, authors concluded that NFTs are not sufficient to cause cognitive dysfunction or neurodegeneration in this Tau pathology model. This model revealed a different approach to study Tau pathology and clearly shows that different models should be applied to different study targets. More recently, an interesting inducible mouse line was developed based on P301L mutation. In order to study early spreading of Tau pathology outside of the entorhinal cortex (EC), Liu et al., generated a transgenic mice line (h0N4RP301L) with a neuropsin-tTa activator, driven by the promoter of the neuropsin gene, crossed with the Tg4510 inducible line previously described[75,100]. This model resulted in the specific expression of Tau in the EC and, then, to other brain regions, accordingly to what was described by Braak et al[25]. Moreover, the authors were able to propose the model of trans-synaptic spreading of Tau pathology.

Several more transgenic Tau models have been developed throughout the years and this review only focused in the more remarkable ones. Although these models allowed the understanding of some concepts, the development of more specific models is essential to unveil several questions related to Tau pathology. Therefore, the road ahead should be to define a model for each step of the neurodegeneration pathway, including Tau phosphorylation, Tau aggregation and Tau spreading. Thus, the collection of all the data from each specific model could provide great insight about Tau pathology and, consequently, facilitate the development of new therapeutic strategies.

1.2.6 Therapeutic strategies

Taken together, the above findings suggest a central role of Tau in neurodegeneration and, thus, the interest in Tau-targeted treatments for Alzheimer's disease is growing. Recently, several therapeutic approaches to prevent Tau pathology have been developed, such as: inhibition of Tau phosphorylation; inhibition of Tau aggregation; inhibition of Tau spreading.

As referred, Tau hyperphosphorylation seems to be an early crucial event in Tau pathology, namely by detaching Tau from microtubules, allowing Tau to form aggregates. Intuitively, inhibition of Tau kinases and/or activation of Tau phosphatases seem to be good therapeutic targets. However, since these enzymes have several substrates, this strategy may not be safe due to unspecific targets of Tau kinases and phosphatases. Nevertheless, beneficial effects of protein kinase inhibition were reported in a study with a P301L mouse transgenic line, namely using inhibitors of the kinase GSK-3β[101]. In contrary, no clinical benefit have been reported using inhibitors of GSK-3β in patients with AD[102,103]. The other strategy consisting in the activation of Tau phosphatases was more promising, since, in mouse models of Tauopathy, the antidiabetic drug metformin, as well as, chronic low doses of sodium selenate reduced Tau phosphorylation by increasing PP2A activity[104,105]. Moreover, Tau aggregation and neurodegeneration were decreased and cognition was improved[19].

Following, inhibition of Tau aggregation during the pathway that leads from soluble and monomeric Tau to hyperphosphorylated, insoluble and filamentous Tau, also seems to be a promising therapeutic strategy. Particularly, the design of an amino acid peptide based on the atomic structure of the amino-terminus of the third repeat of Tau (Val-Gln-Ile-Val-Tyr-Lys) was effective in inhibiting Tau filament formation[106]. Phenothiazine methylene blue inhibited Tau aggregation *in vitro*, by modification of the cysteine residues in the repeats of Tau[107]. *In vivo* studies confirmed this effect of methylene blue. Furthermore, phase 2 clinical trials for mild to moderate AD treated cognitive deficits[108] and currently phase 3 trial of methylene blue are in progress.

Prevention of Tau spreading has been a recent focus of intense research by different approaches: blocking of Tau release; inhibition of Tau uptake; reducing levels of extracellular Tau by preventing accumulation or oligomerization (Fig.5). Targeting Tau release seems to be a good strategy, since it will reduce extracellular Tau levels and reduce its availability to be internalized and spread Tau pathology. However, the possibility of constitutive release of Tau *in vivo* and the

presence of extracellular Tau in absence of neurodegeneration may suggest unknown functions of Tau outside the cell. Therefore, blocking Tau release could interfere with these physiological processes. Reduction of extracellular Tau concentration can be achieved by several strategies. Both decreasing of Tau expression and increased Tau clearance could be effective approaches. Particularly, enhancement of proteasome function revealed effective in stimulating degradation of soluble Tau[109]. On the other hand, increased activity of autophagy-lysosome pathway induced degradation of Tau aggregates[110,111]. A different study suggested that inhibition or reversion of extracellular Tau oligomerization might interfere with Tau spreading and could also be a successful strategy[112]. However, this strategy may result in the formation of other toxic soluble Tau species. Nevertheless, to date, the most promising therapeutic strategy has been the passive or active Tau immunization. Passive immunization consists in the use of specific antibodies of Tau aggregates in order to block Tau spreading from the extracellular space, whereas active immunization uses Tau peptides as antigens. Several studies have shown beneficial effects in transgenic mouse models of Tauopathy[113,114,115]. Recently, some studies have been evaluating passive immunization as a strategy to sequester different extracellular Tau species, namely by targeting oligomeric and phosphorylated Tau. First, one study using a tau oligomer monoclonal antibody administered in mice expressing mutant human Tau, showed reversion of locomotor and memory deficits as well as a decrease of Tau oligomers. However, neither phosphorylated NFTs nor monomeric Tau was reduced, suggesting the essential role of Tau oligomers in the disease progression[116]. Then, a study using two independent mouse models of Tauopathy, tested different antibodies, either specific for full-length or phosphorylated Tau (pS404)[117]. Expectedly, only the antibody specific for phospho-Tau was able to reduce hyperphosphorylated Tau and tangle burden in both transgenic mouse models, alleviating Tau pathology, and, thus, validating the feasibility of a passive immunization approach for AD. Accordingly, in a more recent study, it was demonstrated that other phospho-Tau antibodies, targeting pT231 and pS396, reduced brain and CSF phospho-Tau levels and improved cognition in Tau transgenic mice[118]. Moreover, when an anti-Tau antibody specific for the N-terminal region

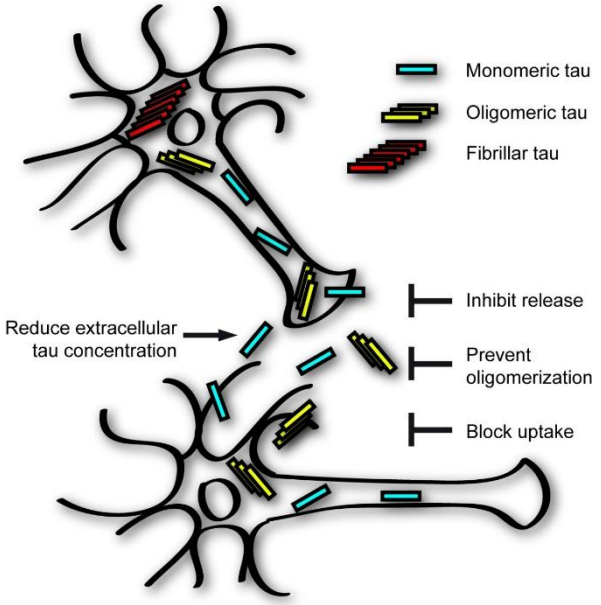

Fig.5: Potential therapeutic targets to slow or prevent the spread of Tau pathology in the brain. From: Pooler et al., 2013[82]

(HJ8.5) was administered in Tau transgenic mice by intraperitoneal injection, a great reduction of insoluble Tau was observed[119]. Consistently, also a decrease in abnormal phosphorylated Tau was seen, as well as, reduced brain atrophy and improved motor behavior. Taking into account all these recent studies, it is clear that passive immunotherapy may be an effective therapeutic approach for alleviating Tau pathology. Nevertheless, a study in which three phospho-Tau antibodies specific for different epitopes were tested, not all antibodies showed the same efficacy, with only one having a real effect in preventing the development of Tau pathology[120]. Thus, draws attention to the importance of screening multiple Tau antibodies not only in different brain regions, but also in distinct *in vitro* and animal models, before proceeding to clinical trials. Consequently, ongoing clinical trials will confirm whether anti-Tau immunotherapy may slow or reverse disease progression in AD[121].

Since extracellular Tau has been found in ISF, one possibility to show target engagement for anti-Tau immunotherapy could be by measuring ISF Tau levels, as well as, characterizing which Tau species are present in ISF. Thus, if an alteration in ISF Tau concentration of pathogenic Tau species occurs after applying the treatment, it can suggest that Tau immunization is being successful.

1.3 CSF AND ISF TAU

Tau is a cytoplasmic protein, but several studies (see Tau pathology) revealed that it can be secreted to the extracellular space. CSF is a brain fluid produced in the choroid plexus of the ventricles of the brain and occupies the subarachnoid space. This fluid functions as: 1) buoyancy system protecting the brain from injury; protection against rises in intracranial pressure; major source of entrance of substances into the brain; 2) clearance system, having a sink action that helps to maintain needed low concentrations of several substance in the brain; 3) route of communication from the brain to the rest of the body, playing a role in signal transduction[122]. ISF is composed of water, solutes and structural molecules of the cellular microenvironment in the brain or extracellular matrix (ECM) components[123]. The ISF transports nutrients and waste products between cells and blood capillaries but it is also an important route of signaling molecules and cells of the immune sytem[124]. The CSF-ISF connection is thought to play an

important role in the clearance of interstitial solutes, likely including soluble extracellular Aβ and Tau. Thus, a system called "glympathic pathway" was proposed, in which influx of CSF into the brain interstitium and ISF efflux along large-caliber draining veins are responsible for interstitial solutes clearance[124].

Tau is present in CSF of healthy individuals, suggesting a physiological release of Tau. However, CSF Tau levels could increase under certain pathological conditions such as stroke[125], prion diseases[126] or AD (moderately)[127]. Interestingly, in forms of FTD due to Tau mutations, CSF Tau is not increased[128]. Until recently, very little information was known about ISF Tau levels, since no methods to assess ISF Tau in living animals existed. Thus, Yamada et al[78], in 2011, applied a microdialysis technique to analyze monomeric ISF Tau levels based in a technique previously used to assess ISF Aβ[129]. Microdialysis consists in an invasive method that allows *in vivo* sampling and collecting of molecules from the interstitial space. This method uses a probe, which captures molecules from the extracellular space by diffusion through a concentration gradient. Each microdialysis probe has a dialysis membrane through which a solution is constantly perfused, resembling the ionic composition of ISF. The dialysis membranes can differ in pore size and used material[130]. During the process, the samples are continuously collected into microvials[131]. In this study, they validated a microdialysis method in wild-type and P301S Tau transgenic mice, using probes with 100 kDA cutoff membranes, allowing them to measure only monomeric Tau, since large molecules cannot bypass microdialysis membrane with this size. After validation, the authors evaluated the method before the appearance of insoluble Tau and neurodegeneration. ISF Tau was detected in both wild-type and P301S tg mice, but the monomeric ISF exchangeable Tau (e-Tau) was fivefold higher in P301S tg mice than in wild-type mice, consistent with the expression level of human Tau being also fivefold higher in P301S than in wild-tipe[93]. Furthermore, e-Tau concentrations in wild-type mice were 10-fold higher in ISF than CSF[78]. Since these mice did not show any signs of neurodegeneration at this age, it can be suggested that Tau is normally released from cells into the extracellular space, namely to ISF, under physiological conditions. Furthermore, Yamada and colleagues verified whether ISF Tau levels were influenced by the appearance of Tau aggregates during aging. As expected, only P301S tg mice started to accumulate filamentous Tau, at about 6 months of age, and it continued to increase in an age-dependent manner. On the other hand, ISF Tau levels decreased with age, suggesting that the increased formation of intracellular Tau aggregates may be related to the dramatic drop in monomeric ISF Tau levels in P301S tg mice. In another experiment, CSF Tau levels in P301S tg mice increased in an age-dependent manner. No correlation between ISF and CSF levels was verified, suggesting an independent regulation. Thus, an equilibrium between intracellular Tau aggregates

and ISF monomeric Tau was proposed in this work, namely that the formation of Tau aggregates was decreasing soluble Tau. Supporting this hypothesis, evidences emerged suggesting that the increase in insoluble brain Tau (Tau aggregates) results in a decrease in brain soluble Tau, which corresponds to a reduction of ISF Tau, since Tau aggregates may sequester P301S soluble monomeric Tau with higher aggregation potential. Taking into account all findings revealed in this study, the authors proposed a model regarding Tau release in the absence/presence of Tau aggregates (Fig.6).

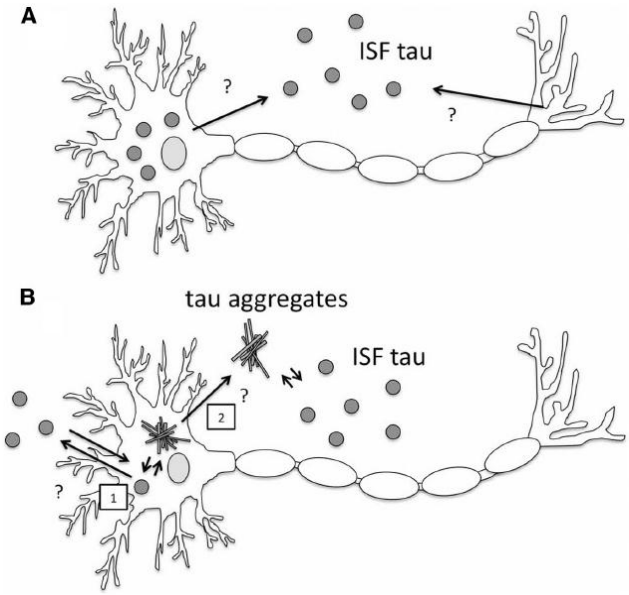

Fig.6: Proposed model of Tau release in the presence or absence of Tau aggregates. Under normal conditions, that is, in the absence of Tau aggregates, Tau is constitutively released to ISF (A). In contrast, once Tau intracellular Tau aggregates are formed, Tau is sequestered inside the cells, leading to a decrease in ISF Tau. Since Tau aggregates can potentially be formed outside, they can also decrease ISF Tau directly by sequestering extracellular soluble Tau (B)[73]. . From: Yamada et al., 2011[78]

Nevertheless, this model may have some limitations. First, since the dialysis cutoff probe was 100 kDA, it did not assess the presence of dimers, oligomers, or even Tau in exosomes in ISF. Thus, the decrease in monomeric soluble Tau could be related to an increase in Tau oligomerization for example. Moreover, recent findings suggest that Tau oligomers could be the most pathogenic Tau species[90]. Second, an increased activity of clearance mechanisms could also account for the decrease of ISF Tau[78]. Thus, it is important to test this in future studies in order to rule out this hypothesis. Third, increased phosphorylation of extracellular Tau at AT-8 epitope could explain the decrease in ISF Tau levels since the antibody used was the BT-2, which has an epitope proximal of the AT-8 epitope but binds preferential to non-phosphorylated Tau, and, thus, increased phosphorylation at AT-8 could result in reduced availability of the BT-2 epitope.

Therefore, further studies should use specific antibodies to detect ISF phosphorylated Tau. In summary, although the findings reported here are promising, the great novelty of this study is the development of an *in* vivo technique to assess Tau-mediated pathogenesis and target engagement for treatments that influence extracellular Tau levels, such as immunotherapy.

Taking advantage of this technique, numerous recent studies have been investigating the role of extracellular Tau and how it changes in response to increased Tau pathology. For example, a paper from the same group[132], investigated whether antisense reduction of extracellular Tau in adult mice protected against seizures. To follow brain ISF Tau levels *in vivo*, a modified method of the one previously described was used. In this case the probe used had 1000 kDA cutoff membrane. Indeed, they demonstrated that the treatment with antisense oligonucleotides (ASOs) delivered directly to the CSF, reduced CSF and ISF Tau levels. Moreover, the authors established a correlation between total Tau protein levels in the brain and seizure severity, since Tau levels reduction was protective against induced global seizures. Recently, other study[133] used *in vivo* microdialysis to verify whether increased neuronal activity promoted Tau release. Using this technique, authors were able to assess, hourly, endogenous ISF Tau levels in awake and freely moving mice, simulating the normal functioning of neuronal networks. In response to increased neuronal activity, using a high K^+ perfusion solution, the ISF Tau levels increased, altering the steady-state levels of pre-existing ISF Tau. Furthermore, was verified that pre-synaptic glutamate release is sufficient to increase ISF Tau levels, suggesting that presynaptic excitatory neuronal activity is linked to Tau release. Importantly, this microdialysis technique was already adopted to assess aβ and Tau dynamics in ISF of human patients with suspected normal pressure hydrocephalus[134]. In that study, AD-related biomarkers such as ISF $a\beta_{1\text{-}42}$, total Tau and phospho Tau, were investigated without causing significant brain injury or adverse effects due to the microdialysis procedure, enabling this method as a manner to follow AD pathogenesis in the living brain during the different stages of the disease. In summary, these findings highlight the importance of the advent of this technique, and further studies may use it, since the value of *in* vivo microdialysis has been proven in important investigations regarding the role of extracellular Tau.

1.4 CONCLUDING REMARKS

As stated above, one of the intriguing tasks will be the unravelling of the biochemical and biological processes involved in Tau release from neuronal cells. This will also be crucial to evaluate the spreading components of TG Tau mouse models and for the development of target engagement assays for therapeutics interfering with extracellular Tau species. The development of a variety of transgenic mice provided evidence supporting the hypothesis that Tau pathology proceeds via trans-synaptic transport of a pathogenic species of Tau[73]. This hypothesis is further strengthened by the finding that injection of ALZ17 mice (a strain expressing normal human Tau) with brain homogenates derived from different Tauopathies induce the formation of Tau inclusions with a morphology that resembles the Tauopathy in human brain[87,135]. Despite the variety of mouse Tau aggregation (spreading/seeding) models, the species of Tau responsible for Tauopathy spread is elusive. An even more compelling question is to know what is the relationship between this form of Tau and the species detected in CSF. A recent study performed in P301S mice demonstrated that soluble Tau is released *in vivo* and the levels drop under conditions of elevated Tau aggregation[78]. Furthermore, studies showed that secreted Tau is phosphorylated and cleaved at the C-terminus[83] and that an important fraction of this form of Tau is enriched in exosomes[79]. This would imply that the increase in phosphorylated Tau in CSF is not only a result of passive release from dying neurons but that also an active transport component contributes to Tau signals in CSF. Methodology applied in the Yamada study on brain ISF of P301S mice[78] should be repeated with other models. Preferentially in one model where Tau aggregation can be induced by injection and in another where Tau aggregation is exacerbated by amyloid pathology. Finally, similar analysis should be performed in mice having amyloid pathology without overt signs of Tau pathology as described in Maia et al[136].

In this project we will focus on the characterization of Tau in ISF from Tau transgenic mice which will be collected by different probes (1 MDa), using the methodology applied by Yamada et al[78]. The main objective is to quantify and characterize Tau species or aggregates in young P301S transgenic mice, at very early stages of Tau pathology.

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