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Altered neuronal activity in the hippocampal CA3 region in an in vitro model of tauopathy

Ânia Gonçalves

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE
Sciences de la Vie et de la Santé
Spécialité: Neurosciences

Par Ânia GONÇALVES

**Altered neuronal activity in the hippocampal CA3 region in
an *in vitro* model of tauopathy**

Sous la direction de : Sandrine POUVREAU

Soutenue le 17 décembre 2020

Membres du jury :

Dr. Giovanni MARSICANO	Directeur de Recherche, INSERM	Président
Dr. Alexis BEMELMANS	Chargé de recherche, CEA	Rapporteur
Dr. David BLUM	Directeur de Recherche, INSERM	Rapporteur
Dr. Ilse DEWACHTER	Professeur, University Hasselt	Examineur
Dr. Olivier NICOLE	Chargé de recherche, CNRS	Examineur
Dr. Juan PITA-ALMENAR	Senior Scientist, Janssen Pharmaceutica	Invité
Dr. Sandrine POUVREAU	Chargé de recherche, CNRS	Directeur de thèse

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Para a minha irmã, Diana.

Por nunca parar de fazer perguntas, por não me deixar parar, nunca, de procurar respostas e por acreditar que eu sou 'tao inteligente que até irrita'.

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Résumé

La maladie d'Alzheimer (MA) est une maladie neurodégénérative caractérisée par des dépôts cérébraux de plaques amyloïdes et de neurofibrilles tau. La propagation de la tauopathie est fortement corrélée au déclin cognitif chez les patients, mettant en évidence le rôle critique de tau dans la MA. Cependant, les mécanismes reliant le développement de la tauopathie et l'altération progressive des fonctions cognitives restent méconnus. Différents modèles *in vitro* et *in vivo* ont été utilisés pour étudier les caractéristiques pathologiques liées aux tauopathies, telles que le dysfonctionnement synaptique, l'altération de l'activité neuronale et la perte neuronale. Néanmoins, il reste difficile de trouver un modèle qui permette d'évaluer la toxicité de tau dans des circuits neuronaux complexes avec une résolution spatio-temporelle appropriée.

Par conséquent, le but de ce travail de thèse a été de développer un modèle *ex vivo* de tauopathie liée à la MA, basé sur des coupes organotypiques d'hippocampe, pour l'investigation à haute résolution spatio-temporelle des altérations progressives de la physiologie neuronale. Nous avons profité de la capacité d'ensemencement des fibrilles de tau pour induire une tauopathie dans les coupes organotypiques d'hippocampe, une structure particulièrement vulnérable dans la MA.

Nous avons pu observer une accumulation progressive de tau hyperphosphorylé dans ces tranches et le développement des principales caractéristiques de la maladie telles que l'accumulation d'autres formes pathologiques de tau, la neurodégénérescence et la vulnérabilité cellulaire sélective. Notre modèle récapitule développement progressif de la pathologie observé chez des souris transgéniques âgées. De plus, notre modèle présente une altération de l'activité neuronale aux stades avancés de la pathologie, ainsi que l'accumulation de tau hyperphosphorylé et la perte neuronale dans CA3. Cette altération, consistant en une hyperactivité neuronal et une hypersynchronie, est reproduite dans les tranches WT par inhibition simultanée de la transmission GABAA et GABAB. Nos données suggèrent que l'altération de la signalisation GABAergique sous-tend les altérations de l'activité neuronale observées dans la tauopathie.

Mots clés : tau protein, maladie d'Alzheimer, réseau neuronal

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterised by brain depositions of amyloid plaques and tau tangles. The propagation of the tauopathy correlates strongly with the cognitive decline in patients, highlighting the critical role of tau in AD. Yet, the mechanisms linking tauopathy and impaired brain functions remain elusive. Different *in vitro* and *in vivo* animal models have been used to study the pathological features related to tauopathies, such as synaptic dysfunction, impaired neuronal activity and neuronal loss. Nevertheless, it is still challenging to find a model that allows the evaluation of the tau toxicity in a complex neuronal circuit with proper spatio-temporal resolution.

Hence, in this work, we developed a physiologically relevant *ex vivo* model of AD-linked tauopathy, based on organotypic hippocampal slices, for the investigation at a proper spatiotemporal resolution of progressive impairments in neuronal physiology. We took advantage of the seeding properties of misfolded tau to induce tau pathology in organotypic hippocampal slices, considering that the hippocampus is particularly vulnerable in AD.

The hippocampal organotypic slices model shows a progressive accumulation of hyperphosphorylated tau and the main features of the disease such as accumulation of other pathological forms of tau, neurodegeneration and selective cellular vulnerability. Our *in vitro* model recapitulates the development of the disease observed in old transgenic mice. Additionally, our model displays altered neuronal activity at late stages of the disease, along with the accumulation of hyperphosphorylated tau and neuronal loss in CA3. Remarkably, the pathological pattern of neuronal activity, including hyperactivity and hypersynchrony, could be reproduced in WT slices by simultaneous inhibition of GABAA and GABAB transmission. Our data suggest that impairment of GABAergic signalling underlies the altered neuronal activity found in tau pathology.

Keywords: tau protein, Alzheimer's Disease, network activity

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Abbreviations

AAV - Adeno-associated virus
AD - Alzheimer's disease
AED - antiepileptic drugs
ApoE - apolipoprotein E
APP - amyloid precursor β protein
A β - amyloid beta
CA1 and CA3 - *Cornu Ammonis* regions
CBD - corticobasal degeneration
CDK5 - cyclin-dependent kinase
CSF - cerebrospinal fluid
DG – dentate gyrus
DIV - days *in vitro*
EC – entorhinal cortex
EOAD - early-onset AD
FAD - familial AD
FDG - fluorodeoxyglucose
fMRI - functional MRI
FTDP - 17 - frontotemporal dementia and parkinsonism linked to chromosome 17
GABA - acid γ -aminobutyric
GFAP - glial fibrillary acidic protein
GSK-3 - glycogen synthase kinase
HSPGs - heparan sulfate proteoglycans
LOAD - late-onset AD
MAPK - mitogen-activated protein kinase
MAPT – microtubule-associated protein
MCI - Mild cognitive impairment
MF – mossy fiber
MMSE) - Mini-Mental State Exam
MT- microtubules
MTBD - microtubule-associated domain
NeuN – Neuronal nuclear protein
NFT - neurofibrillary tangles
PET - positron emission tomography
PFA - paraformaldehyde

PHF - paired helical filaments

PiD - Pick's disease

PPs - Protein Phosphatases ()

PSP - progressive supranuclear palsy

p-tau - phosphorylated tau

PV – Parvalbulin

Ser - serine

Thr - threonines

TLE - temporal lobe epilepsy

Tyr - tyrosine

Introduction

1. Alzheimer's disease (AD)

Dementia is a syndrome characterised by a cognitive decline that affects mostly the elderly. However, it is not part of normal ageing. In 2018, approximately 50 million people worldwide were living with dementia. It is estimated that this number will triple by 2050. Alzheimer's disease (AD) is the most common form of dementia, contributing to approximately 60-70% of the cases (WHO, 2019). The most evident clinical symptoms of AD are memory loss, impairment of learning and thinking followed by later deterioration in communication, motor function, and behaviour (Kaj Blennow et al., 2006; McKhann et al., 2011). As a consequence of the progression of the disease, the patients lose the ability to perform daily activities and increasingly need assisted self-care.

1.1. AD diagnosis

Alzheimer's disease was described for the first time by Alois Alzheimer in 1907. The patient was Auguste D., a 50 years old woman who showed sleep disorders, failures in memory retrieval, especially for recent events, unpredictable behaviour, disorientation and aphasia. Besides describing the symptoms and the progression of the illness, Alzheimer also investigated her brain after her death in 1906. The morphological and histological investigation reveals brain atrophy, massive loss of cells, amyloid plaques and neurofibrillary tangles (NFT). Alzheimer described for the first time what today are known as the histopathological hallmarks of AD (Alzheimer et al., 1995; Dahm, 2006; Hippus & Neundörfer, 2003).

There is no single or a combination of features exclusive for AD, however several macroscopic and microscopic features that can lead to a diagnosis. There is significant brain atrophy, especially in the frontal and temporal cortices than in the primary motor and somatosensory cortices. Consequently, a frontal and temporal horns enlargement occurs, and there is a loss of brain weight. Still, moderate cortical atrophy and volume loss of the brain can be found in older people without any disorder. This temporal cortices atrophy along with medial temporal atrophy affecting the amygdala and the hippocampus is widespread in AD, but also other age-related disorders exhibit these features (DeTure & Dickson, 2019). Thus, the presence of the main hallmarks of the disease is necessary to establish a precise AD diagnosis. The criteria should be centred on three morphological features: amyloid plaques, NFTs and neuritic plaques. Most of the time, a microscopic examination is required post mortem to assess these

microscopic features, which aids to identify the stages of progression of the disease (M. S. Albert et al., 2011).

Regarding a clinical diagnosis, physicians use different and test and approaches such as interviews with the patient and relatives to understand behaviour changes; neuropsychological tests to evaluate cognitive functions — episodic memory, executive function, language, visual and spatial skills, and attention; biomarkers and genetic tests.

The psychiatric history, as well as the family history of relatives who have been diagnosed with AD or other forms of dementia, may help to identify the cause of the symptoms.

The evaluation of the performance in neurological tests allows to assess to the brain state and possible issues and to consequently screen not only for AD but also for other brain disorders. (Alzheimer's Association). The most common mental status test is the Mini-Mental State Exam (MMSE) which tests thinking and memory skills. The MMSE evaluates the degree of cognitive impairment and is crucial to the clinical diagnosis (Folstein et al., 1975). Additionally, there are also neuropsychological tests not only to evaluate possible dementia but also to assess the ability of the patient to conduct the daily tasks safely.

Along with these validated neuropsychological tests, there are even more recent diagnostic tools that can be used. Through the analysis of tau and amyloid β ($A\beta$) present in the cerebrospinal fluid (CSF) and positron emission tomography (PET) imaging it is possible to identify a preclinical phase of AD during which the cognitive function is maintained. Several studies demonstrate that early amyloid depositions and neuroinflammatory changes can precede the appearance of clinical symptoms by 10-20 years (Vermunt et al., 2019) (Figure 1)

Eventually, genetic tests can be performed, considering genes that increase the risk of developing AD. However, according to the clinical diagnostic criteria guidelines, this is recommended for research purposes rather than for diagnostic (M. S. Albert et al., 2011).

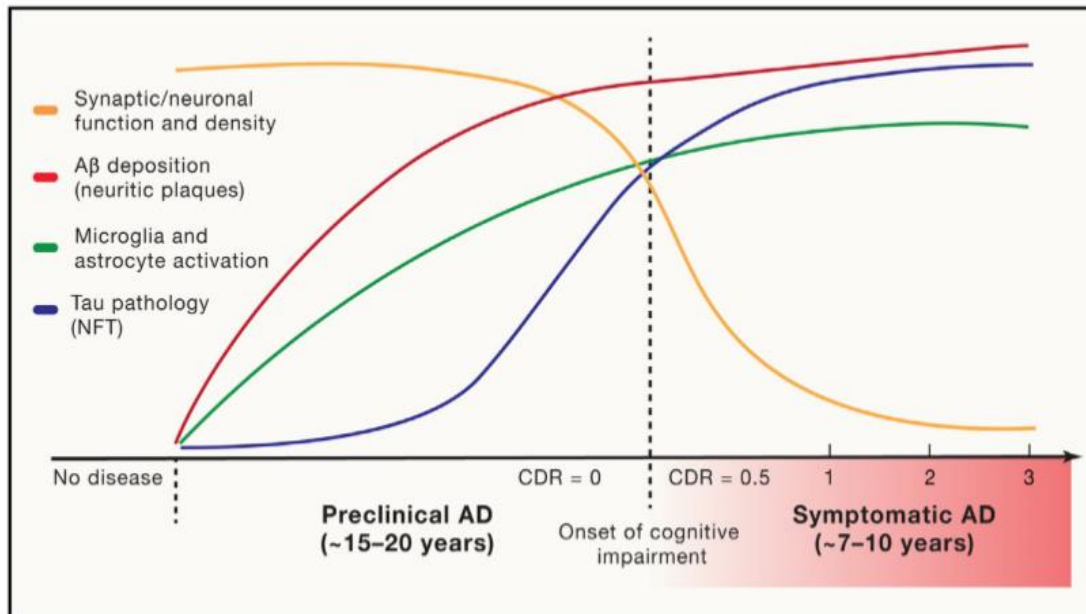


Figure 1. Timing of Main AD Pathophysiological Events in Relation to Clinical Course.

A prolonged preclinical phase of the disease is characterised by the early onset of amyloid deposition, which is detected by a reduction in CSF and plasma levels of A β 42 or increased global signal on amyloid PET imaging. Simultaneously, there are early neuroinflammatory changes (such as microglial activation). This is followed by the spread of neurofibrillary tangle (NFT), progression of tau pathology, from the medial temporal lobes into the neocortex. This is detected by the increased signal on tau PET imaging and increased CSF phosphorylated tau levels in patients. Synaptic dysfunction, synapse loss, and neurodegeneration accumulate with pathologic spread of tau aggregates. Onset and progression of cognitive impairment correlate with accumulation of tau and hippocampal volume loss but not an amyloid deposition. The severity of clinical symptoms in AD can be staged by use of the Clinical Dementia Rating (CDR) scale, where a score of 0 indicates normal cognition and scores of 0.5, 1, 2, and 3 indicate questionable, mild, moderate, and severe dementia, respectively (Long & Holtzman, 2019).

1.2. Classification of AD

AD can be divided into familial AD (FAD), early-onset AD (EOAD) and late-onset AD (LOAD). Specific mutation types can cause FAD. Patients who inherited this rare form are less than 1% of the cases. Usually, they exhibit the first symptoms from a very early age (20 years old) and with much faster progression of the disease (DeTure & Dickson, 2019). When it affects people before the age of 65, the disease is characterised as EOAD. These cases represent less than 5% and are associated with delays in diagnosis. The patients present an aggressive clinical course. The greater number of cases occurs after the age of 65 and are classified as LOAD. These cases are considered sporadic despite the existence of risk factors such as lifestyle, age, family history and genetic risk factor, the most prevalent being the apolipoprotein E 4 (APOE4) gene. The individuals who inherited the two copies of the APOE4 polymorphism have odds of ratio for AD of 12 compared to the non-carriers (Lane et al., 2018).

AD is a multifactorial neurodegenerative disorder making the diagnosis a challenging task. The progression of the disease varies between patients. The life expectancy after diagnosis is on average between four to eight years but can reach 20 years depending on several factors. According to the clinical diagnostic criteria, AD can be described in three stages: 1) *Preclinical*— At this stage, there are no significant symptoms, but the impairments of the brain, such as amyloid depositions, might have started. 2) *Mild cognitive impairment (MCI)*- At this stage, the memory and/cognitive capacities are significantly altered compared to a healthy person of similar age. However, the daily life of the patient is still not impacted. The MCI might progress to AD. 3) *Alzheimer's dementia* – This is the final stage of the disease. It is characterised by symptoms of dementia, such as memory loss, word-finding difficulties, and visual/spatial problems. At this point, the “independent living skills”, such as personal hygiene, home and money management, cooking and eating are compromised (M. S. Albert et al., 2011).

1.3. Available treatments

Currently, there is no medication available to slow or stop the progression of the disease. Thus, AD is ultimately fatal. Over the past 20 years, more than 100 drugs were tested, but only four were approved. Three of these drugs are cholinesterase inhibitors: donepezil, galantamine and rivastigmine, which are approved for mild-to-moderate AD treatment. These drugs aim to overcome the cholinergic deficit caused by the loss of cholinergic neurons. This loss has a significant effect on learning and memory in AD. The three drugs prevent the breakdown of acetylcholine, increasing the levels of this neurotransmitter at the synapse. Another drug,

memantine, an antagonist of the NMDA receptor, is indicated to treat patients with moderate-to-severe AD. The mechanisms by which memantine acts are still not completely understood. However, it is thought that it may act by inhibiting the toxicity caused by the increase of glutamate, which results in neuronal death (Rammes et al., 2008).

Of note, these drugs only help to ameliorate the symptoms of the disease, and their efficiency can vary from patient to patient. There is no effect on long-term disease progression.

In recent years several clinical trials have failed at early phases of drug development (Mehta et al., 2017). Most of the investment was focused on the amyloid cascade, but the results showed that the clearing of A β plaques is not enough to rescue the brain function neither to stop the worsening of the disease. As AD is a multifactorial disease, new targets are already starting to emerge (Figure 2), and it is increasingly believed that effective treatment implies a combination of therapies rather than a single medicine. However, the animal models available have some limitations that can make it difficult to extrapolate the results to humans. Therefore, it is important to develop better models not only to improve preclinical tests of drug candidates but also to provide new evidence that can lead to the understanding of AD pathobiology.

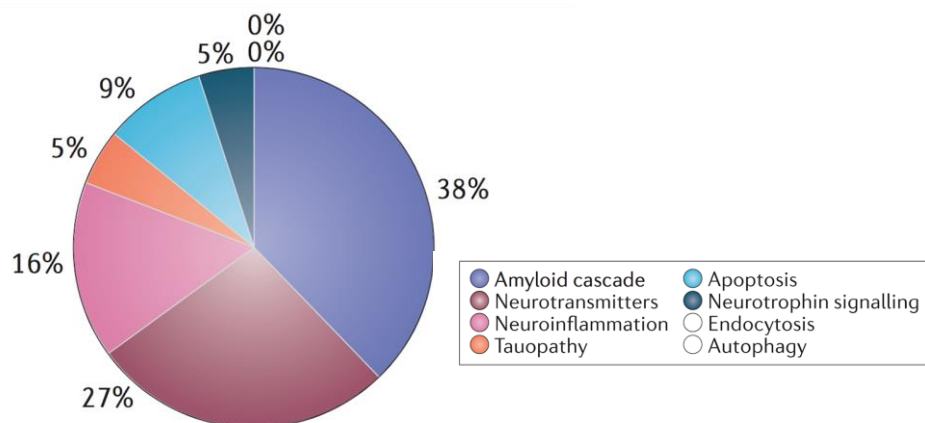


Figure 2. Investment in drug development for Alzheimer's disease (adapted from (Kodamullil et al., 2017)).

1.4. Histopathological hallmarks of AD

Alzheimer's disease is characterised by the progressive accumulation of insoluble proteins in the brain: the senile plaques composed by amyloid fibrils of A β peptide (Glennner & Wong, 1984; Masters et al., 1985) and the intraneuronal NFTs, paired helical filaments of the microtubule-associated protein tau (Wood et al., 1986) (Figure 3).

A β is a small peptide (about 40 aminoacids) which results from the cleavage of amyloid precursor β protein (APP) by β -secretase (BACE1) and γ -secretase. APP is a transmembrane protein whose physiological function is still unclear. APP has been associated with neuronal development, neurite outgrowth and axonal transport (Kang et al., 1987). Tau proteins constitute a family of six isoforms with the range from 352-441 amino acids. Tau is a microtubule-associated protein that stabilises the microtubules and regulates their dynamics and axonal transport (Weingarten et al., 1975).

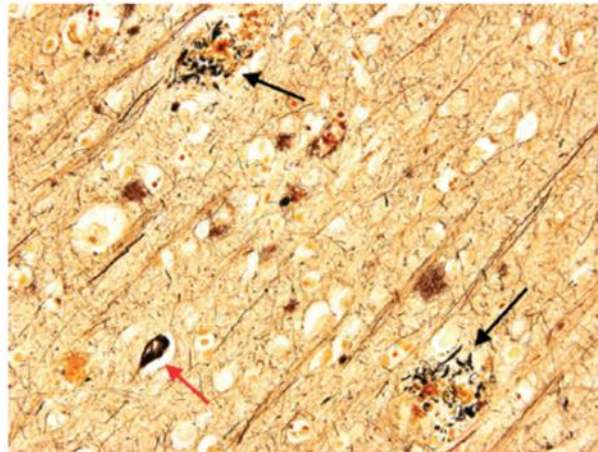


Figure 3. Photomicrograph of the temporal cortex of an AD patient. The presence of both histopathological hallmarks, extracellular A β plaques (black arrows) and intracellular NFTs (red arrow) stained with a modified Bielschowski stain and imaged at 100x. (Perl, 2010).

1.5. The Amyloid Hypothesis

Over the last 30 years, the amyloid hypothesis has been the conventional explanation for the pathogenesis of AD.

In physiological conditions, A β is cleaved by β -secretase and γ -secretase and released in the extracellular space where it is rapidly degraded or removed. However, in older people or under pathological conditions, the ability to clear A β decreases and the A β peptides accumulate. The primary forms present in these accumulations are A β 40 and A β 42. The increase in the level of A β 42 results in the formation of amyloid fibrils which ultimately develop into senile plaques.

(Huang et al., 2012; Pannee et al., 2013). The fact that genetic mutations of the APP gene cause FAD support the amyloid hypothesis. These mutations are close to the cleavage sites of β -secretase and γ -secretase and correlate with an increase in the production of A β 42.

Additionally, mutations in presenilin 1 and presenilin 2 genes were identified in FAD. These proteins are subunits of the γ -secretase complex. Both APP and presenilin mutations are strongly associated with the process of A β production corroborating the hypothesis that A β production and fibril formation play an essential role in the pathogenic cascade of AD (J. A. Hardy & Higgins, 1992; J. Hardy & Allsop, 1991; Selkoe & Hardy, 2016).

Based on this hypothesis, several transgenic mouse models were produced which developed amyloid plaques in the brain. However, these models do not exhibit NFTs and neuronal cell death (Bryan et al., 2009). These observations seem to indicate that the A β fibrils are neither toxic nor induce tau accumulation. The fact that A β is not toxic in physiological conditions gave rise to the theory that maybe the pathological species were the A β oligomers (reviewed (Hayden & Teplow, 2013). Thus, several studies were carried out to test this hypothesis (Haass & Selkoe, 2007). It was observed that the injection of A β oligomers from AD patients' brain extracts in the mouse hippocampus result in a decrease of dendritic spines, inhibition of long-term potentiation and impairment in memory and learning, which seemed to confirm the hypothesis (Shankar et al., 2008).

Although all of the AD patients develop amyloid pathology, there is no substantial evidence that demonstrates the correlation between the accumulation of A β in the brain and cognitive decline (Mormino & Papp, 2018).

1.6. The Tau hypothesis

The tau hypothesis proposes that the tau pathology is the crucial event in AD pathophysiology and the leading cause of neurodegeneration.

The function of tau depends on the phosphorylation/dephosphorylation balance. Under physiological conditions, tau has few phosphorylation sites that negatively regulates the binding to microtubules (Lindwall & Cole, 1984). Under pathological conditions as AD, tau becomes hyperphosphorylated, detaches from microtubules, and aggregates into paired helical filaments (PHF) (Grundke-Iqbal et al., 1986a; K. Iqbal et al., 1986; Kosik et al., 1986), which leads to the formation of NFT and accumulation in the cell bodies. Thus, tau phosphorylation and aggregation might be the primary event which triggers the formation of A β plaque and neuroinflammation in AD pathological cascade subsequently.

As mentioned before, amyloid depositions in the absence of tau pathology are not enough to produce dementia and can be found in normal aged individuals (Arriagada et al., 1992;

Katzman et al., 1988). Contrariwise, the accumulation of NFTs and consequent neurodegeneration are present in other neurodegenerative disorders known as tauopathies, such as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease (PiD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (Kametani & Hasegawa, 2018). Additionally, mutations of the tau gene have been identified for FTDP-17 inherited cases (Strang et al., 2019). These mutations result in tau abnormalities causing the loss of the ability of tau to bind the microtubules and consequently leading to tau aggregation and accumulation in the brain (Poorkaj et al., 1998a). All of this evidence appeared to support the tau hypothesis.

In 1991 Braak conducted a study that aimed to characterise morphological changes in AD brains and define stages of the disease. In this study, they evaluated extracellular amyloid deposits and NFTs changes in post-mortem brain samples from demented and non-demented individuals. The results demonstrated that the cases with severe NFT consistently show high densities of amyloid plaques. However, the patients with amyloid plaques burden do not necessarily display NFT changes. Also, the distribution of amyloid depositions revealed to be constant at late stages but much more variable at early stages of the disease.

In contrast, NFTs showed a typical pattern of distribution with only a slight inter-individual variation. This well-defined patterns allowed the identification of six stages: Stage I and II are characterised by the evident accumulation of NFTs in the transentorhinal region and by its slight presence in the hippocampus (CA1). The progression to the Stage III and IV is defined by the transentorhinal and entorhinal areas visibly affected and the hippocampus being mild to moderated involved. Finally, at Stage V and VI, the cortex is drastically affected by the pathology, along with all the regions mentioned before (Figure 4). Nowadays, tau pathology is still staged, according to Braak (H. Braak & Braak, 1991).

These significant findings were a milestone in the history of AD as they allowed to establish that the progression of tau pathology is strongly correlated with the severity of the disease than the accumulation of amyloid plaques. However, these studies were limited to a post mortem evaluation.

The development of new imaging techniques over the last decade allowed to defined the relationship between to spatial pattern of A β and cognition *in vivo* (Klunk et al., 2004; Mintun et al., 2006). However, the relationship between tau pathology and other markers of AD patients was only established in 2016 (Brier et al., 2016). This study found a positive correlation between tau accumulation in the temporal lobe, a region involved in memory processing, and higher levels of tau in CSF measurements. Furthermore, in these cases, patients revealed deficits in the performance of memory and attention tests. Thus, the

combination of tau PET imaging with the existent approaches demonstrated the link between tau pathology and cognitive dysfunction corroborating the existing hypothesis (Brier et al., 2016) (Brier et al., 2016).

In the last years, tau became an attractive target for therapies not only because of the link with cognitive decline but also because of the consecutive fails of a long list of A β -targeting treatments in clinical trials. Several therapies anti-tau that emerged were inhibitors of kinases and tau aggregation stabilisers of microtubules and immunotherapeutic drugs (Congdon & Sigurdsson, 2018). However, the majority of these approaches have been discontinued because of toxicity, lack of efficacy and adverse effects (Barten et al., 2012; Bollag et al., 1995; Gauthier et al., 2016). Currently, the most promising therapeutically approaches in clinical trials are immunotherapies which have shown promising results in terms of safety and immune response in AD patients (Novak et al., 2017).

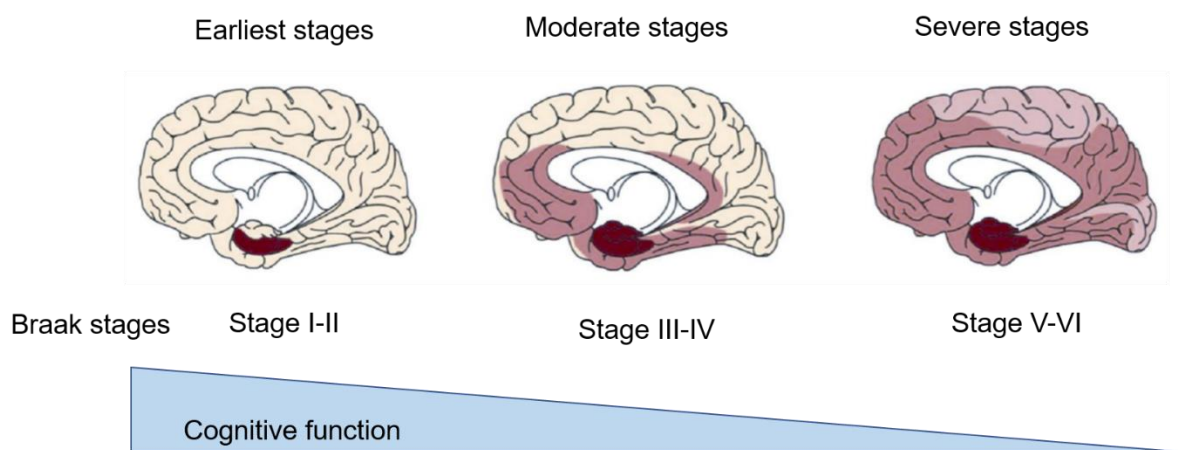


Figure 4. Stages in the development of AD-associated tau pathology. The progression of tau pathology described by Braak stages. NFTs Stages I and II (dark red); NFTs Stages III and IV (medium red); and NFT/NT Stages V and VI (light red) (Adapted from Braak, 2011).

1.7. Relation between A β and tau

In AD, the two main histopathological hallmarks are the appearance of NFTs and amyloid plaques. However, whether they are independent or linked is still unknown.

Several studies have shown the interaction between the two proteins at different levels.

The amyloid hypothesis suggests that A β deposits trigger tau pathology, which propagates progressively through the brain and consequently leads to neurodegeneration and dementia (Hardy & Selkoe, 2002). The fact that the accumulation of A β does not seem to be sufficient

to cause cognitive decline in LOAD supports this idea. The post-mortem, CSF and amyloid PET imaging studies have shown not only that A β plaques do not correlate with cognitive decline but also that they could be found in older people with normal cognitive function (Katzman et al., 1988; Mormino & Papp, 2018). Moreover, A β plaques seem to facilitate the conversion of wild type tau into pathological forms of tau which could be the starting point to the propagation of pathological form of tau in the brain (T. Li et al., 2016). Another hypothesis that has been proposed is that the amyloid plaques might act synergistically to enhance tau pathology. Several *in vitro* and *in vivo* studies have been conducted to provide evidence to corroborate this hypothesis. For instance, the use of double transgenic models, displaying both amyloid and tau pathology showed that the presence of cortical amyloid depositions accelerates tau propagation and toxicity, including neurodegeneration. Indeed, the double transgenic mice exhibit a much more aggressive phenotype than the parental tau transgenic mice at the same age (Pooler et al., 2013; Spiess-Jones & Hyman, 2014). Also, the injection of synthetic A β fibrils into the brain of a young transgenic tau model increases tau accumulation (Götz et al., 2001). Furthermore, the combination of A β and tau results in a synergistic impairment of mitochondrial function and energy homeostasis in AD mouse models (Rhein et al., 2009).

Taken together, these results, along with the longitudinal studies using Amyloid and tau PET imaging, showed that the presence or accumulation of A β is a predictor of more severe tau-associated cognitive impairment (Aschenbrenner et al., 2018; Hanseeuw et al., 2019). These findings support the idea that the tau pathology primarily drives cognitive decline and neurodegeneration.

1.8. Metabolism impairment in AD

Epidemiological studies have pointed to a relationship between type 2 diabetes and an increase in the risk of developing AD. Data from the patient registry of Mayo Clinic shows that 80% of AD patients display an impairment in glucose tolerance or have diabetes (Janson et al., 2004). Moreover, type 2 diabetes induces a cognitive decline and that these patients are 1.5 to 2 fold more likely to have dementia (Biessels et al., 2014).

The supporters of the metabolism hypothesis consider the metabolic alterations as a potential cause of AD occurring before the appearance of A β plaques and NFT.

The improvement of brain imaging techniques contributed most to the development of the study of metabolism impairment in AD patients. These techniques can also be used in presymptomatic stages, providing insights into the AD pathogenesis.

Currently, PET and functional MRI (fMRI) are used to investigate metabolism alterations in the brain. As glucose is the primary source of energy in the brain, PET imaging of fluorodeoxyglucose (FDG), a glucose analog, is a reliable indicator of brain metabolism. fMRI, on the other hand, is a non-invasive imaging technique that assesses the neuronal activity through the measurement of changes in blood oxygen levels. Through the combination of FDG-PET and fMRI imaging techniques, it has been possible to identify alterations in glucose metabolism in the brain of AD patients. For instance, a reduction in hippocampal volume alongside with hypometabolism during normal ageing has been shown to predict cognitive decline before the appearance of clinical symptoms (Mosconi et al., 2008). Additionally, patients with AD display increased lactate levels in CSF, resulting from metabolic dysfunction (Liguori et al., 2016).

As a conclusion, a consensus around the impairment of the metabolism as an early event in AD and an important part of the pathogenesis cascade has emerged. Metabolism imaging can be used as a tool for diagnosis, and metabolism is a potential target for new treatments. However, the evidence are not sufficient to determine whether metabolism impairment is a cause or a consequence of AD pathogenesis.

1.9. Epilepsy in AD

Epileptic seizures are common in AD patients and can be detected at early-onset and more advanced forms of AD. Nonetheless, the incidence of seizures is also higher in other tauopathies such as FTD, CBD, and progressive PSP than in the healthy population reviewed in (reviewed in (Sánchez et al., 2018)).

The seizures were first studied in patients with advanced AD. However, seizures and aberrant network activity can occur in the early stages of AD and contribute to cognitive decline (Palop & Mucke, 2010; Vossel et al., 2017).

The specific cause of the generation of epileptic seizures in AD patients has not been entirely determined. Some studies suggest that seizures can anticipate AD or begin simultaneously with the onset of cognitive decline (Cretin et al., 2012; Vossel et al., 2017). This latter might reflect the potential key role of A β to give rise to epileptiform neuronal activity (Villemagne et al., 2013). Epileptiform activity is defined as spikes and sharp waves on EEG lasting 20 to 200ms, that perturb background activity. This detected activity is known as interictal epileptiform discharges. On the other hand, when these seizures are silent, the used term is subclinical epileptiform activity (Vossel et al., 2017).

In AD patients, 90% of epileptiform discharges occur during sleep. There is no clinical difference between patients with AD and epileptiform activity and those who do not display such activity at the time of monitoring. However, over time, patients with epileptiform activity presented a faster decline in cognitive tests and executive function.

This evidence suggests that this abnormal network activity might accelerate cognitive decline directly or by associations with silent seizure. A follow-up study revealed that the risk for epilepsy patients to develop dementia is not significant unless the seizures start within ten years of AD diagnosis (Breteler et al., 1995). Besides, a hallmark of AD, A β plaques are also correlated with age. The brain analysis of samples from older patients with temporal lobe epilepsy (TLE) and controls found a positive correlation between the incidence of A β plaques and age.

Additionally, a post-mortem study which assessed the long-term pathological effects of chronic epilepsy found an increased tau NFTs formation at mid-Braak stages in patients aged 40-65years (Thom et al., 2011). Furthermore, the examination of excised temporal lobe samples from refractory temporal lobe epilepsy patients revealed hyperphosphorylated tau in 94% of the samples. In these patients, tau pathology was associated with a decline in verbal learning and recall over one year after lobe resection (X. Y. Tai et al., 2016).

AD and epilepsy show similarities not only in pathological features but also in the functional neuronal network. Results from functional MRI of patients with either temporal lobe epilepsy or AD suggest shared regions of network dysfunction (Luo et al., 2011).

Despite the presence of seizures in AD, similar to the ones seen in epilepsy, the decision to treat AD patients with antiepileptic drugs (AED) can be difficult. The exact mechanism by which most of the AED act is unknown and might differ on the dose. Currently, there are no guidelines available to treat silent forms of aberrant activity in patients with AD. Hence, and with the lack of randomised clinical trials for AD-associated seizures, the therapeutic approaches using AED must be based on evidence provided from studies in older adults with or without dementia (Vossel et al., 2017). Among other AED, Levetiracetam has been used in AD patients with epilepsy. The results of an observational study showed that 72% of the patients were seizure-free for at least one year after the treatment one year or longer (Belcastro et al., 2007).

Moreover, a different study used Levetiracetam to treat patients with AD and epilepsy showed that the patients treated with this drug revealed an improvement in the performance of cognitive tests along with 71% effect on seizure reduction (Cumbo & Ligori, 2010). This drug also showed promising results in FAD mouse models where the seizures and epileptiform can occur before the formation of A β plaques. The treatment revealed to ameliorate A β -induced abnormal network activity, synaptic dysfunction, and reverse the deficits in learning in memory (Sanchez et al., 2012). This drug is in Phase 3 clinical trials to evaluate the ability to improve cognitive function and slow the progression of the disease in MCI and AD (Privitera, 2001). Patients with AD and seizures can benefit from treatments with antiepileptic drugs. Nevertheless, more clinical studies are required to evaluate the efficiency of AED to treat abnormal network activity in AD.

Animal models of Alzheimer's disease are widely used to understand the relation between network dysfunction and AD pathogenesis. The transgenic mouse model of familial AD typically overexpresses APP, PS1 or both proteins. Most seizures in these mice are silent except in the double transgenic APP/PS1, which usually present frequent motor seizures. (Minkeviciene et al., 2009; Palop & Mucke, 2010). The seizures or epileptiform activity typically appear before the formation of amyloid plaque (Sanchez et al., 2012).

The evidence provided by these animal models points to different triggers for the mechanisms by which seizures occur in AD models and epilepsy models. The hypothesis that oligomers species of A β could cause the seizures found in AD is supported by the high incidence of seizures in patients with FAD (Shea et al., 2016). However, besides the extensive studies, it is not entirely clear the contributions of A β , APP and other APP metabolites to the network hyperexcitability (Heather A. Born et al., 2014; Minkeviciene et al., 2009; Vogt et al., 2011).

Concerning the involvement of tau in the generation of seizures, it seems that tau facilitates epileptogenesis in AD. In both, AD and epilepsy models, the amount of wild-type tau correlates with neuronal and network excitability in a dose-dependent manner (DeVos et al., 2017; Holth et al., 2013; L. M. Ittner et al., 2010a; Kaufman et al., 2016a).

Moreover, transgenic mice overexpressing human wild-type tau or mutated tau, which mutation is a risk modifier for AD and other tauopathies, have epileptiform activity and higher seizure susceptibility than wild type littermates (Maeda et al., 2016). However, the mechanisms underlying tau-dependent network hyperexcitability is still poorly investigated. Lastly, the neuronal activity has been associated with A β secretion and the enhancement of tau propagation and tau pathology. Thus, persistent epileptic activity in AD could lead to an increase of abnormal aggregation and spreading of these two proteins (Wu et al., 2016; Yamamoto et al., 2015).

In summary, several pieces of evidence both from patients and animal models highlight the role of tau in epileptogenesis in AD. However, the mechanisms underlie the link between tau pathology and aberrant network activity remain largely unknown.

2. Tauopathy in AD

2.1. Background

Tau was first identified as an essential protein for the assembly and stabilisation of microtubules (Weingarten et al., 1975), but later gained notoriety when it was found to be the main component of NFTS in AD (Wischik et al., 1988; M. Goedert et al., 1988). Following studies found that tau aggregates are not exclusive to AD but a common pathological feature of a heterogeneous group of neurodegenerative disorders. Thus, the term tauopathy is currently used to identify a set of progressive neurodegenerative diseases which are characterised by the abnormal accumulation of phosphorylated tau (p-tau) protein in the brain. Studies of familial forms of tauopathies, such as FTDP-17, and consequent identification of associated mutations have been crucial to providing evidence that helps to understand the pathophysiological function of tau, as well as the effect of tau aggregation in neurodegeneration in both inherit and sporadic tauopathy context. Given that tauopathy is the hallmark of AD which better correlates with the cognitive deficit, studying the mechanisms through which tau becomes phosphorylated and aggregated as well as those which determine the progression of tauopathy is key to design better therapies for AD.

2.2. The tau protein

In the brain, tau is mainly expressed in neurons and commonly found in the axonal compartment (Binder et al., 1985) but can also be present in neuronal somatodendritic compartments (Tashiro et al., 1997), astrocytes (Papasozomenos & Binder, 1987) and oligodendrocytes at low levels (LoPresti et al., 1995).

Human tau is encoded by the MAPT gene located on the chromosome 17q21, which contains 16 exons (M Goedert, Spillantini, Potier, et al., 1989). In the adult human brain are produced six tau isoforms (352–441 amino acid residues) by alternative splicing of exons 2 (E2), 3 (E3) and 10 (E10). Tau isoforms can be categorised according to the N-terminal inserts, which are encoded by E2 and E3: tau isoform with 0,1 and 2 N-terminal inserts (0N, 1N, 2N respectively). However, the alternative splicing of E10 generates tau isoforms with 3 or 4 carboxy-terminal repeated domains (3R or 4R, respectively) (Lee et al., 1988) (Figure 5). Each isoform shows a different affinity for microtubules depending on the number of microtubule repeated domains—the presence of these isoforms and their level of expression increase during brain development. For instance, the isoform 3R is the only one expressed in the fetal brain. In the adult human brain, the ratio between 3R and 4R is 1:1, but it could be changed by the alternative splicing of E10. MAPT gene mutations that affect the E10 splicing, and consequently alter the ratio between 3R and 4R, have been associated with different tauopathies. However, in AD patients, no tau mutations have been found to date (Yipeng Wang & Mandelkow, 2016).

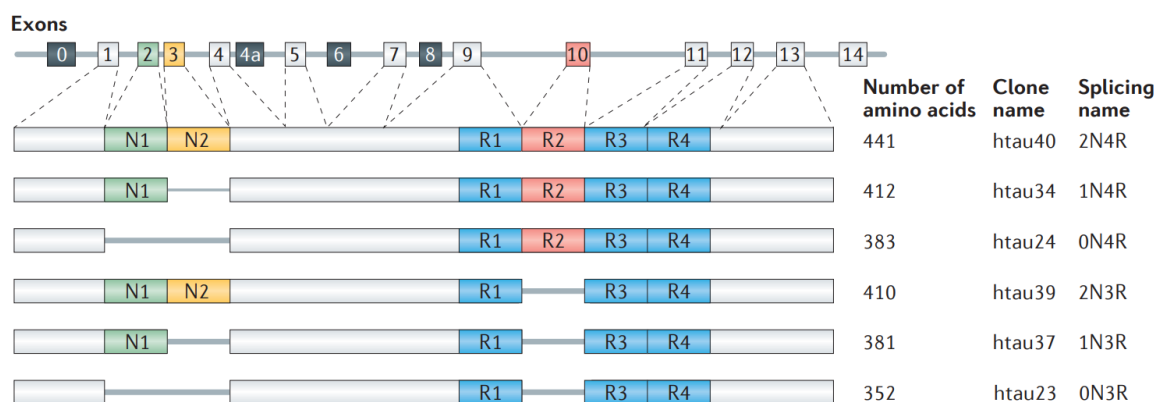


Figure 5. The human MAPT gene and the splice isoforms of tau in the human brain.

MAPT, the gene encoding human tau, contains 16 exons 224. Exon 1 (E1), E4, E5, E7, E9, E11, E12 and E13 are constitutive, whereas the others are subject to alternative splicing. Alternative splicing of E2, E3 and E10 leads to six human brain tau isoforms. These tau isoforms differ according to the presence of 0, 1 or 2 near-amino-terminal inserts (0N, 1N or

2N, respectively) and the presence of repeat R2, yielding 3 or 4 carboxy-terminal repeat domain (3R or 4R, respectively) tau species (Adapted from (Yipeng Wang & Mandelkow, 2016).

Tau is a hydrophilic protein, and it is natively unfolded, showing a little propensity for aggregation (Jeganathan et al., 2008). The structure of tau holds four domains which differ in their amino acid character: the acidic N-terminal projection domain and the neutral C-terminal assembly domain (Steiner et al., 1990; Hirokawa et al., 1988). This asymmetry of charges is essential for the interactions with the microtubules and other partners as well as for the internal folding and aggregation (Mandelkow & Mandelkow, 2012). The N-terminal projection domain projects away from the surface of the microtubule. This domain has been related with roles in the regulation of microtubule spacing (Chen et al., 1992), interaction with the plasma membrane (Brandt et al., 1995) and other elements in the cytoskeleton. In the middle region of tau there is the proline-rich domain (150-240 residues) which links the two main domains. This sequence can bind to signalling proteins with the SRC homology 3 (SH3) domains such as the tyrosine kinase FYN (Lee et al., 1998) and also proline-directed kinases including glycogen synthase kinase 3 (GSK-3), cyclin-dependent kinase 5 (CDK5) and mitogen-activated protein kinase (MAPK) (Yipeng Wang & Mandelkow, 2016). Finally, the C-terminal segment contains the repeated domains R1-R4 referred as the microtubule-associated domain (MTBD) and the flanking regions which are responsible for binding the microtubules (Goedert, Spillantini, Potier, et al., 1989; Goedert, Spillantini, Jakes, et al., 1989; Himmler et al., 1989) and for tau aggregation (Hanger & Wray, 2010). The number of repeated domains are strongly related to the binding affinity of tau for microtubules. It is thought that the positive charge of the MTD interacts with the negative charge of the tubulin residues promoting the direct binding (Jho et al., 2010; Kar et al., 2003; Lee et al., 1988). The affinity to bind the microtubules is higher for the 4R tau isoform than 3R tau isoform (Panda et al., 2003). This can perhaps be explained by the existence of specific sequences in MTBD that have strong interactions that are coded by exon 10

2.2.1. Tau under physiological conditions

2.2.1.1. Functions of tau

The primary function of tau protein is the assembly and stabilisation of microtubules (Weingarten et al., 1975). Microtubules are protein polymers of the cytoskeleton which play an essential role in many cellular processes counting, maintaining cell shape, intracellular trafficking, organelle positioning and organisation and cell division (de Forges et al., 2012;

Kapitein & Hoogenraad, 2015). They are composed of two subunits α -tubulin and β -tubulin heterodimers. Tau binds at the interface between α - β tubulin through the MTBD, promoting the microtubule assembly (Mandelkow & Mandelkow, 2012).

Microtubules are highly dynamic structures alternating between phases of growth and shrinkage in a process known as dynamic instability (Howard & Hyman, 2009). This dynamic is regulated by tau allowing the reorganisation of the cytoskeleton (Mandelkow & Mandelkow, 2012).

Additionally, in the axons, tau can regulate the axonal transport by an active role in the function of the motor proteins dynein and kinesin which transport cargos towards the cell body (retrograde transport) and the axonal terminus (anterograde transport) (Stamer et al., 2002).

Tau act as a competitor of dynein and kinesin for binding microtubules slowing the axonal transport of these motor proteins which can result in the accumulation of cargos like mitochondria in the cells' soma (Dixit et al., 2008). Also, tau interferes with axonal transport of cargos, reducing the number of motors that are engaged with cargos (Vershinin et al., 2007).

Although tau is mostly found in axons, a small amount of tau is present in dendrites and dendritic spines under physiological conditions (L. M. Ittner et al., 2010a; Mondragón-Rodríguez et al., 2012). The dendritic localisation of tau has been extensively studied in disease context as AD and FTD: hyperphosphorylated tau accumulates in the dendrites and leads to the formation of NFTs (Baner et al., 1987). However, to date, its physiological function has not been well characterised. Several studies suggest that dendritic tau might be involved in the regulation of synaptic function. In cortical neurons was demonstrated that endogenous tau is translocated from the dendritic to the postsynaptic compartment after pharmacological synaptic activation. The same results were observed when acute hippocampal slices were exposed to long-term potentiation (Frändemiché et al., 2014).

Additionally, the involvement of tau in neurotransmission through the FYN pathway has been reported. Tau seems to be essential in targeting the tyrosine kinase FYN to the dendrite and associates with the postsynaptic density complex. In this complex, the interaction of NMDA receptors with the scaffolding protein PSD-95 is regulated by the phosphorylation activity of FYN (L. M. Ittner et al., 2010a; Trepanier et al., 2012). This interaction between FYN and tau is highly dependent on the phosphorylation state of tau (Bhaskar et al., 2005; Usardi et al., 2011; Lee et al., 1998). Moreover, the reduction of tau expression results in a synaptic loss (Q et al., 2011) this seems to indicate that tau may play a critical role in maintaining synaptic integrity.

In neurons, tau has also been detected in the nucleus (Loomis et al., 1990; Y. Wang et al., 1993). Both *in vitro* (Sultan et al., 2011) and *in vivo* (Violet et al., 2014) studies have demonstrated the essential function of tau protecting neuronal DNA integrity in physiological

conditions and under ROS-producing stress. The absence of tau makes the neuronal cells more susceptible to heat stressed-induced DNA damage.

2.2.1.2. Post-translational modifications of tau protein

Tau can undergo various types of post-translational modifications, including ubiquitination truncation, glycation, N-glycosylation, nitration, O-GlcNAcylation, acetylation and phosphorylation. This suggests that tau is regulated by several mechanisms (M. Morris et al., 2015).

Tau phosphorylation has been extensively studied due to its involvement in the regulation of tau binding to microtubules, but mainly due to its implications in AD and other tauopathies. In these diseases, the phosphorylation state can induce the ability to template normal tau in a prion-like manner, to disrupt microtubule assembly and self-aggregate into tangles (Alonso et al., 1994; Wang et al., 1996; Wang et al., 2007). Phosphorylation is the process of adding a phosphate group by esterification at three types of aminoacids: serine, threonine and tyrosine. The longest tau isoform contains 45 serine (Ser), 35 threonines (Thr) and 5 tyrosine (Tyr) residues which can be phosphorylated (Yipeng Wang & Mandelkow, 2016).

In physiological conditions, a molecule of tau contains approximately two phosphates while in pathological conditions, tau can contain as many as seven to eight phosphates (Köpke et al., 1993a). The different states of tau phosphorylation are due to the balance of the activity of specific protein kinases and phosphatases, which promotes and decreases tau phosphorylation, respectively.

Kinases involved in tau phosphorylation are part of three main groups: proline-directed protein kinases (PDPK), non-proline-directed protein kinases and tyrosine protein kinases (TPK).

The PDPK include the proline-directed Ser/Thr kinase, GSK-3 and CDK5. Their activity is regulated by phosphorylation, and it seems to be related to AD and tauopathies (Bhat et al., 2000; Giese, 2009). Protein Phosphatases (PPs) are responsible for the dephosphorylation of tau. PP2A is accountable for the majority of phosphatase activity in the brain, and its inhibition increases tau phosphorylation (Alonso et al., 2004).

The phosphorylation states are not constant, and they change during development. In fetal stages, the degree of phosphorylation is high and decreases with age due to the activation of the phosphatases (Mawal-Dewan et al., 1994). Moreover, the degree of phosphorylation also changes according to the cell compartment where tau is found (Riederer et al., 2009). It is known that tau binds microtubules (MT) through MTBD and the microtubule assembly is regulated by the degree of phosphorylation of tau. Therefore, the phosphorylation of MTBD

shows a decrease of tau affinity to bind MT, which can lead to the accumulation of free tau in the cytosol and increase tau propensity to aggregate (E. M. Mandelkowitz et al., 1995).

Truncation may also contribute to the formation of NFTs, given that it seems to promote tau aggregation through oligomerisation of the microtubule-binding repeats (M. Pérez et al., 1996; Zilka et al., 2006). However, the precise role of these post-translational modifications in neurofibrillary degeneration is still unknown. Moreover, O-GlcNAcylation has also received attention in AD research. This post-translational modification of tau occurs on serine/threonine residues by β -linked N-acetylglucosamine and the O-GlcNAcylation sites identified on tau are also phosphorylated in AD (Arnold et al., 1996; Yuzwa et al., 2011). Reciprocal regulation between tau O-GlcNAcylation and phosphorylation has been observed both *in vitro*, in cell cultures, and *in vivo* (F. Liu et al., 2004, 2009). Additionally, O-GlcNAcylation levels have been found decreased in the brains of AD patients and also an inverse correlation between O-GlcNAcylation and phosphorylation at multiple hyperphosphorylation sites (F. Liu et al., 2009). Altogether, this suggests that a reduction of O-GlcNAcylation might contribute to the hyperphosphorylation of tau.

2.2.2. Tau in pathological conditions

Tau is centrally involved in the etiopathogenesis of AD and other tauopathies. Despite clinical and histopathological differences between these diseases, tau hyperphosphorylation and aggregation observed in all of them. Hyperphosphorylated tau detaches from the microtubules, accumulates and becomes toxic which results in a destabilisation of the microtubule network, synaptic dysfunction and mislocalisation of tau in the somatodendritic compartment.

Tau pathology has been an object of intense investigation. However, the mechanisms by which tau mediates neurodegeneration remain unclear. In familial tauopathies, different mutations in the MAPT gene seem to underline tau aggregation. On the other hand, in sporadic tauopathies, such as AD, the mechanisms behind the pathogenesis are poorly understood. Nevertheless, there is considerable evidence that abnormal and hyperphosphorylated tau precedes tau aggregation.

2.2.2.1. Tau mutations

In 1998 autosomal dominant mutations in the MAPT gene were discovered. These mutations were found to cause some forms of FTDP-17 demonstrating that the dysfunction of tau protein can have a significant impact in the CNS and is sufficient to cause neurodegeneration and dementia (Hutton et al., 1998a; Poorkaj et al., 1998b; Spillantini et al., 1998). Usually, these mutations are associated with FTDP-17, but they have been observed in different tauopathies such as Pick's disease, PSP, CBD and Globular glial tauopathy (Murrell et al., 1999; M et al., 2003; Bronner et al., 2005; Tacik et al., 2015).

Over 50 different pathogenic MAPT missense, silent and intronic mutations have been identified so far. (Ghetti et al., 2015) (Figure 6). While these mutations can give rise to different phenotypes and neuropathological features, the similarities found among the various tauopathies aids to comprehend how tau pathology may develop. Thus, some mechanisms by which these mutations might cause the disease to have been pointed out. Such as loss of function, including MT binding and assembly; changes in alternative splicing; shifts in protein-aggregation kinetics and more recently prion-like "seeding".

The majority of mutations reside at exons 9-13, which encodes for the repeat regions, and adjacent introns (Figure 6). They affect the protein level or the alternative splicing of pre-mRNA (Spillantini & Goedert, 2013). Intronic mutations mainly reside in intron 10 and disrupt mRNA. The most common intronic mutation is IVS10+16 and alters the 3R:4R ratio, increasing the 4R tau expression by disrupting the mRNA (B Ghetti et al., 2015). The silent pathogenic mutations found in tau are located in Exon 10 (L284L, N296N or S305S) or exon 11 (L315L). These mutations could also affect splicing through the disruption of mRNA, which alters the normal 1:1 3R:4R ratio (D'Souza & Schellenberg, 2005). The majority of missense mutations are located in or near the microtubule-binding domain and alter the sequence of tau, making it more prone to have a reduced affinity for microtubules and an increased tendency to aggregation (Figure 7). For instance, it has been demonstrating that some mutations (G272V, P301L, P301S, V337M, G389R, R406W) cause a decrease the affinity to microtubules and diminish the capability to promote microtubule assembly *in vitro* (D'Souza et al., 1999; Hasegawa et al., 1998; Hong et al., 1998; Kouri et al., 2014; Murrell et al., 1999; Pickering-Brown et al., 2000). Some mutations (V337M, G272V, P301L) convert tau in a favourable substrate for tau protein kinases which leads to abnormal tau hyperphosphorylation (A. D. Alonso et al., 2010; Alonso et al., 2004). Additionally, other mutations (N279K, K280, N296H,

P301L, P301S, G303V) cause the impairment of splicing of exon 10 resulting in a shift of the physiological 3R:4R ratio

Finally, some missense mutations located in exon 10 (N296H, N296, K280) affect the expression level of tau mRNA and induce tau accumulation (D'Souza et al., 1999; Rizzu et al., 1999; Yoshida et al., 2002).

Overall, the missense type of mutations alters the sequence of tau and promote the assembly of tau filaments (Barghorn et al., 2000; M. Goedert et al., 1999; von Bergen et al., 2001). This mutated form of tau turns to hyperphosphorylated faster, and self-aggregate promptly compare to the wild-type tau (A. del C. Alonso et al., 2004; Bhaskar et al., 2005). This faster kinetics of the hyperphosphorylation might explain a relatively early onset, severity and autosomal dominance of disease in the inherit FTDP-17 (Iqbal et al., 2010). On the other hand, the splicing mutations which affect the 3R:4R ratio results in increased levels of unbound, free-floating tau. Nevertheless, how this imbalance triggers tau aggregation remains unknown, but it is probably related to the different microtubule-stabilizing capacities of the different isoforms.

Interestingly, there are also mutations outside the MT region binding which might interfere with general axonal transport (R5H or R5L) (Magnani et al., 2007), or impairs the binding of tau to MT and makes the MT assembly less efficient (A152T). Also, A152T enhances the formation of oligomers but not fibers, and it is a risk modifier in AD (Coppola et al., 2012).

MAPT Mutations Associated with FTDP-17

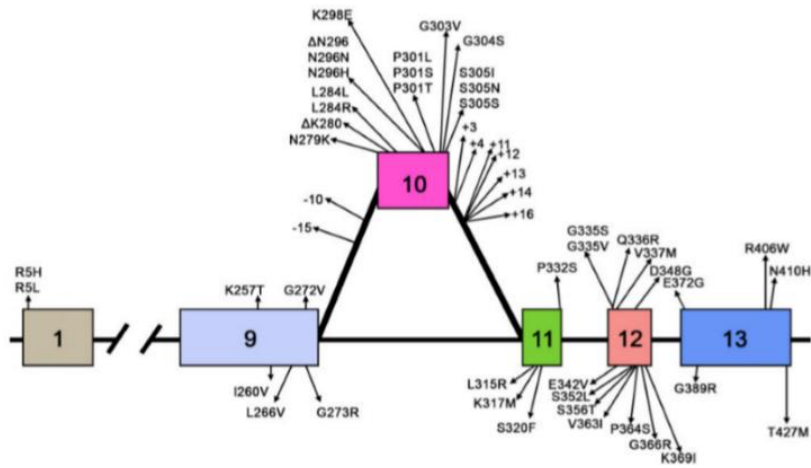


Figure 6. Schematic representation of the exons and introns of the MAPT gene. 53 mutations were identified as causing FTDP-17 (from (Bernardino Ghetti et al., 2015)).

Table 1.

Summary of the reported effects of mutations within the *MAPT* coding regions on *in vitro* tau amyloid aggregation, tau's ability to promote MT assembly, tau MT binding, altered exon 10 splicing and the major tau isoforms present as aggregates in human brains.

Mutation	Genomic region	Tau aggregation	MT assembly	MT binding	Exon 10 inclusion	Major aggregated tau isoforms	References
R5H	Exon 1	↑	↓	ND	↔	1N/3R, 0N/4R, 1N/4R	(126)
R5L	Exon 1	↑	↓*	ND	ND	1N/3R, 0N/4R, 1N/4R (cortical region); 0N/4R, 1N/4R (subcortical region)	(13,112,127)
G55R	Exon 2	ND	↑ (4R only)	ND	ND	ND	(128)
A152T	Exon 7	↔	↓	↓	↔	Variable	(17,129,130)
K257T	Exon 9	↑ (3R)*	↓	ND	ND	All six isoforms or increased 3R	(15,131,132)
I260V	Exon 9	↑ (4R)	↓(4R)	ND	↔	0N/4R, 1N/4R, 2N/4R	(131)
L266V	Exon 9	↑ (3R)	↓	ND	↔	0N/3R, 0N/4R, 1N/4R, 2N/4R	(133,134)
G272V	Exon 9	↑	↓*	↔	ND	0N/3R, 1N/3R, 2N/3R	(112,135,136)
N279K	Exon 10	↑	↔	↔*	↑	0N/4R, 1N/4R	(135,137–140)
Δ280K	Exon 10	↑	↓	↓	↓	0N/3R, 1N/3R	(79,135,138,141–143)
S285R	Exon 10	ND	ND	ND	↑	ND	(80)
Δ296N	Exon 10	↔	↓*	ND	↑*	ND	(112,144,145)
N296H	Exon 10	↔	↓	ND	↑	4R isoforms	(144,145)
K298E	Exon 10	ND	↓	ND	↑	ND	(146)
P301L	Exon 10	↑	↓	↓	↔	4R isoforms	(8,112,135,138,140,147–149)
P301S	Exon 10	↑	↓	↔	ND	4R isoforms	(141,146,150–152)
P301T	Exon 10	ND	ND	ND	ND	ND	(153)
G303V	Exon 10	↑	↓	ND	↑	4R isoforms	(112,154)
S305I	Exon 10	ND	ND	ND	↑	0N/4R, 1N/4R	(155)
S305N	Exon 10	ND	↔	↔	↑	ND	(137,156)
L315R	Exon 11	↔*	↓*	ND	ND	1N/3R, 2N/3R, 0N/4R, 1N/4R, 2R/4R	(112,157)
K317M	Exon 11	ND	ND	ND	ND	ND	(158)
K317N	Exon 11	↓(3R) ↑(4R)	↓	ND	ND	4R tau isoforms	(159)
S320F	Exon 11	↑	↓	ND	ND	1N/3R, 2N/3R, 0N/4R, 1N/4R	(112,116)
P332S	Exon 11	ND	ND	↔	ND	All six isoforms	(150,160)
G335S	Exon 11	↔	↓	ND	ND	ND	(161)
G335V	Exon 11	↑	↓	ND	ND	ND	(161,162)
Q336H	Exon 11	↑(3R greater)	↑	ND	ND	1N/3R, 2N/3R	(83)

Mutation	Genomic region	Tau aggregation	MT assembly	MT binding	Exon 10 inclusion	Major aggregated tau isoforms	References
Q336R	Exon 11	↑(3R greater)	↑	ND	ND	ND	(82,83)
V337M	Exon 12	↑*	↓*	↓	ND	All six isoforms	(112,147,149,163,164)
E342V	Exon 12	↔	↑	ND	↑	0N/3R, 0N/4R, 1N/4R, 2N/4R	(112,151,165)
S352L	Exon 12	↑	↓	↔	ND	ND	(112,166)
S356T	Exon 12	ND	ND	ND	ND	ND	(167)
P364S	Exon 12	↑	↓	ND	ND	ND	(168,169)
G366R	Exon 12	↔	↓	ND	ND	ND	(168)
K369I	Exon 12	↓	↓*	ND	ND	All six isoforms	(112,170)
E372G	Exon 13	↑	↓	ND	ND	ND	(171)
G389R (G→A)	Exon 13	↑	↓*	ND	↔	0N/3R, 0N/4R, 1N/3R, 1N/4R	(112,132,172)
G389R (G→C)	Exon 13	↑	↓*	ND	ND	0N/3R, 0N/4R, 1N/3R, 1N/4R	(112,172,173)
R406W	Exon 13	↔*	↓	↓	ND	All six isoforms	(127,140,148,149)
N410H	Exon 13	↑	↓	ND	↑	ND	(14)

↑ and ↓ arrows indicate an increase or decrease compared to WT tau, while ↔ means no difference. ND indicates no data. An * indicates that studies have shown differing results; thus, a "↑*" indicates an overall trend towards an increase compared to WT based on available data.

Figure 7. Effects of reported mutations within the MAPT gene on *in vitro* tau aggregation, tau's ability to promote MT assembly, tau MT binding, altered exon 10 splicing and the major tau isoforms present as aggregates in human brain (from (Strang et al., 2019)).

2.2.2.2. Hyperphosphorylation of tau

Approximately ten phosphorylation sites were found on soluble tau in brain extracts from healthy patients (Hanger et al., 2007). On the other hand in PSP in brain extracts, at least 16 phosphorylated sites (T. Arai et al., 2004; Wray et al., 2008) and approximately 45 phosphorylated sites (serine, threonine and tyrosine) in AD can be identified (Grundke-Iqbal et al., 1986b; Hasegawa et al., 1992). Hyperphosphorylation of tau is believed to be involved in the pathogenesis of tauopathies and has been pointed as a critical molecular feature of neurofibrillary degeneration in AD. Many of the forms of phosphorylated tau identified in PHF tau were also found in the normal brain. However, at the early stage of AD, tau is hyperphosphorylated before its aggregation (Buée et al., 2000; Hasegawa et al., 1996; Köpke et al., 1993a; Noble et al., 2013) and at late stages, the pathological tau aggregates are three times more phosphorylated than the physiological tau (Köpke et al., 1993b; Ksiezak-Reding et al., 1992).

Phosphorylation of tau protein is regulated by the kinase and phosphatase activity. The imbalance of kinase and phosphatase activity may result in hyperphosphorylation of tau, which seems to be a key factor for the formation of tau aggregates. This might happen due to the increase of kinase protein activity such as Src family kinase, Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), CDK-5, GSK-3 and tyrosin Fyn (Nygaard, 2018). Recently, a study using post-mortem tissue from AD and non-demented patients reported that the hippocampus and temporal cortex regions have high levels of casein kinase 2 (CKII) in AD compared to controls (Rosenberger et al., 2016). Fyn has also been associated with neurodegeneration. Fyn phosphorylates tau and plays an essential role in amyloid signalling. Also, a Fyn inhibitor reduces memory deficits in the triple transgenic mice (with the APP/PS/TauP301L) (Kaufman et al., 2016a).

Moreover, GSK-3 seems to be also associated with the progress of neurodegeneration. The analysis of brains from AD patients indicates that GSK-3 levels are increased, and its activity correlates with the appearance of NFTs (Leroy et al., 2002; Pei et al., 1997). *In vitro* studies demonstrate that after phosphorylation by GSK-3 the specific site epitope AT100 becomes phosphorylated (Zheng-Fischhöfer et al., 1998).

Conversely, hyperphosphorylation of tau may be due to the decrease of phosphatase activity. As seen before, among the PP, PP2A is the main phosphatase and accounts for 70% of tau phosphatase activity in the human brain. In AD brains PP2A activity is reduced by approximately 20% and 40% in the grey and white matters respectively (Gong et al., 1993).

This reduction can be due to decreased of PP2A expression or the increase of the endogenous PP2A inhibitors (Chen et al., 2008; Sontag et al., 2004).

Several pieces of evidence show that phosphorylation of particular residues on tau can affect tau function considerably. Although, whether hyperphosphorylation contributes to the pathogenesis of the disease, it is not fully understood.

2.2.2.3. Tau aggregation

Under physiological conditions, tau is an unfolded and soluble protein in the brain. However, in AD and other tauopathies, tau becomes insoluble and aggregates. In the aggregation process, the misfolded protein clumps together and form well-structured fibrils which in turn form filaments. These filaments are rich in β -sheet structures, and each filament is organized perpendicular to the long axis of the fibrils (Makin & Serpell, 2002; Nelson et al., 2005).

Several factors affect the ability of tau to aggregate, starting with its conformation. Normal tau has a paperclip-like fold, which makes the N- and C-terminal ends bend around the MT, like this preventing the self-aggregation of tau (Mandelkow et al., 2007). In tau sequence, there are two hexapeptides motifs (VQIVYK and VQIINK) that are essential for aggregation and formation of tau filaments (Pérez et al., 1996; von Bergen et al., 2000). These hexapeptides, VQIVYK and, VQIINK, are present at the beginning of R2 and R3 of the MTBDs, respectively and show a propensity for forming B-sheet structures (von Bergen et al., 2000). Given that both hexapeptides are only present in the 4R isoforms, this may explain why these isoforms are highly prone to aggregate. Another critical factor that affects the ability of tau to aggregate is the tau mutations. For instance, the P301L mutation, found in patients with FTDP-17, is located close to one of the hexapeptides and promotes aggregation (Hutton et al., 1998a). Additionally, the disturbance of these motifs by artificial mutations like proline mutations reduce the propensity for tau to aggregate. Conversely, the introduction of mutations such as deltaK280 or P301L enhance the beta-structure and accelerates tau aggregation both *in vitro* and *in vivo* (Khlistunova et al., 2006).

As mention above, among the several post-translational modifications that regulate tau function, phosphorylation has been an object of interest due to its putative role in the pathological processes. It has been established a strong connection between abnormal phosphorylation and self-aggregation (von Bergen et al., 2000). However, to date, no complete pathological phosphorylation pattern was identified.

The accumulation of insoluble tau in the brains patients with tauopathies or transgenic mice are always composed by hyperphosphorylated tau (M. Goedert & Jakes, 1990; Grundke-Iqbal et al., 1986a; Hanger et al., 2009; Ksiezak-Reding et al., 1992). Also, several studies suggest that tau hyperphosphorylation precedes aggregation (Braak et al., 1994). Thereby, phosphorylation has been pointed as the initiator of tau aggregation, but this is not fully consensual in the tau field. On the one hand, hyperphosphorylated mouse and human tau can self-aggregate *in vitro* (Alonso et al., 2001; Chohan et al., 2005) and dephosphorylation of soluble tau from AD brains inhibit its polymerization and re-establishes the ability of tau to stabilize MT (K. Iqbal et al., 1998). Studies using a transgenic mouse in which tau kinase activity is increased showed a rise in tau phosphorylation which precedes the appearance of tau aggregates (Cruz et al., 2003; Kelleher et al., 2007; Noble et al., 2003). Furthermore, the treatment of transgenic mice with kinase inhibitors leads to a reduction in tau phosphorylation and tau aggregates (Klafki et al., 2006; Noble et al., 2005).

On the other hand, there is a high and heterogeneous number of phosphorylation sites. Some of them might be prone to aggregation in the brain (Alonso et al., 2001) but whether other factors are involved in tau aggregation, it is still not known. AD-like hyperphosphorylation of tau happens in a physiological context during animal hibernation and anaesthesia-induced hypothermia. This process is reversibly, and there is no aggregates formation (Arendt et al., 2003; Planel et al., 2007). Additionally, it is possible to induce tau aggregation efficiently *in vitro* through cofactors like polyanions such as RNA and heparin that compensate for the repulsive positive charges of tau irrespective of phosphorylation (M. Goedert et al., 1996; Kampers et al., 1996).

The mislocalization of tau is an additional factor that may affect aggregation. Once hyperphosphorylated detaches from MT, this leads to the increase of cytosolic tau, which becomes more susceptible to aggregation (Wegmann et al., 2018). Moreover, abnormally phosphorylated tau can sequester other MAPs which cause even worse microtubule destabilization (Alonso et al., 1997). Hence, one possibility that emerged is that unknown factors may trigger tau aggregation in AD brain, whereas phosphorylation may accelerate aggregation indirectly, for example, detaching tau from the MT (Yipeng Wang & Mandelkow, 2012).

Despite the several factors that affect aggregation, such as isoform composition, mutations, phosphorylation and localization, the mechanism by which tau form fibrils is not fully understood. *In vitro* studies have shown that tau aggregation follows a nucleation-elongation mechanism (von Bergen et al., 2000). This mechanism consists in two phases: the nucleation when the soluble monomers suffer conformational alterations or misfolding and form β -structures which aggregate and generate the assembly-competent initial nucleus. Through

monomer addition, the initial nucleus grows in dimers/trimers and small oligomers. This is followed by a rapid elongation phase when a critical nucleus size is reached, and the monomers and higher-order oligomers (insoluble granular tau oligomers - GTOs) elongate the nucleus and form PHFs which are the main component of NFTs. However, it is not clear whether the sequence always follows this linear way, or whether monomers and GTOs can form PHF (Figure 8).

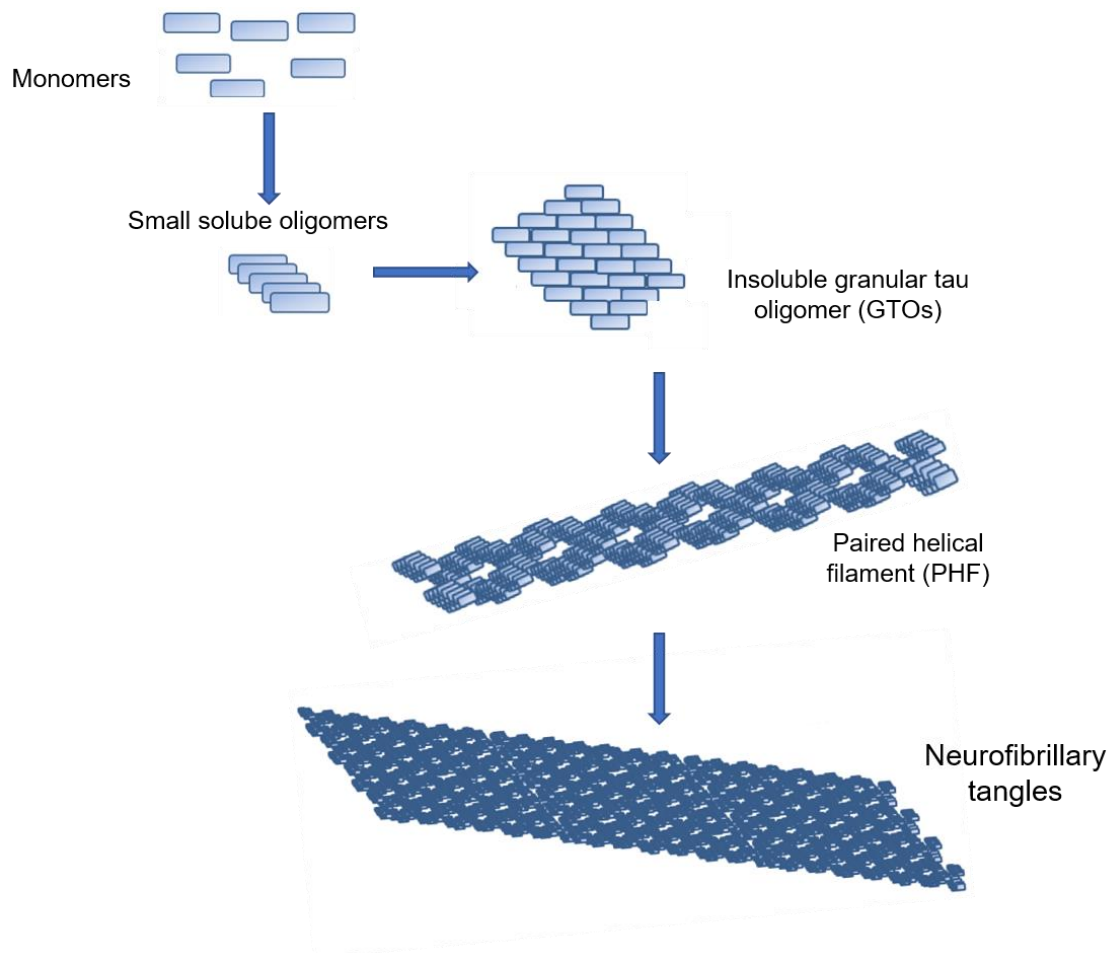


Figure 8. Proposed sequence of stages leading to NFTs formation (adapted from (Mietelska-Porowska et al., 2014))

2.2.2.4. Toxic tau species

Physiological and pathological tau species include monomers, dimers/trimers, small soluble oligomers, insoluble granular tau oligomers, filaments, PHFs, NFTs and ghost tangles (Brunden et al., 2008) (Table 1).

Since their discovery, NFTs have always been considered as the most toxic species and indicators of cell death, mainly because their progression and number correlate well with the harshness of cognitive decline in AD (Arriagada et al., 1992; H. Braak & Braak, 1995; Heiko Braak & Del Tredici, 2012; Gómez-Isla et al., 1997). It has been suggested that NFTs disturb essential cell functions either by physical damage/lesion (Ballatore et al., 2007; Gendron & Petrucelli, 2009a) or by abnormal quantity or distribution of essential molecules, like synaptic proteins (Adalbert et al., 2018; Spires-Jones et al., 2011) and calcium-binding proteins (Bezprozvanny & Mattson, 2008; Furukawa et al., 2003; Mattson, 2003, 2007). The toxicity of tau aggregates is also supported by shreds of evidence from cell culture models in which tau aggregation leads to activation of caspase cascades and cell death (Spires-Jones et al., 2009). Nevertheless, a body of evidence supports the opposite hypothesis: that NFTs are silent witness indirectly implicated in the pathological process and are not sufficient for tau-induced toxicity. As AD is diagnosed when the NFT is already spread throughout the brain, NFT are unlikely the specie responsible for the major toxicity (H. Braak & Braak, 1995, 1997). FTDs, known to be caused by tau mutations, and several animal models demonstrate severe neuronal cell loss and dysfunction in the absence of obvious NFT pathology (Andorfer et al., 2005, 2005; Gómez-Isla et al., 1997; Kimura et al., 2010; Spires-Jones et al., 2009; Wittmann et al., 2001).

Moreover, several studies using the mouse model rTg4510 in which the expression of the human mutant form of tau can be reversed show that suppressing the tau transgene stops the neuronal loss though NFTs continue to accumulate (SantaCruz et al., 2005; Spires et al., 2006). Indeed, in these animals, the appearance of NFT occurs following caspase activation. However, the neurons persist longer than expected after the activation of cell death signalling cascades (de Calignon et al., 2010; Spires-Jones et al., 2008). Altogether, these findings give rise to the theory that the formation of NFT might be a failed neuronal protective mechanism of sequestering toxic tau aggregates rather than a marker of cell death (Spires-Jones et al., 2009, 2011). Thus, it has been proposed that maybe the toxic species are produced in the process of tau aggregation. Given that, the soluble tau species have been pointed as suitable candidates, though this still a matter of debate.

It is known that native tau protein is not prone to aggregate, but changes in tau conformation, phosphorylation status, and localization may induce this ability. Thus, the abnormally phosphorylated monomers may self-aggregate and result in dimers and trimers.

Dimeric tau forms were identified *in vitro* as well as in transgenic P301L mutation (Sahara et al., 2007) and they are believed to be critical intermediate species in the formation of PHFs (Schweers et al., 1995).

Toxicity of tau trimers has been demonstrated at a nanomolar concentration in human neuronal cells, while monomers and dimers are harmless at such low concentration (H. Tian et al., 2013). Also, the same study revealed that trimeric form is the minimal size of tau assemblies that could be spontaneously internalized by cell and induce intracellular aggregation, which is the basis of tau propagation. This event was confirmed for both recombinant tau aggregates and tau assemblies purified from AD patients with the size three or more units. These trimers were internalized by primary neurons and by HEK293 cells which express two AD-associated mutations (P301L and V337M) (Mirbaha et al., 2015).

Small soluble tau oligomers have been described and detected in transgenic tau animal models and isolated from synapses in AD brains. It was shown that these oligomers might develop from soluble dimeric tau (Sahara et al., 2007). Their toxicity has been proposed in several studies. For instance, transgenic *Drosophila* expressing mutated tau show soluble tau oligomer and their formation was associated with degeneration (Ali et al., 2012; T.-H. Wu et al., 2013). In the transgenic mice expressing P301L mutation, the small soluble oligomers appear at very early stages of the disease, when memory deficits are apparent. However, there is no formation of NFTs or neuronal loss (Berger et al., 2007). These oligomers are also detectable in the brains of AD and FTDP-17 patients. Subsequently, tau may aggregate into insoluble forms such as granular oligomers, straight filaments (SF), PHFs and NFTs (Sahara et al., 2007). However, it is not clear whether aggregation into larger oligomers and insoluble forms affect the toxicity of the small tau oligomers.

Finally, the toxicity of tau oligomers was demonstrated when injected into the brain of wild-type mice and led to cognitive, synaptic, and mitochondrial abnormalities rather than tau monomers or fibrils (Castillo-Carranza et al., 2014; Lasagna-Reeves et al., 2011). Noteworthy that small aggregated tau species have been gathering interest because they can induce endogenous tau to misfold and propagate from affected to unaffected brain regions in mice, whereas fibrils do not (Lasagna-Reeves, Castillo-Carranza, Sengupta, Guerrero-Munoz, et al., 2012; Lasagna-Reeves, Castillo-Carranza, Sengupta, Sarmiento, et al., 2012). This piece of evidence suggests that these species may be involved in the propagation of tau pathology.

Table 1. Summary of tau species and their toxicity (Adapted:(Cowan & Mudher, 2013; Mroczko et al., 2019))

Tau species	Description	Toxicity
Monomers	Highly soluble proteins Size: 55–74 kDa	No toxic - Native form Probably toxic - Aberrantly phosphorylated form
Dimers/trimers	Composed of 2 tau monomers in anti-parallel orientation; minimal propagation unit size: 120–180 kDa	Yes, some types Toxic for neurons at nanomolar levels
Small soluble oligomers	Developed from soluble dimeric tau; detected in mice model of tauopathy, AD and FTDP-17 patients Size: 300–500 kDa	Yes, some types
Insoluble granular tau oligomers	Intermediate step in the formation of PHFs and SFs; isolated from AD brains Size: 1800 kDa	Yes, some types / Not always
Straight Filaments (SF)	Tau hyperphosphorylation may induce its self-assembly into NFTs consisted of PHFs/SFs Size: >50 nm length, 10 nm width	Not always
Paired helical filament (PHFs)	Highly insoluble and resistant to proteolysis Size: 10–20 nm width, with 80 nm periodicity, length > 220 nm	Probably not toxic
Neurofibrillary tangles (NFTs)	Large bundles of fibres consisting of both PHFs and SFs	Probably not toxic
Ghost tangles	Remnants of degenerated neurons within which NFTs have been formed	Unlikely

2.2.3. Extracellular tau and propagation of tau pathology

Tau is predominantly an intracellular protein. However, a body of evidence shows that tau is physiologically released into extracellular space by cells *in vitro* and by neurons *in vivo*. Moreover, this process is regulated by neuronal activity, meaning that stimulating neuronal activity enhances tau release (Pooler et al., 2013; Yamada et al., 2014).

Initially, it was thought that extracellular tau was the result of release from degenerating neurons since high levels of total and phosphorylated tau are found in the CSF of AD patients compared to controls (K. Blennow et al., 1995). However, tau was later detected in the CSF of healthy individuals (Hampel et al., 2010) and CSF of normal mice (Barten et al., 2011; Yamada et al., 2011). Additionally, full-length of tau was found in the brain interstitial fluid (ISF) in the absence of neurodegeneration in wild-type mice and transgenic mice expressing the P301S mutation suggesting a physiological role for extracellular tau (Yamada et al., 2011). To date, the mechanisms by which tau is released in extracellular space are still not precise. Of note, in cultured primary neurons, tau release is, at least in part, dependent on presynaptic vesicle secretion mechanisms and neuronal activity (Pooler et al., 2013). However, the function of physiological extracellular tau remains elusive.

Although the relevance of extracellular tau is not well understood, it has been associated with the mechanisms of progression of tau pathology in the brain. The progression of tau pathology through the brain would occur in a prion-like manner (Clavaguera et al., 2009; Frost et al., 2009; Kaufman et al., 2016b; Kfoury et al., 2012). This hypothesis comprises at least two steps: 1) misfolding induction and 2) transfer of misfolded protein which contact with still native protein, known as seeding and propagation, respectively.

According to this concept, misfolded tau must be present in the extracellular space. This can happen passively due to neuronal death or secrete as several pieces of evidence demonstrate (Yanamandra et al., 2013, 2017). This secretion step leads to the presence of tau in extracellular fluids, ISF and CSF, (in a vesicular or non-vesicular form) (Mudher et al., 2017a; Mar Pérez et al., 2019). Once in the extracellular space, pathological tau, monomers, oligomers or aggregates, ought to be taken up by cells and transfer between them. *In vitro* studies have shown that extracellular tau aggregates and small tau oligomers but not tau monomers can be taken up by cultured HEK293 cells and neuronal stem cells (J. W. Wu et al., 2013). Tau protein seems to be internalized via endocytosis (Evans et al., 2018; Guo & Lee, 2011a; Polanco et al., 2018; J. W. Wu et al., 2013) and mediated by heparan sulfate proteoglycans (HSPGs) (Holmes et al., 2013; Rauch et al., 2018; Stopschinski et al., 2018).

Subsequent cellular uptake of pathological tau (tau seeds) can convert healthy intracellular tau into a pathological form: this process is known as tau seeding. It is possible to induce tau pathology in mice expressing human wild-type tau by injecting tau aggregates isolated from transgenic mice expressing human P301S mutation (Clavaguera et al., 2009).

Similarly, injecting brain lysates from different tauopathies not only induce tau pathology but reproduces the same morphology of inclusions seed in human diseases (Clavaguera et al., 2013). This study supports the concept that tau seeds can induce a templated misfolding of tau and consequently, propagation. Furthermore, *in vivo* models have been created to investigate the propagation of tau pathology. Some models use neuropsin promoter (de Calignon et al., 2012a; Li Liu et al., 2012; Wegmann et al., 2017) or viral vectors (Caillierez et al., 2013; Dujardin et al., 2018; Wegmann et al., 2017) to get the expression of tau in neurons. Even though both models display cell-to-cell transfer of tau it is necessary to take in account reliable controls to exclude transgene leakage or viral diffusion across the brain as the cause of these observations (Dujardin et al., 2014; Yetman et al., 2016). Likewise, transgenic mice which express tau only in the entorhinal cortex also show the propagation of tau pathology to distant, connected brain regions (de Calignon et al., 2012a; Li Liu et al., 2012). Other *in vivo* models are based on intracranial injections (Mudher et al., 2017b) or peripheral administration (Clavaguera et al., 2014) in transgenic or wild-type mice of a pathological form of tau such as recombinant oligomeric or fibrillar tau, brain extracts from AD or tauopathy patients or transgenic mice. The results observed in these models suggest cell-to-cell propagation given that tau pathology appears progressively in distant and anatomically connected regions. Apart of cell-to-cell transfer, other mechanisms for tau pathology propagation have been proposed such as synaptic transfer, interstitial diffusion and even the involvement of glial cells in tau transfer (Asai et al., 2015; Kovacs et al., 2018; Martini-Stoica et al., 2018; Narasimhan et al., 2017).

The tau propagation hypothesis is also supported by the Braak stages, which describe the progression of tau pathology through an anatomical connected pathway and the presence of increased concentrations of extracellular tau in CSF of AD patients. However, to date, it has not been possible to prove that active cell-to-cell transfer arises in the human brain. However, some PET and MRI studies suggest that it may happen.

2.2.4. Cellular vulnerability to tau pathology

According to a different hypothesis, the pattern of neurodegeneration could be explained by the possible existence of chemically defined neuronal subpopulations that are highly vulnerable in AD (Hof et al., 1996). Vulnerability to pathology refers to the fact that one or more types of neurons may be susceptible to accumulate pathology-associated proteins and consequently be lost due to the toxicity (Mattsson et al., 2016). In the case of AD, the selective loss of vulnerable neurons is intricately linked to tau pathology. Likely, there are two types of selective cell vulnerability: the primary vulnerability where the vulnerable cells are affected at the initial stage of the disease and the secondary vulnerability where the cells are affected in the regions where the pathology has already propagated (Fu et al., 2019). In the light of this concept and considering the progression of tau pathology according to the Braak stages, maybe tau accumulates in regions of primary vulnerability and propagates to regions of secondary vulnerability along with anatomical connections. Different types of neurons have been reported to be specifically vulnerable in AD including large pyramidal neurons in layer II of EC, subiculum, the CA1 region of the hippocampus (B. T. Hyman et al., 1984; B. M. Morrison et al., 1998; J. H. Morrison & Hof, 2002; Stranahan & Mattson, 2010); cholinergic neurons in the basal forebrain (P. Davies & Maloney, 1976; Whitehouse et al., 1982), and noradrenergic neurons in the locus coeruleus (Bondareff et al., 1982; Greenamyre & Young, 1989).

At early stages of AD, the cells within EC and hippocampus, which are primarily affected regions, accumulate pathological forms of tau and progressively degenerate. Based on their morphology and location, it suggests that they are excitatory neurons (H. Braak & Braak, 1991; B. T. Hyman et al., 1984). Conversely, some studies suggest that inhibitory neurons which express calcium-binding proteins (parvalbumin, somatostatin, calbindin-D28k, and calretinin) are less vulnerable in AD or animal models of AD (Hof et al., 1993; Fu et al., 2017). However, this is still a matter of debate, and some studies suggest that tau pathology induces the loss of inhibitory neurons (Levenga et al., 2013). Remarkably, granule cells from the dentate gyrus (DG), parts of layer III and layers V and VI of EC, and cortical interneurons are somewhat affected in early AD (B. T. Hyman et al., 1984; Mattson & Magnus, 2006).

There is no irrefutable evidence so far that the various subpopulations of neurons display differences in vulnerability. However, animal models of AD have been providing a notable piece of evidence which might help to explain the selective vulnerability among different cell types.

In this sense, some lines of investigation have been trying to identify the reason why the excitatory neurons are, supposedly, the most vulnerable cell type to pathology in AD and other

neurodegenerative disorders. Among others, the causes for the neuronal vulnerability that have been pointed include cell size and location within neural circuits, protein homeostasis dysfunction, mechanisms on the regulating calcium and energy homeostasis.

Some protein homeostasis genes are modified in pretangles-bearing neurons during the progression of AD (Johnson et al., 2017; Tiernan et al., 2016) indicating that protein homeostasis might be compromised before clinical symptoms of the disease. Moreover, a transcriptional analysis of healthy brains at early ages (before the onset AD) revealed a protein homeostasis signature related to protein aggregation and predicted Braak staging (Freer et al., 2016). The protein homeostasis signature count in a set of aggregation-prone proteins (Ciryam et al., 2016) and another set of components such as co-aggregators, aggregation promoters, and aggregation protectors (Kaushik & Cuervo, 2015). The expression of the protein homeostasis protein signature is significantly increased in neurons compared to other cell types, pointing out that neurons have a cellular environment more predisposed to protein aggregation (Freer et al., 2016). Indeed, more recently it was reported that excitatory neurons are more prone to accumulate pathological tau rather than inhibitory neurons in the EC, which is affected at early stages of AD but also in regions that are affected later such prefrontal cortex (ref). It was found, by analyzing RNA transcripts that excitatory neurons display a raised expression of a specific subset of aggregation-prone proteins and tau aggregation promoters and a decreased expression of tau aggregation protectors. On the other hand, inhibitory neurons show a high expression of tau aggregation protectors (Fu et al., 2019).

It is thought that the disturbance of cellular Ca^{2+} regulation plays a crucial role in the vulnerability of neurons in AD and other neurodegenerative disorders. The neurons expressing calcium-buffering proteins are resistant, or less vulnerable, in the neocortex of AD patients (Hof et al., 1993; Iwamoto & Emson, 1991; B. M. Morrison et al., 1998). As mention before, CA1 neurons are selectively vulnerable to degeneration in AD compared to CA3 neurons and granule cell in DG (Mattson & Magnus, 2006; Simonian & Hyman, 1995). Studies in a mouse model of AD suggest that the excessive Ca^{2+} influx through L-type voltage-gated calcium channels (L-VGCC) at CA1 synapses maybe explain the selective vulnerability of CA1 neurons (Yue Wang & Mattson, 2014).

Furthermore, large and long projection neurons in the EC and CA1 are characterized by high energy demand for their function and are vulnerable to decreased glucose and oxygen, meaning energy deficiency (Mattsson et al., 2016). In agreement with this notion, disturbance in calcium homeostasis and mitochondrial dysfunction have been associated with the pathogenesis of AD (Roselli & Caroni, 2015; M. T. Lin & Beal, 2006).

Interestingly, the intrinsic firing properties seem to be related to a selective neuronal vulnerability in neurodegenerative diseases. It has been proposed that the elements that lead to neurodegenerative diseases might compromise the pathways involved in neuronal excitability (e.g. calcium and/or energy homeostasis) and that the neurons in which excitation-regulation is highly sensitive are selectively vulnerable to the disease (Roselli & Caroni, 2015). However, in AD, it is not clearly understood how the firing properties of vulnerable neurons are related to their degeneration.

2.2.5. Tau induced cell dysfunction

The importance of tau function has been demonstrated over the past four decades. The discovery of inherited tauopathies and the identification of mutations in the tau gene MAPT (Hutton et al., 1998b; Poorkaj et al., 1998c; Spillantini et al., 1998), definitely demonstrated that tau dysfunction could drive neurodegeneration. Although tau deletion is not lethal and can be in part compensated by other microtubule-associated proteins, for example, MAP1A, in aged tau-knockout mice (~12-month-old), behavioural impairments are observed, showing that tau is essential for normal neuronal and brain function (Lei et al., 2014). On the other hand, transgenic mouse models of tauopathy, which express human wild type or mutant tau, display progressive neuronal loss (LaFerla & Green, 2012; Mhatre et al., 2013). These animal models allowed to identify and characterize several cellular processes in tauopathies caused by tau dysfunction and that might lead to neurodegeneration, such as destabilization of the microtubule network and cytoskeletal dysfunction, impaired axonal transport, mitochondrial dysfunction and oxidative stress and synaptic dysfunction.

2.2.5.1. Microtubule instability

Although the role of tau in neurodegeneration is not fully understood, it is widely accepted that loss of function precedes degeneration. Tau-induced neuronal dysfunction is usually related to microtubule instability and can be caused by mutations (Hutton et al., 1998a; Spillantini et al., 1998) or post-translational modifications (Köpke et al., 1993b; Min et al., 2015). Different models of tauopathy have shown microtubule disruption, counting transgenic mice overexpressing human wild-type tau (T44 model) or mutated tau (P301S and P301L) (B. Zhang et al., 2005; Yoshiyama et al., 2007).

Phosphorylation has been associated with the decrease in the interaction between tau and the microtubules. For instance, the hyperphosphorylation of tau in the repeat domain (Ser262 and Ser356) significantly reduce the affinity of tau to microtubules (Biernat et al., 1993). Also,

phosphorylation sites in proline-rich (Ser205, Ser212, Ser214, Thr231, Ser235) and C-terminal (Ser396 and Ser404) domains have shown some effects on microtubule association (Cho & Johnson, 2004; Haase et al., 2004; Sun & Gamblin, 2009).

Isolating abnormal and regular phosphorylated tau from AD brain in solution (Köpke et al., 1993a) it was possible to assess the impact of hyperphosphorylation in microtubule assembly. The results showed that regular phosphorylated tau has a physiological microtubule-promoting activity *in vitro*. Inversely, hyperphosphorylated tau did not promote microtubule assembly. However, the regular activity can be restored by dephosphorylation with alkaline phosphatase treatment (A. C. Alonso et al., 1994). Thus, hyperphosphorylation causes a weak binding between tau and microtubules, which lead to microtubules depolymerizing and disassembling. Furthermore, the self-aggregation reduces the soluble functional tau, also contributing to the network destabilization, which affects crucial neuronal functions.

2.2.5.2. Cytoskeletal dysfunction

Tau protein interacts not only with microtubules but also with the actin cytoskeleton (Henríquez et al., 1995). Their proper organization and dynamics are essential for cellular function. However, in tauopathies, actin dynamics seems to be considerably distorted.

The link between actin dysfunction and neurodegeneration was suggested after the discovery of intracellular aggregates composed by actin, named Hirano bodies, in post-mortem AD brains (Galloway et al., 1987). The Hirano bodies can also be observed in different models of tauopathy such as mouse and *Drosophila* and can colocalize with tau. The increase of actin stabilization reduces actin turnover and dynamics, which significantly constrain the organelle transport mediated by myosin (Semenova et al., 2008). Moreover, in transgenic *drosophila* models which express either wild type or mutated human tau, the actin stabilization correlates with the degree of toxicity (Fulga et al., 2007).

Additionally, synaptic activation increases the interaction between tau and filamentous actin at the postsynaptic density, which emphasizes the role of tau in the regulation of synaptic plasticity (Frandemiche et al., 2014).

Thus, the excess of stabilized actin and the impaired actin dynamics affect axonal transport and may have an impact at a synaptic level, suggesting their contribution to neurotoxicity in tauopathies.

2.2.5.3. Impaired axonal transport

Axonal transport is vital for the cellular function, supplying the distal synapse with proteins and lipids synthesized in the somatodendritic compartment, mitochondria and other organelles. The impairment of axonal transport contributes to a diversity of neurodegenerative disease, including AD and tauopathies (S. T. Brady & Morfini, 2017).

The transport in the axon occurs in both directions through microtubules (Kreutzberg, 1969) which are stabilized by MAPs, such as tau, and form tracks along which motor proteins can transport different cargoes. The two major motor proteins families are the kinesins and dyneins responsible for the anterograde and retrograde transport respectively. The conventional kinesins are involved in the transport of various organelles, including mitochondria, synaptic vesicles and among other cargoes toward the synaptic terminal (Leopold et al., 1992; Elluru et al., 1995). On the other hand, the complex cytoplasmic dynein is responsible for the returning of signalling complexes and organelles carrying degradation products from synaptic terminals and axons to the soma (Susalka & Pfister, 2000; Delcroix et al., 2003).

The extensive study on tau lesions in AD allowed collecting a significant body of evidence determining axonal degeneration as a conspicuous hallmark of the disease (Kanaan et al., 2013). The analysis of post-mortem AD brains patients reported a reduced number of microtubules and an accumulation of vesicles containing pathological forms of tau within dystrophic neurites (Praprotnik et al., 1996; Dessi et al., 1997). Remarkably, in FTDP-17 brains patients was found that mutant tau aggregates localize within dystrophic axons (Delisle et al., 1999; Lippa et al., 2000; Kouri et al., 2014). Together with human data, several models of tauopathy exhibit axonal degeneration including, axonal spheroids and degenerated myelinated axonal tracts (Krajewski et al., 2000; W.-L. Lin et al., 2003), recapitulating the axonopathy observed in the human brain. Pathogenic tau might contribute to axonal transport impairment either by causing the disassembly of microtubules or by interaction with the motor proteins.

As mention before, hyperphosphorylation or specific mutations decrease the affinity of tau for the microtubules, which cause their destabilization. This structural disruption and instability most likely lead to the impairment of axonal transport.

Pathogenic tau can inhibit axonal transport in different ways of interaction. For instance, the excessive level of monomeric tau can compete directly with the motor protein to bind with the microtubules, which limit their movement along the microtubules (Seitz et al., 2002). Another way that tau manage to prevent the binding of kinesin to the microtubules is by, in its

phosphorylated form, trap the kinesin adaptor molecule JIP1 (c-Jun N-terminal kinase interacting protein 1). Once trapped, JIP1 is unable to load cargoes onto kinesin for subsequent transport down the axon. Moreover, in the AD brain JIP1 is relocalized from the axon to the soma (L. M. Ittner et al., 2009).

Additionally, tau can promote the detachment of kinesins from its cargoes via kinesin's phosphorylation by GSK3 (Pigino et al., 2009; Kanaan et al., 2011). Tau contains, at the N-terminal, a phosphatase activating domain (PAD) which triggers the PP1-GSK3 signalling pathway. Briefly, PP1 activates GSK3, which in turn phosphorylates kinesin leading to the release of its transported cargoes (Morfini et al., 2002). There is various form of pathological forms of tau, such as tau aggregates, phosphorylated tau (at the sites Ser202/Thr205) and mutant tau, where PAD can be exposed. Thus, these forms may exacerbate this cascade of events, consequently resulting in the inhibition of kinesin-dependent axonal transport. Likewise, tau might play a role in dynein-mediated retrograde transport. In the transgenic mice expressing mutated tau (P301S) dynactin complex, which is necessary for the cargo to dynein motor proteins, was found abnormally disturbed in the retinal ganglion cell axons (Magnani et al., 2007). In the same model was found that tau inclusions have an impact on axonal viability. However, in 1-month-old animals, which there is less phosphorylated tau, the axonal transport was not impaired (Gasparini et al., 2011). This data supports the idea that tau aggregates may also interfere in intracellular trafficking due to the physical space that they occupy.

Hence, these shreds of evidence suggest that the impairment of axonal transport may result from the action of pathogenic forms of tau, such as the overexpression at high levels, tau aggregates or altered isoform composition or phosphorylated state.

2.2.5.4. Mitochondria dysfunction

Mitochondria are essential for cellular viability, neurotransmission and synaptic plasticity. Their main functions are energy supply, regulation of intracellular calcium homeostasis, production of free radicals and regulation of apoptotic pathways (Gleichmann & Mattson, 2011; Jacobson & Duchon, 2004; MacAskill & Kittler, 2010).

The first analysis of AD brains reported the presence of mitochondria abnormally shaped in dystrophic neurites (Kidd, 1964). Postmortem morphological and morphometric studies, which analysed different brain regions, also verified mitochondrial alterations in AD (Baloyannis, 2006). Moreover, data from tissue samples and iPSC-derived neurons from patients with AD and FTDP-17, respectively, indicate that atypical mitochondrial morphology correlates with mitochondria dysfunction (Schägger & Ohm, 1995; Esteras et al., 2017).

Subsequently, several studies suggest that mitochondria dysfunction not only play a vital role in the pathogenesis of the disease but it is an early event in the pathogenesis of AD and other tauopathies, supporting these observations (Gibson & Shi, 2010; Cabezas-Opazo et al., 2015a). Regarding tau pathology, the mitochondrial function can be affected at different levels: (i) mitochondrial transport, (ii) dynamics (morphology) and (iii) bioenergetics.

2.2.5.4.1. Mitochondrial Transport

Given the mitochondrial importance for different cellular processes, their movement along the axons guarantees a proper distribution in the neurons and at the synapse. Mitochondria are usually generated within the soma and then transported to specific locations in response to alterations in energetic demand (Hollenbeck, 1996; Cai & Sheng, 2009). In this sense, it is conceivable that the impairments of mitochondrial transport can lead to a decrease in ATP levels at these locations and result in a synaptic impairment (Gendron & Petrucelli, 2009b). As already mentioned, pathological forms of tau can disrupt axonal transport in different ways. For instance, in cell cultures, the overexpression of tau induces a decrease in mitochondria movement (Shahpasand et al., 2012; Schulz et al., 2012). However, it is the hyperphosphorylated form of tau (Ser202/Thr205) that presents a more significant effect in the inhibition of mitochondrial transport, likely through the increase the space between the microtubules (Shahpasand et al., 2012). Also, in transgenic mice overexpressing the P301L mutation, cells which accumulate misfolded tau in the soma display a perinuclear mitochondrial clumping. This evidence suggests that tau-induced transport deficits lead to improper distribution of mitochondria (Kopeikina et al., 2011). On the other hand, in cortical neurons from the knockin P301L mice, the expression of mutated tau at physiological levels reveal a significant 50% reduction in the number of mitochondria in the axons and an increased volume of individual moving mitochondria, but without differences on the speed, the direction of travel or chances of movement of mitochondria (Rodríguez-Martín et al., 2016).

Altogether, this indicates that changes in tau levels and the posttranslational modifications could impact the mitochondrial transport in neurons and ultimately affect the synaptic process.

2.2.5.4.2. Mitochondrial Dynamics

Mitochondria are highly dynamic cellular organelles, and they are capable of changing size, shape and position rapidly. The two active processes, fission and fusion, are responsible for regulating all these changes and for keeping the proper mitochondrial function (Suen et al.,

2008; Itoh et al., 2013). The soluble cytosolic protein dynamin-related GTPase (Drp1) is the primary mediator of mitochondria fission. This protein can form spiral filaments around mitochondrial tubules which compress them, resulting in mitochondrial fission (Kageyama et al., 2011; Tamura et al., 2011).

Contrariwise, the process of mitochondrial fusion is mediated by the dynamin-related GTPases mitofusin 1 and 2 (Mfn1/2) and optic dominant atrophy 1 (Opa1) (Tamura et al., 2011). Affecting the equilibrium, one of those proteins leads to mitochondrial fragmentation (Itoh et al., 2013).

Several models demonstrated the impact of tau in those processes showing that mitochondrial dynamics are compromised in tauopathies. The overexpression of human tau in HEK 293, primary neurons and neuronal cultures from transgenic mice boosts retrograde mitochondrial transport and fusion. The proteins involved in this process, Mfn1/2 and Opa1 are increased, but there is no alteration in the fission proteins (X.-C. Li et al., 2016). Moreover, studies used mutated forms of tau (P301L and R406W) showed a reduction in fusion and fission factors and an enhanced in elongation of mitochondria in both, *Drosophila* and mouse neurons (Schulz et al., 2012; DuBoff et al., 2012). The formation of elongated mitochondria results from the mislocalization of Drp1 induced by hyperphosphorylated tau blocking mitochondrial fission. Interestingly, mitochondrial dysfunction exacerbated production of reactive oxygen species (ROS), and cell cycle-mediated cell death go along with mitochondrial elongation. This impairment was rescued *in vivo* by genetically reestablishing the proper balance of mitochondrial fission and fusion (DuBoff et al., 2012). Noteworthy, in postmortem brain of AD patients reveal diminished DRP1 levels in pyramidal neurons (X. Wang et al., 2009). Also, in different models of AD phosphorylated tau interacts with Drp1 at late stages of the disease, which seems to aggravate mitochondrial and synaptic deficiencies eventually resulting in neurodegeneration (Manczak & Reddy, 2012).

2.2.5.4.3. Mitochondrial Bioenergetics Failure

Mitochondrial bioenergetics dysfunction has been reported in tauopathies and might lead to neuronal degeneration. For instance, neuronal cultures, overexpressing the P301L mutation, display a reduction in ATP levels, a slight mitochondrial depolarization and a decrease in metabolic activity (Schulz et al., 2012). Studies in P301L mice revealed downregulation of complexes I and V activity of the mitochondrial electron transport chain. Moreover, the worsening of tau pathology during ageing results in an impairment of mitochondrial respiratory activity and a significant reduction in ATP levels accompanied by an increase of ROS (David et al., 2005).

Interesting, AD triple transgenic mice (expressing APP, PS and tau mutations) exhibit mitochondria dysfunction before the appearance of Ab plaques. These mice presented altered expression of complexes I and IV as well as mitochondrial depolarization, reduced ATP synthesis and increased ROS production (Rhein et al., 2009; Yao et al., 2009). This evidence suggests that the synergistic action of Ab and tau on mitochondria regulation may have an essential role in AD pathogenesis.

2.2.6. Synapse loss

The synapse loss can occur either by neuronal death or because the neurons are not able to maintain functional axons and dendrites (reviewed in (Bloom, 2014)). The analysis of AD patients showed an initial decrease in the number and the density of synapse not proportional to the loss of neuronal cell bodies, suggesting that the disintegration of synaptic endings precedes neuronal loss (Davies et al., 1987). Additionally, synapse loss is described often as one of the first events in pathogenesis as can be found in patients with mild cognitive impairment and first AD (Scheff et al., 2006; 2007). Contrary to amyloid pathology, the pattern of accumulation of hyperphosphorylated tau is a much better hallmark to track cognitive decline in AD patients (Jack et al., 2010), which may indicate tau relevance in synapse dysfunction.

Small amounts of tau are present in the dendritic compartment in healthy neurons. However, in AD and other tauopathies, these levels increase and are one of the first and critical pathological defects (A. Ittner & Ittner, 2018).

Hyperphosphorylation, mutations and aggregation of tau can drive the mislocalization of tau into postsynaptic spine leading to loss of spines and synaptic dysfunction (Hoover et al., 2010; Thies & Mandelkow, 2007; H.-C. Tai et al., 2014). Tau mislocalization can likely result in the increase of interaction between FYN and tau (Bhaskar et al., 2005) and AMPA receptor clustering deficits (E. C. Miller et al., 2014) which ultimately may jeopardise synapse integrity. Besides, in approximately one-third of the synapses of AD patients, A β colocalizes with tau (Fein et al., 2008). Also, a recent study has demonstrated that several synaptic proteins, including tau, are phosphorylated in the presence of A β (H.-Y. Wu et al., 2018).

Together these studies may suggest that in AD context A β can enhance the level of hyperphosphorylated tau in the synapse, which increases the probability of synaptic loss and lead to cognitive decline.

Nonetheless, several factors highlight the role of tau in mediating synaptic pathology. Besides the progression of tau pathology correlates well with the cognitive decline in human AD,

synapse loss is accompanied by NFTs formation and appear in the same regions of the brain (Terry et al., 1991; C. A. Davies et al., 1987; Serrano-Pozo et al., 2011). The presence of a higher amount of tau tangles is associated with lower levels of presynaptic proteins (Honer, 2003) and the neurons containing NFTs accountable to the selected synaptic deficits in AD (Callahan et al., 2002). Strikingly, synaptic dysfunction is observed in FTLD and other tauopathies (Bigio et al., 2001; X. Liu et al., 1996; Suzuki et al., 1995), as well as in tau transgenic models (P301s and P301L for example) which exhibit deregulation in the synaptic proteome, impairment of synaptic transmission, loss of synapses and dendritic loss (Crescenzi et al., 2017; J. A. Harris et al., 2012; Yoshiyama et al., 2007). Altogether, the body of evidence establishes a strong correlation between synaptic defects and tau pathology.

3. Network dysfunction in AD and tauopathies

The activity of neuronal networks is linked with the ability to form memories which involves encoding, storage and retrieval of the new information acquired. The effectiveness of this process is necessary for learning and memory. The brain activity is tightly associated with changes in network synchrony, the degree of correlated neuronal activity among populations of neurons, which vary significantly with behaviour and brain states (Jasper, 1936). For instance, during non-active states, such as natural awake and sleep states, the network activity is characterized by synchronized slow-frequency and high-amplitude fluctuations. On the other hand, during active states is characterized by desynchronized fast-frequency and low-amplitude fluctuations (Destexhe et al., 1999). This way, the increase in synchrony seems to be a mechanism for 'switching off' specific brain regions and desynchronization a mechanism which allows neurons to respond to different inputs (Poulet & Petersen, 2008).

The patients with AD and other neurological diseases that cause cognitive impairment, often show alterations in the network activities even before the appearance of the symptoms (Fahoum et al., 2013; R. A. Sperling et al., 2009). Among these alterations are abnormal oscillatory rhythmic activity and network hypersynchrony. Network hypersynchrony can be defined as a pathological state of excessive synchronization of neuronal activities resulting in seizures or epileptiform discharges which are observed in AD mice models and AD and FTLD patients (de Waal et al., 2012; Goutagny et al., 2013; Irizarry et al., 2012; Lozsadi & Larner, 2006; Palop & Mucke, 2010, 2016; Vogt et al., 2011). The balance between excitatory and inhibitory transmission (E/I balance) is crucial to maintain the brain oscillations (Boyce et al., 2016; Amilhon et al., 2015) and normal cognitive function (Zhou & Yu, 2018). Given this strong association, it is likely that the disturbance of E/I balance and consequent changes in excitatory

and inhibitory synapses might constitute an essential mechanism that leads to AD (Busche & Konnerth, 2016; Frere & Slutsky, 2018a; Palop et al., 2006; Palop & Mucke, 2016).

3.1. Hippocampal circuit

The hippocampus is a structure localized in the medial temporal lobe, and it was described for the first time in 1587 by Julius Caesar Aranzi. The study of the hippocampus' function in memory started with the case of patient Henry Molaison who exhibited severe memory deficits after a bilateral hippocampal resection (Scoville & Milner, 1957). Subsequently, several studies of patients and animal models with hippocampal lesions were performed contributing to the characterization of hippocampal function not only in learning in memory (Squire, 1992) but also in spatial navigation (J. O'Keefe & Dostrovsky, 1971; R. G. Morris et al., 1982). Besides the memory deficits and spatial disorientation exhibited by AD patients, hippocampus atrophy is one of the most reliable features in AD. It is the earliest region to be severely affected.

3.1.1. Hippocampus anatomy

The hippocampus is composed of two interconnected regions: the dentate gyrus (DG) and Ammon's horn (cornu ammonis (CA)), which can be further subdivided in CA1, CA2 and CA3. In humans, there is another subdivision, CA4, which is located between the two cell layers of the DG. In the hippocampus, there are two main classes of neurons: the principal neurons, which account for the majority of neurons, and the interneurons. The principal neurons are excitatory (glutamatergic), and the interneurons are inhibitory (GABAergic)

The principal neurons of the hippocampus include the granule cells of the DG, the mossy cells of the dentate hilus and the pyramidal neurons of CA. In turn, the interneurons are found in all regions of the hippocampus (reviewed in (Amaral et al., 2007; Freund & Buzsáki, 1996; Bezaire & Soltesz, 2013). These cells are involved in the intrinsic circuit inside the hippocampal formation, also known as a tri-synaptic circuit (Ramon y Cajal, 1899). The hippocampus received its primary input from EC, which is composed of six layers. Thus, in this excitatory pathway, the DG received inputs from the axons of layer II of EC through the perforant pathway (PP). The granule cells from DG connect to the CA3 pyramidal cells via mossy fibers, which are unmyelinated axons. CA3 also receives direct projections from layer II of EC through the PP and can establish interconnections CA3-CA3, known as the associative/commissural (A/C) loop. Subsequently, CA3 project to CA1 through the Schaffer collaterals and receives direct input from the layer III of the EC. Finally, CA1 pyramidal neurons send back-projections into to the deep layers V and VI of EC. Moreover, the DG's granule cells also project to the mossy

cells and interneurons in the hilus, which send excitatory and inhibitory projections, respectively, back to the granule cells. Likewise, the pyramidal cells of the CA1 and CA3 regions contact local interneurons.

3.2. Neuronal types of CA3

3.2.1. CA3 Pyramidal cells

In recent years, the CA3 region has gained significant attention due to its particular role in memory processes and to its susceptibility to seizures and neurodegeneration.

The CA3 pyramidal cells have their cell body located in the pyramidal cell layer; its apical dendrites extend towards stratum radiatum and arborise in the stratum lacunosum moleculare, and its basal dendrites ramify into the stratum oriens.

CA3 received excitatory inputs directly from layer II of the EC via the perforant path (Woodhams et al., 1993) from DG granule cell through mossy fibers (Blackstad et al., 1970; Swanson et al., 1978) and can also exhibit a great interconnection with other CA3 pyramidal cells (Myers & Scharfman, 2011).

One particularity of these cells is the presence of mossy fiber synapses, as known as thorny excrescences (Lorente De Nó, 1934). The mossy fiber connections receive strong input from the DG and display a wide dynamic range of short-term plasticity, which vary according to post-synaptic cell type. Another defining feature that characterizes the CA3 circuits is the multiple interconnection CA3-CA3 which have been implicated in associative memories storage and recall (Henze et al., 2002; Rebola et al., 2017).

Apart from the recurrent connection from other CA3 pyramidal cells, they also receive inhibitory inputs from CA3 interneurons which regulate neuronal excitability and synchronisation of network activity (Lorente De Nó, 1934; Buzsáki & Wang, 2012). This circuit is implicated in encoding spatial representations (John O'Keefe & Nadel, 1978) and episodic memories (Scoville & Milner, 1957).

In the disease context, the loss of associative LTP of CA3-CA3 connections has been shown in a mouse model of AD (Viana da Silva et al., 2019). Additionally, mossy fiber plasticity is impaired due to structural changes and Ca²⁺ dysregulation in a mouse model of tauopathy (Decker et al., 2015). Also, the use of high-resolution fMRI permits to identify different subregions of the hippocampus, as CA3, in humans. These studies have contributed mainly to revealed the structural alterations associated with mild cognitive impairment in early Alzheimer's disease (Deuker et al., 2013).

3.2.2. Interneurons

Interneurons represent only 10% of all neurons; however, they constitute a heterogeneous group in the hippocampus. They can regulate the activity of excitatory neurons contributing to the typical neural circuitry and neural network activities (Palop & Mucke, 2016; Verret et al., 2012). Interneurons are crucial for the fine balance between excitation and inhibition thereby play an essential role in the generation or maintenance of network oscillations present in the hippocampus such as theta, gamma and sharp-waves (Klausberger et al., 2003). In CA3, interneurons are present in all the layers, and they can receive information from DG granule cells via mossy fiber and CA3 pyramidal cells generating feed-forward and feed-back inhibition, respectively controlling and shaping the firing of excitatory pyramidal cells (Stark et al., 2015). Interneurons discharges are rhythmic and are responsible for periods of increased and decreased excitability on the neuronal network, thereby oscillating at the same frequency (Buzsáki, 2002; Mizuseki et al., 2014).

Interneurons can be classified by morphological, anatomical, neurochemical, or physiological features. Although there is no widely accepted taxonomy of hippocampal interneurons, according to their differential in neuronal molecular expression, they are divided into at least five categories, including parvalbumin (PV) neurons, neuropeptide somatostatin (SST/SOM) neurons, neuropeptide Y (NPY) neurons, vasoactive intestinal peptide (VIP) neurons, and cholecystinin (CCK) neurons (DeFelipe et al., 2013). The two significant populations are PV and SST interneurons, account for 40–50% and 20–30%, respectively (Wonders & Anderson, 2006). Different studies showed that PV and SST interneurons have an essential role in controlling the rate, burst and timing of hippocampal pyramidal cells concerning theta oscillations in the hippocampus (Royer et al., 2012). Furthermore, a recent study highlights the critical role of these neurons in slow hippocampal gamma oscillations in CA3 (Antonoudiou et al., 2020).

3.3. Excitatory synapse

Glutamate is the primary excitatory neurotransmitter in the brain.

The excitatory activity of glutamate is mediated by the glutamate receptors, which can be distinguished in two main subtypes: the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors (mGluR).

The iGluRs, 5-methyl-4-isoxazole propionate (AMPA), kainate (KA), and N-methyl-D-aspartate (NMDA) receptor are ligated-coupled ion channels (Hollmann & Heinemann, 1994). Different combinations of subunits GluA1-4 compose the AMPA receptors, and each combination

determined the channel properties. For instance, the receptors that express only one GluA2 subunit are permeable to Na^+ and the ones lacking this subunit are permeable to the Ca^{2+} (Sommer et al., 1991). Four subunits composed KA receptors out of GluK1-5. NMDARs are composed of subunits GluN1, GluN2A, GluN2B, GluN2C, and GluN2D. These receptors must contain one GluN1 subunit in order to be functional (Herguedas et al., 2013).

Glutamate is released from presynaptic terminals of pyramidal neurons after stimulation, and it binds to glutamate receptors that are located in the postsynaptic neurons. The concentration of glutamate in the synapse is transiently high during the activation of the receptors. The excitatory activation ceases when the neurotransmitter is reduced by diffusion and by uptake by glutamate transporters. Glutamatergic signalling is implicated in the regulation of crucial processes that mediate the synaptic transmission and plasticity in learning and memory (reviewed in (Willard & Koochekpour, 2013)). At the synapse, glutamate concentrations are low and tightly controlled by several mechanisms. Thus, the disturbances of this regulation can lead to an exacerbated release of glutamate, which can induce hyperexcitability in post-synaptic neurons, causing excitotoxicity and cell death (Choi, 1994; Doble, 1999).

3.4. Inhibitory synapse

γ -aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in CNS, and it is essential in the regulation of excitability in cortical networks (McCormick, 1989; Faingold et al., 1989) and the synchronization of neuronal activity by cortical interneurons activity (Cobb et al., 1995; Tamás et al., 2000). Three different GABA receptors mediate GABA's inhibitory function: GABAA, GABAB and GABAC (Y. Li et al., 2016).

The receptors GABAA and GABAC are ligand-gated chloride (Cl^-) channels, whereas GABAB receptors are G-protein coupled metabotropic receptors (Chebib & Johnston, 1999)

The GABAA receptors are composed of five subunits $\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, $\rho 1-3$, θ , δ , π and ϵ . The subunits are pentamerically assembled and form the ligand-gated Cl^- ion channel. These channels are widely expressed in CNS. GABAC receptors are composed of $\rho 1-3$ subunits, forming homomeric or heteromeric channels which make them different from GABAA in pharmacology and function (Johnston, 1994; Lüscher & Keller, 2004). GABAC receptors are often identified as a subgroup in GABAA receptors (Barnard et al., 1998). Unlike the GABAA, these receptors are mostly expressed on the retina. GABAB receptor is formed by two subunits GABAB1 and GABAB2 which interact allosterically between for optimal regulation of receptor function (Kaupmann et al., 1997; Galvez et al., 2001).

The synthesis of GABA occurs in the pre-synaptic terminal from glutamate by the enzyme glutamic acid decarboxylase (GAD). GABA is transported along the axon and then recruited

into synaptic vesicles by the vesicular GABA transporter (vGAT) (Glykys & Mody, 2007; Gonzalez-Burgos et al., 2009). Following the action potential which induces the membrane depolarization, GABA is released from the presynaptic vesicles into the synapse, which results in an increase of GABA concentration in the synaptic cleft. GABA binds to GABA receptors in the postsynaptic membrane, decreasing postsynaptic neuron excitability (Farrant & Nusser, 2005).

GABA inhibitory activities can be divided into two classes: phasic inhibition and tonic inhibition (Farrant & Nusser, 2005; McQuail et al., 2015).

GABAA receptors primarily mediate phasic inhibition resulting from the transient activation of GABA receptors containing $\gamma 2$ subunits postsynaptically (Schweizer et al., 2003; Farrant & Nusser, 2005).

Tonic inhibition describes the continuous form of inhibition. Besides the binding to the postsynaptic receptors, GABA also activates extrasynaptic GABA receptors results in a persistently neuronal inhibition (Y. Li et al., 2016). Extrasynaptic GABAA receptors mainly mediate tonic inhibition. GABAA receptors containing $\alpha 5$ subunit regulates inhibition, particularly in hippocampus (Glykys & Mody, 2007). Approximately 75% of the inhibitory charges received by the hippocampal neurons is from tonic inhibition (Mody & Pearce, 2004). This form of inhibition is crucial to regulate excitation due to the capacity to sustained long-lasting hyperpolarization (Schipper et al., 2016).

Among other processes, tonic inhibition is responsible for mediating neuronal excitability, network oscillations (Mann & Mody, 2010).

3.5. Hippocampal Hyperexcitability

In the years before the AD diagnosis, it is possible to detect hyperactivity or excessive activation of the memory network, comprised of the hippocampus, medial temporal lobe, and several cortical regions (R. A. Sperling et al., 2009). Additionally, in numerous studies using fMRI, subjects at genetic risk for AD show hippocampal hyperactivity during memory tasks (Bookheimer et al., 2000; Dickerson et al., 2005; Quiroz et al., 2010) and impaired default network activity (Filippini et al., 2009). Recently, a study combining amyloid and tau PET with (fMRI) found an association between increased hippocampal activity and tau accumulation in the inferior temporal cortex. These data suggest that the pathogenesis of hippocampal hyperactivity and spread of tau pathology from the entorhinal cortex to the neocortex take place simultaneously and prior the appearance of the clinical symptoms of AD (Huijbers et al., 2019). Together, this data suggests the presence of network alterations at early stages.

The hippocampal neurons are usually intact with little neuron loss in age-associated memory deficits, as seen in MCI (Ganeshina et al., 2004; Rapp & Gallagher, 1996). Nevertheless, age-related alterations in the functional connections of the cell population are found within all the hippocampus subregions. For instance, both MCI patients and aged animals with memory deficits exhibit a loss in synaptic strength in the DG (Geinisman et al., 1992). The loss of synaptic connection correlates with the worsening of memory impairments. Furthermore, MCI patients also exhibit fewer synapses in the DG compared to age-matched controls without memory impairments (Stephen W. Scheff et al., 2006a)

On the other hand, CA3 display hyperexcitability in aged memory-impaired rats (Wilson et al., 2005) and aged individuals with poor memory performance, as well as aMCI patients (Dickerson et al., 2005; R. Sperling, 2007). Interestingly, the altered activity in CA3 can predict not only the degree but also the rate of conversion to AD (S. L. Miller et al., 2008). Finally, in the CA1, which has been widely studied in animal models, the memory deficits are thought to result from an increase in the number of silent synapses (or synapses without excitatory glutamatergic AMPA receptors) and calcium channels, leading to long-lasting excitation signals. (Nicholson et al., 2004; Thibault & Landfield, 1996). The latter could maybe be the reason why CA1 neurons are susceptible to excitotoxicity and more susceptible to AD (West et al., 2000).

This increasing body of evidence supports the idea that increased hippocampal activation is a dysfunctional condition. Although the hippocampal hyperexcitability cannot yet be considered as a biomarker of AD development, it is crucial to understand the link between the hippocampal hyperactivity and the development of AD.

4. Imbalance E/I: abnormal network activity

As discussed previously, AD patients show network hyperexcitability even at early stages of AD pathogenesis. Several studies are suggesting that A β might be a key factor for the changes in the E/I balance since 58% of the familial AD patients (with APP or PSEN1 mutations) experience seizures (Amatniek et al., 2006; Friedman et al., 2012; Vossel et al., 2013). Also, AD transgenic mouse models with familial AD mutations exhibit conspicuous neuronal hyperactivity and impaired E/I balance. For instance, in APP mice models A β oligomers severely affect synaptic transmission (Busche et al., 2012) and the dendritic morphology, thereby changing the structure of the circuit that directly causes hyperexcitability (Šišková et al., 2014). Moreover, mice overexpressing APP, PSEN1 mutations or ApoE4, which is a risk factor tightly linked to sporadic AD in humans, display spontaneous seizures and spike-wave discharges (SWDs), suggesting an early amyloid-related E/I imbalance (H. A. Born, 2015;

Minkeviciene et al., 2009; Nuriel et al., 2017). SWDs are episodes of abrupt, transient synchronous hyperactivity, consisting of high voltage deflections with amplitudes 2-fold higher than the baseline of recording, and resemble the waveform of interictal spikes (Bezzina et al., 2015a; Kam et al., 2016). Given that they occur even before the presence of spontaneous seizures, these events can be of particular interest as an early feature of E/I imbalance in AD (Bezzina et al., 2015b; Heather A. Born et al., 2014; Verret et al., 2012). However, despite extensive studies about the role of A β in abnormal neuronal activity, very little is known regarding the incidence of seizures in tau models. Strikingly, it is frequent that patients with epilepsy present tau aggregates (X. Y. Tai et al., 2016) and in experimental models of epilepsy, reducing tau attenuates neuronal network hyperexcitability (Holth et al., 2013). Remarkably, in mouse models of AD, endogenous soluble tau seems to promote APP-dependent network hypersynchrony, and cognitive decline (Roberson et al., 2007; L. M. Ittner et al., 2010b) and the reduction in tau prevents against seizures (DeVos et al., 2017). Studies in models of tauopathy are limited; however, the FTDP-17 mice have spontaneous seizures at early stages (5 months of age), before tau aggregates are present (García-Cabrero et al., 2013). Taken together, this piece of evidence suggests that A β and tau might contribute to the E/I imbalance in AD. However, the precise neuronal circuits that are responsible for hyperexcitability observed in AD patients remain largely unknown. It is widely accepted that the glutamatergic system plays an essential role in this neuronal hyperexcitation. AD pathogenesis is associated with the impairment of glutamatergic and cholinergic system. Besides the neuronal network dysfunction, AD patients show changes in the levels of these neurotransmitters (Dickson & Murray, 2015; Ferreira-Vieira et al., 2016; Rossor et al., 1981). Based on these studies, currently, the prescribed treatments are based on acetylcholine inhibitors, which enhance residual cholinergic activity and glutamatergic targets (NMDAR antagonist). Notwithstanding, these treatments are not efficient and only ameliorate the symptoms in a modest and temporarily fashion. Contrariwise, initial studies showed that the GABAergic system was relatively less vulnerable in AD (Pike et al., 1993; Reinikainen et al., 1988; Rossor et al., 1982). However, the interest in the dysfunction of GABAergic system in an AD context has increased as well as the emergence of evidence that supports the idea that the E/I imbalance might have a central role in AD pathogenesis (Frere & Slutsky, 2018a; Palop et al., 2006; Palop & Mucke, 2016).

4.1. Excitatory dysfunction

In AD, the implication of glutamate in the synaptic dysfunction has been investigated over the last decades. Thus, excitatory dysfunction was pointed out as the primary cause for the E/I imbalance found in the disease. The alterations of excitatory synaptic transmission may contribute as a critical factor in the abnormal hyperactivation and hypersynchrony of the neuronal network, which results in epileptic discharges.

The first studies on the pathological alterations and cognitive impairment focussed on the impact of A β on excitatory neurons. *In vitro* studies showed that A β reduces synaptic transmission by activating NMDA receptors (Kullmann & Lamsa, 2007; Lidong Liu et al., 2004). However, many studies have shown the opposite and that A β can also induce aberrant neural network activity. For instance, the administration of soluble A β *in vivo* (Busche et al., 2012), *in vitro* (Minkeviciene et al., 2009) and neuronal cultures (Cuevas et al., 2011) can induce hyperexcitability in hippocampal neurons and circuits. Several mechanisms associated with glutamatergic transmission could be responsible for the changes in neuronal excitability. Several studies demonstrated that A β could enhance hippocampal NMDAR currents *in vitro* (J. Wu et al., 1995), *in vivo* (Molnár et al., 2004). A β can act directly in glutamate transporter expression and/or function, causing hyperexcitability by disrupting glutamate uptake function (Fernández-Tomé et al., 2004; M. E. Harris et al., 1996). The impairment of glutamate transporters increases spontaneous excitatory postsynaptic currents, population spike frequencies, and impairments in long-term potentiation (Masliah et al., 1996). This impairment in glutamate transporter expression has been found in AD patients, and likely contribute to to reductions in glutamate uptake, E/I imbalance and neurodegeneration (Masliah et al., 1996; Scott et al., 2011).

Lastly, the A β oligomers induce the abnormal accumulation and overstabilization of mGluR5, which consequently increase intracellular calcium and may lead to synaptic failure (Renner et al., 2010).

Apart from A β , tau it is also implicated in the excitatory dysfunction in AD. Through the abnormal association with Fyn, tau can stabilize NMDARs in synapses and increase the currents dependent on this receptor, leading to the strengthening of glutamate neurotransmission (L. M. Ittner et al., 2010b). Remarkably, the reduction of tau counteracts excitotoxicity by diminishing NMDA receptor-dependent Ca²⁺ influx *in vitro* (Miyamoto et al., 2017) and decrease the hyperexcitability and epileptiform activity in AD models (Roberson et al., 2007). Recently, a study demonstrated that tau pathology leads to excitatory cell loss in the MEC, with grid cell dysfunction, and alterations in cell firing properties can contribute to spatial navigation deficits (Fu et al., 2017).

Finally, glutamate is also a GABA precursor, and the disturbance of glutamate reuptake could result in a reduction of GABAergic synthesis which enhanced E/I imbalance and consequently might lead to hyperexcitability (Sepkuty et al., 2002).

Hence, the extensive research focused on the excitatory dysfunction suggests that these alterations may have a direct impact on the inhibitory dysfunction in AD. There is evidence showing that glutamatergic signalling can modulate post-synaptic GABA_A receptor expression and clustering (Bannai et al., 2015) and the plasticity of the inhibitory transmission (Mapelli et al., 2016; McLean et al., 1996; Moreau & Kullmann, 2013). Notwithstanding, a significant piece of evidence has arisen, indicating that the impairment of the GABAergic synaptic transmission per se has an essential part in AD pathogenesis.

4.2. Inhibitory dysfunction

The inhibitory dysfunction in AD has been controversial since the beginning. On the one hand, early studies show that GABA inhibitory interneurons are resilient to A β -induced damage (Rossor et al., 1982; Pike et al., 1993). On the other hand, contemporary studies reveal impairments of inhibitory neurons (P. Davies et al., 1980; Chan-Palay, 1987). Nonetheless, GABAergic dysfunctions in AD have been widely neglected, and the research has mainly focused on the effect of A β on excitatory neurons. During recent years, the GABAergic system has gained attention and accumulating evidence suggests that inhibitory transmission may also suffer significant pathological changes in AD. For instance, several studies have shown that the memory and cognitive impairment in AD patients can be in part due to hippocampal hyperactivity caused by GABA inhibitory interneuron dysfunction (Huang et al., 2012; Govindpani et al., 2017; Selkoe, 2019; Villette & Dutar, 2017). However, the role of GABAergic dysfunction in the pathogenesis of AD is a still poorly understood and yet matter of debate. The available data, when taken together, are often inconsistent and difficult to interpret.

4.2.1. Human AD

4.2.1.1. EEG alterations

Collecting and analysing data from human AD patients is particularly challenging due to the available techniques. The EEG test is used to detect the electrical activity in the brain and abnormalities in brain waves. In AD patients, the EEG often describe abnormal brain activities at early stages which be might be linked with periods of transient epileptic amnesia

(Rabinowicz et al., 2000). Though, these alterations are subtler than those found in epilepsy patients which make them more challenging to detect and consequently to interpreted as a diagnostic marker (Vossel et al., 2017). Recently, a study using long-term EEG recordings identified a population of AD patients who display subclinical epileptiform activity. When compared with age-matched controls, these patients present a quicker cognitive decline. This unknown neural network hyperexcitability might be necessary for the patient evaluations and in clinical trials but still requires further investigation (Vossel et al., 2016).

Besides the EEG, MRI and functional MRI might be a possibility as a diagnostic tool. However, establish a link between fMRI signals and neuronal activity is still challenging and require careful interpretation (Dickerson et al., 2005; R. A. Sperling et al., 2009).

Not long ago, a study found changes in resting-state networks of AD dementia cases compared with healthy controls. Regardless, the diagnostic accuracy is still inferior to the one found in MRI measurements of hippocampus volume (Teipel et al., 2018).

The GABAergic system dysfunction seems to play an important role contributing to network dysfunction in AD (Kurudenkandy et al., 2014; Marczyński, 1998), which might be underlying unforeseen variations in episodic memory of the patients. In the last decade, the neuronal network dysfunction hypothesis has emerged. The changes in excitatory and inhibitory mechanisms are likely to be the cause epileptiform activity observed in AD and is a critical factor to the development of AD (Palop & Mucke, 2010).

Indeed, initial clinical trials using anti-epileptic drugs, targeting GABAergic transmission to increase inhibition, demonstrated positive effects in AD patients (Bakker et al., 2012; Sanchez et al., 2012). Although alterations in the neuronal network are evident, the causes and mechanisms underpin this event remain unclear.

4.2.1.2. GABAergic neuronal loss in AD

The attempt to relate impaired inhibitory neurotransmission, neuronal network dysfunction and memory impairment started in the early 80s. The analysis of AD brains first revealed a reduction in somatostatin and GABA, which are markers of some inhibitory neurons (P. Davies et al., 1980; Rossor et al., 1980, 1982). After this, a study investigated the relation between somatostatin in CSF, the EEG measurements and neuropsychological tests. The results found that low levels of somatostatin in CSF correlated with abnormalities in EEG and the neuropsychological tests assessing cognitive dysfunction (Soininen et al., 1988). The development of immunohistochemistry techniques allowed the labelling of somatostatin positive neurons, which unveil a loss of these inhibitory neurons in brain regions affected by

tau pathology in AD brains (Chan-Palay, 1987; Gabriel et al., 1993). Also, a decreased in GABA uptake was found, suggesting the GABAergic synapse loss in AD (J. Hardy et al., 1987; Simpson et al., 1988).

Over time other markers of inhibitory neurons have been investigated as well as their presence in different brain regions.

The expression of high levels of calcium-binding proteins allows to distinguish the three primary interneurons populations in the cortex: Calretinin (CR), calbindin D28k (CB) and parvalbumin (PV) (Klausberger & Somogyi, 2008; Tremblay et al., 2016). The results that find these populations reduced or unaltered are often dependent on the brain region analysed.

For instance, the analysis of the prefrontal, and temporal cortex did not show any alteration in CR-positive neurons between AD brains and controls (Fonseca & Soriano, 1995; Hof et al., 1993). However, in the hippocampus and EC, brain regions typically affected by A β - and tau-pathology, a constant loss of CR neuron was found (Brion & Résibois, 1994; Kaufmann et al., 1998; Mikkonen et al., 1999a). Similarly, changes in the CB and PV positive neurons were described. In visual cortex, CB-positive neurons were found unaltered (Leuba et al., 1998), while in the EC and hippocampus they were reduced in AD cases compared to healthy control brains (Ferrer et al., 1993; Mikkonen et al., 1999b; Palop et al., 2003). PV positive neurons were first found unaffected in the temporal, visual and prefrontal cortex (Ferrer et al., 1991; Hof et al., 1991; Leuba et al., 1998). Later, the brain regions affected by the disease, like entorhinal cortex and hippocampus, were shown with a decreased in the number of PV-positive neurons (H. Arai et al., 1987; D. R. Brady & Mufson, 1997; Inaguma et al., 1992; Mikkonen et al., 1999b; Solodkin et al., 1996)

Remarkably, neuronal network dysfunction such as altered hippocampal oscillations seems to be related to PV-positive inhibitory neuron dysfunction in AD and mouse models of AD (Verret et al., 2012).

4.2.1.3. GABA and GABAR levels in AD and tauopathies

An increasing body of evidence suggests that neurotransmission at GABAergic synapses is significantly affected in AD, however, whether GABAergic dysfunction is involved in the pathophysiology of AD is poorly understood and a matter of debate. Thus, several studies have been conducted to measure of GABA and GABA receptors levels in post-mortem AD brains. Overall, the majority of studies in post-mortem brain samples from patients with AD or other AD-like pathology indicate moderate-to-significant reductions in GABA levels in several brain regions, counting the temporal, frontal, parietal and occipital cortices. However, some

studies are challenging to interpret due to their contradictory results, showing an increase of GABA levels instead of a reduction (reviewed in (Govindpani et al., 2017)).

In post-mortem hippocampal regions the results are also controversial. Some groups have reported a decrease in the GABA levels measured (Rossor et al., 1982, 1984; Perry et al., 1987) and others showed that the neurotransmitter levels remain unaltered (Ellison et al., 1986; Sasaki et al., 1986; Yew et al., 1999).

Indeed, it exists a lack of consensus in the literature regarding the alterations in GABA levels in different regions of the AD brain. This can perhaps be explained by the fact that these studies might be considerably different in several parameters of the study design such as the sample size, the age and gender of the subjects, the post-mortem interval time (PMI), the stage of the disease, cause of death, and the use of medications by patients before death that interfere in GABAergic system.

For instance, the patient age and the stage of the disease are essential aspects to consider in this type of studies. A previous study reported that hippocampal GABA levels were only decreased by about 24% in a group of 49 AD patients compared with controls. However, analysing the differences between the age, the data showed a decrease of 41% in AD patients below 79 years old ($n = 23$) and only 9% for those above 79 years (Rossor et al., 1984). Another critical factor to take into consideration is the PMI; the matching of cases by PMI is crucial. The possible changes that can exist between antemortem and post-mortem in GABA levels could be problematic to analyse the dysregulation in GABA levels that might occur in AD. Several studies were conducted to measure the GABA levels in cerebrospinal fluid (CSF) to overcome the limitations mentioned above. Still, the results obtained were controversial. Most studies pointed to the maintenance of GABA levels in the CSF of AD patients when compared with healthy controls while others seem to demonstrate a reduction in GABA concentrations in CSF of AD patients (reviewed in (Govindpani et al., 2017)).

Concerning to GABAARs levels in the AD brain, various biochemical and molecular changes in the GABAergic system have been reported. In particular, the structures, distributions and compositions of GABAARs have been studied in different brain regions.

Of note, potential changes in the protein and mRNA expression levels of specific GABAAR subunits have been investigated. For example, it was shown using immunohistochemistry, that the expression of the $\alpha 1$ subunit in CA1, CA2 and subiculum fields is decreased in the human AD hippocampus (Mizukami et al., 1998). On the contrary, in a study which examined the expression levels of GABAARs subunits, $\alpha 1$, $\beta 1$ or $\beta 2$, in the hippocampus at different stages of the disease, no significant changes were found. But the expression of $\alpha 5$ subunit was found slightly decreased in the CA1, CA2 and CA3 regions of the hippocampus when comparing between mild/moderate and severe AD cases (Rissman et al., 2003).

Hence, even the controversial results obtained from the studies in AD brains, the body of evidence provided suggests the existence of changes in GABA_A receptors during the development of AD. These changes probably impact the pharmacological properties and sensitivities of the GABA_AR, and consequently, the inhibitory neurotransmission in the AD brain.

Interestingly, some studies suggest the link between NFT formation and GABAergic dysfunction in AD, in particular with changes in the GABA_AR subunit distribution within the human hippocampus. It has been shown that the density of subunits $\gamma 1/3$ is reduced, and $\alpha 1$ is increased in Braak stages V to VI, while mean $\gamma 2$ and $\beta 2/3$ labelling appear to be preserved even at this advanced stage of NFT pathology (Mizukami et al., 1998; Iwakiri et al., 2009). Additionally, in the hippocampus, the $\beta 2$ mRNA levels are stable while $\beta 3$ mRNA levels are reduced in Braak stages V to VI (Mizukami et al., 1998). Still, it is not clear whether the worsening of tau pathology is responsible for the changes in the expression of GABA system components.

In summary, the investigation in AD brains showed that the number of interneurons, GABA concentrations and GABA receptors tended to be reduced. Though, it is not clear how GABAergic dysfunction contributes to AD pathogenesis. However, this could likely be an essential object of further investigation for potential therapeutic approaches. In other tauopathies such as FTD and PSP, these values also tend to be decreased (Ferrer et al., 1993). Nevertheless, further investigation is required to understand the relationship between tau pathology and GABAergic dysfunction since, to date, the current evidence is not enough to establish the precise contribution of GABAergic dysfunction in the pathophysiology of tauopathy and consequently whether GABAergic approaches to treatment could benefit to FTD patients (Murley & Rowe, 2018).

4.3. Mouse models of AD and tauopathy

Establish an association between clinical symptoms of AD and pathological mechanisms that ultimately lead to network alterations in humans is particularly challenging. Given that, different experimental models, which reproduce crucial aspects of the AD pathogenesis, were developed to help to elucidate this question. With the insights from the studies in AD models, the neuronal network dysfunction hypothesis gained a more complex understanding, including the molecular, synaptic and neuronal changes which ultimately are responsible for network impairment (Palop & Mucke, 2010).

4.3.1. GABAergic neuronal loss in AD and tauopathies models

The fate of inhibitory neurons in AD mouse models is as debated as in AD human brains.

Animal models bearing the familial-associated mutations APP/PS1 or triple mutations, APP/PS1/MAPT, have been commonly used to evaluate the inhibitory neuronal loss, through stereological examinations and quantitative mRNA expression analyses.

Different studies have shown that there is a reduction in inhibitory interneurons in the transgenic mice hippocampus. The APP/PS1 model shows a loss of PV neurons (40–50%) in CA1-2 and a loss of CR neurons (37–52%) in the DG and hilus (D. R. Brady & Mufson, 1997; H. Takahashi et al., 2010). Also, the same model exhibits a loss of SST neurons (50–60%) in the DG and CA1 area (Ramos et al., 2006). Different studies found in very young mice (4 months) a decrease (35–45%) in CR-positive interneurons in CA1 and CA2/3 regions of the hippocampus (Baglietto-Vargas et al., 2010) and a significant reduction (20%) in PV neurons in the frontal cortex (Cheng et al., 2020). Recently, a study investigated the interneuron loss in the triple transgenic model, 3xTg-AD mice, at 18 months old showed a decrease of 33.7% and 52% of interneurons expressing CR and PV, respectively, in the CA1 hippocampal region (Zallo et al., 2018)

In respect to tau pathology, limited studies were conducted to evaluate the associated interneuron loss. However, the majority of them showed impairment in GABAergic function and a reduction in the number of interneurons. In 2013 Levenga et al. demonstrate for the first time that tau pathology induces GABAergic interneuron loss. They found that the number of both PV and SST interneurons were significantly decreased, about 20% compared to WT in the CA1 area (Levenga et al., 2013). Additionally, the removal of tau prevents loss of hilar GABAergic interneurons in a transgenic mouse of tauopathy (Andrews-Zwilling et al., 2010)

Though few studies presented contradictory analysis, they were showing no difference in the number of interneurons either the hippocampus or the entorhinal cortex in models which display A β and/or tau pathology (Verdaguer et al., 2015; Fu et al., 2019). The different results found related to the interneuron loss might rely on the distinct genetic features of the models, the pathological stage at which the samples are processed, and the brain region analysed. However, overall, the studies mentioned above show that A β pathology might induce a loss of interneurons and an impairment in neuronal activity. Therefore, protecting the interneurons increasing inhibitory connection could be a potential therapeutic approach in AD.

Thus, recent studies suggest that the rescue of PV cell numbers through an enriched environment (Cattaud et al., 2018) or pharmacological (Q. Zhang et al., 2018) could improve

cognitive deficits. For instance, the chronic administration of citalopram in APP/PS1 mice rescued impaired short-term memory and ameliorated non-cognitive behavioural deficits. Citalopram is a selective serotonin reuptake inhibitor and can reduce A β formation *in vitro* and reduce A β plaques in APP/PS1 mice (Dhami et al., 2013). In addition, after treatment with citalopram, the number of PV neurons increased in the cortex. The cortex is significantly affected at 6-month-old in these mice showing a decrease in PV neurons. Thus, the improvements in memory and behaviour might be due to the rescue of PV interneurons in the cortex through the pharmacological treatment (Q. Zhang et al., 2018)

Transplantation of GABA progenitor neurons is another approach to attempt rescue the number of interneurons. This approach revealed an improvement in learning and memory function in a transgenic model overexpressing A β (Tong et al., 2014)

In these experiments, the transplanted progenitors' cells differentiated into GABA inhibitory interneurons with normal function. Also, their integration markedly improves neuronal circuit dysfunction, leading to the improving the cognitive ability of AD mice (Maroof et al., 2013; Nicholas et al., 2013; Southwell et al., 2014; Shetty & Bates, 2016).

These experiments support the idea that the loss of interneurons is implicated in neuronal network dysfunction and ultimately in the cognitive decline.

4.3.2. GABAergic dysfunction and altered neuronal network

The disturbance of the fine balance between GABAergic and glutamatergic signalling might be led to the altered neuronal network activity in AD. Indeed, this disturbance can be precepted in the EEG of AD patients and mouse models in the form of epileptic activity (Busche & Konnerth, 2016; Palop et al., 2006; Palop & Mucke, 2016). Also, the hyperexcitability is a recurrent event, as demonstrated *in vivo* through calcium imaging of cortical neurons in the transgenic mice expressing APP and PS1 mutations (Busche & Konnerth, 2015).

The link of A β pathology in hippocampus and inhibitory neuron dysfunction, behavioural and *in vivo* network aberrations emerged from the observations in rats seeded with A β deposits. The misfolded A β reduces the frequency of the theta oscillations in the hippocampal local field potentials, which are associated with learning tasks and reduce the firing rate of PV neurons that project from the medial septum (Villette et al., 2010).

In hAPP-J20 mice, the granular cells in the hippocampal DG and pyramidal neurons in cortical layers II/III display a decrease in the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) without inhibiting the action potential (Palop & Mucke, 2016; Verret et al., 2012). Also, the amplitude of the action potential, induced in PV inhibitory interneurons in the cortex,

is smaller compared to the control mice. These observations confirm that A β overexpression affects the activity of GABA inhibitory interneurons (Roberson et al., 2011; Verret et al., 2012). Subsequent studies in this model have shown not only that the function of GABA inhibitory interneurons is affected but also that the GABA release is impaired in the hippocampus, leading to the dysfunction of neural network and cognitive impairment during AD development (Villette & Dutar, 2017). Additionally, in the cerebral cortex of APP23 \times PS45 transgenic mice the decrease in synaptic inhibition cause neuronal hyperactivity in the vicinity of amyloid plaques, further suggesting an A β pathology-associated impairment in GABA inhibitory neurons activity (Busche et al., 2008).

Regarding the mechanisms underly the GABAergic neurons dysfunction Verret et al. showed that in transgenic hAPP-J20 mice, PV+ interneurons have lower levels of the Nav1.1 channel (Verret et al., 2012). The Nav1.1 channel is a voltage-gated sodium channel which controls cellular and network excitability by modulating intrinsic excitability in PV interneurons (Ogiwara et al., 2007; Meisler & Kearney, 2005). The lack of this channel in interneurons is linked to the decrease of their action potential amplitude *in vitro* and the reduction of in gamma oscillation *in vivo*, thereby causing the synchronization of excitatory pyramidal neurons and increase in abnormal network activity (epileptiform activity) (Verret et al., 2012). Recently, another study confirms these observations not only for the lower expression of Nav1.1 but also Nav1.6 (Hamm et al., 2017). Thus, increasing levels of Nav1.1 in PV inhibitory interneurons promote gamma oscillation and consequently increase the PV inhibitory activity. The consequent observation is the inhibition of the hypersynchronization of neural network activity, reducing the epileptiform activity and improving the cognitive function (Verret et al., 2012). The following study observed the same effects with the transplant of interneurons overexpressing Nav1.1 into the cortex and hippocampus of hAPP-J20 mice (Martinez-Losa et al., 2018).

Curiously, the tau knockout in hAPP-J20 mice can inhibit epileptiform activity and improve learning and memory (Roberson et al., 2007, 2011). It has been hypothesized that this improvement could be due to the increase of inhibitory activity of GABA interneurons (Roberson et al., 2011). Besides the hippocampal interneurons' loss, tau pathology is implicated in the GABAergic dysfunction, leading to altered synaptic plasticity and severe memory deficits. Remarkably, these mice displayed increased long-term potentiation in hippocampal slices, which could be restored using a GABAAR agonist, zolpidem (Levenga et al., 2013). Moreover, accumulation of phosphorylated tau caused alterations in the metabolism of both excitatory glutamatergic and GABA inhibitory neurons, impairing the E/I balance and leading to abnormal neural network activity in the tauopathy model TauP301L (Nilsen et al., 2013) Finally, a recent study using triple transgenic mice (APPS/ tauP301L/PS1) showed that phosphorylated tau accumulates in PV and SST neurons, suggesting GABA inhibitory interneuron function is affected in AD (Zheng et al., 2020)

Taken together, the growing evidence streaming from the animal models research suggests that A β may cause interneuron loss, downregulate GABA interneurons activity and thereby lead to functional deficiencies in GABA inhibitory interneurons (Lerdkrai et al., 2018; Palop & Mucke, 2016; Verret et al., 2012). Differently, less is known about the impact of tau pathology in the GABAergic system. However, the results seem to point to interneurons loss and impairment caused by tau pathology, which might contribute to the alterations in the neuronal network activity (Levenga et al., 2013; Nilsen et al., 2013). Additionally, few studies found an accumulation of phosphorylated tau in interneurons, suggesting GABA inhibitory interneuron function is disturbed in AD (Soler et al., 2017; Zheng et al., 2020) Nevertheless, the latter is still a matter of debate, and further research is required.

Remarkably, several studies demonstrate that the rescue of GABAergic function seems to be necessary and sufficient to reduce and improve the neuronal network impairments and behavioural deficits. However, further investigation is required to clarify the mechanisms underpinning functional impairment mediated GABA inhibitory interneurons in particular during the progressing of tau pathology.

5. Experimental models

The use of experimental animal models is essential to understand AD pathogenesis and ultimately to perform preclinical testing of new potential therapeutics. The majority of animal models consists in transgenic mice. Different approaches have been used to develop transgenic models that mimic AD symptoms and pathological features.

5.1. *In vivo* - Animal models

To date, none of the existing transgenic mouse models recapitulates all the specific pathological features observed in AD. Regardless, the different animal models display some critical characteristics of the disease, which largely contribute to the study and the identification of mechanisms that underlie the development and progression of AD.

The majority of transgenic mice used in AD research express human APP and/or PS1/PS2 with familial AD mutations. These mice develop significant A β pathology consisting of a robust formation of plaques in brain regions usually implied in AD, such as the cortex and hippocampus. Additionally, these APP and/or PS mice exhibit synaptic dysfunction and moderate behavioural changes. On the other hand, these models often lack the presence of critical pathological features such as neuronal loss (although neuronal loss can be seen in

some models and of tau pathology, characterized by the development NFTs (reviewed in (Sasaguri et al., 2017).

Modelling the tau pathology found in AD is quite challenging since, up to the present, there are no MAPT mutations associated with AD. However, the mutations associated with FTDL are typically used to investigate tau-associated changes in tau in tauopathies. Still, they are also widely used to comprehend the development of AD-linked tau pathology.

The most generally used models are those that express human tau with the P301S (show progressive tau pathology similar to AD) and P301L mutations.

- P301L mutation: Different transgenic mouse models are overexpressing the P301L mutations. They can carry a transgene encoding the human tau isoform 4R0N with the P301L mutation which is the case of the models JNPL3(P3001L) (Lewis et al., 2000), TgTauEC (de Calignon et al., 2012b), rTg(tauP301L)4510 (SantaCruz et al., 2005; Gamache et al., 2019; Ramsden et al., 2005) or the isoform 4R2N such as Tau P301L (Terwel et al., 2005). Recently, using a virus approach, Cook and colleagues generate the TauP301L-AAV model. An adeno-associated viral (AAV) vector encoding human tau with the P301L mutation was injected into the ventricles of neonatal mice to create this model (Cook et al., 2015). All these models progressively develop NFTs. For instance, TgTauEC develops a stereotyped progression of tau pathology with ageing, starting in the EC and extended to the hippocampus (de Calignon et al., 2012a). Additionally, they also show tauopathy-related changes such as neuronal loss, astrogliosis, synaptic loss, changes in LTP/LTD and cognitive impairment.
- P301S mutation: These transgenic mouse models express the human tau isoform 4R0N with the mutation P301S.

The model hTau.P301S express the transgene under the control of the neuron-specific murine Thy-1 promoter and exhibit several features of human tauopathy (Allen et al., 2002a). These mice develop tau pathology during ageing and show accumulation of hyperphosphorylated tau and conformational changes that ultimately lead to NFTs formation in the cerebral cortex, hippocampus, brain stem, and spinal cord. Moreover, they also exhibit neurodegeneration especially in the spinal cord and cortical neurons as well (Hampton et al., 2010) followed by astrogliosis and microgliosis in the brain stem and spinal cord and memory deficits (Bellucci et al., 2004; Xu et al., 2014).

The model Tau P301S (Line PS19) express the mutated human tau under the direction of the mouse prion protein (Prnp) promoter. These mice display neuronal loss and brain atrophy by eight months, mainly in the hippocampus but also in the neocortex and entorhinal cortex. The NFTs are present in the neocortex, amygdala, hippocampus, brain stem, and spinal cord at six months and developed progressively. The NFTs formation is accompanied by microgliosis and astrocytosis (Yoshiyama et al., 2007). Additionally, synaptic loss occurs progressively from three to six months in CA3 region of the hippocampus as well as reduced LTP in CA1 region and spatial learning and memory impairments (Yoshiyama et al., 2007; Takeuchi et al., 2011; Lasagna-Reeves et al., 2016).

Interestingly, before the appearance of tau pathology, these mice reveal tau seeding activity, which means extracellular tau can elicit further tau aggregation possible through a prion-like mechanism (Holmes et al., 2014)

The seeding activity present in these animals is commonly used to accelerate the induction of tau pathology, both *in vitro* and *in vivo*, to investigate the mechanisms underlying the propagation/spreading of pathological tau (Colin et al., 2020). (Table 2).

Finally, there are a few studies using animal models that display both A β plaques and NFTs. These models count on the simultaneous expression of mutated forms of APP, MAPT and sometimes PS1 or PS2 (Bolmont et al., 2007; Grueninger et al., 2010; Lewis et al., 2001; Oddo et al., 2003; Ribé et al., 2005).

Among the models reported, the triple transgenic, 3xTg mouse model has been widely used in AD research. These mice show A β plaques formation in cortex and hippocampus and NFTs appearing first in the hippocampus and subsequently in the cortex. Additionally, 3xTg exhibit synaptic dysfunction and cognitive deficits can show some neuronal loss (Oddo et al., 2003; Saul et al., 2013). Although 3xTg mouse reproduces the key features of AD neuropathology, it has some limitations such as the production of mutated A β and tau, which are not representative of sAD.

5.2. *In vitro* - Cell cultures

In vitro cell cultures are used to model AD and other tauopathies. This *in vitro* approach consists of generating different cell-line-based disease models, such as primary neuronal cultures, non-neuronal cell lines and induced pluripotent stem cells (iPSCs).

These models allow the investigation of pathophysiological mechanisms at the cellular level. Among others, mechanisms such as tau aggregation, seeding and spreading of tau are commonly studied in primary neuronal cultures from transgenic mice. (Table 2).

Although the use of animal models provides reasonably consistent evidence, there is still a need in the research field for improved model systems that better reproduce the development of the disease in humans. However, it is especially challenging to generate human cell-based models given the lack of availability and the low quality of post-mortem tissue. The development of models using human embryonic kidney 293 (HEK 293) cells and the development of iPSCs addresses this limitation.

The HEK 293 cells are commonly used in tau aggregation assays studying the protein-protein interactions. This non-neuronal cell model exhibits ease of transfection, viability, and expresses high amounts of tau in a stable manner. Hence, it can reproduce three primary characteristics of all tauopathies, namely, accumulation, phosphorylation, and aggregation of tau.

Different cell types from human donors, including fibroblasts, blood cells and epithelial cells, are used to generate iPSCs. These cells are indistinguishable from embryonic stem cells. They show the ability to maintain a stable karyotype, express the pluripotency of the genes and more importantly, they can be differentiated into any cell type of interest, including several subtypes of neurons. Thus, iPSCs are an essential tool to investigate disease mechanisms (Tao & Zhang, 2016). The iPSC-derived neuronal cultures display a genetic predisposition to AD (Penney et al., 2020).

Since the first documented generation of iPSCs (K. Takahashi et al., 2007) multiple groups used this technology to developed *in vitro* models of AD and FTD using cells from patients (Almeida et al., 2012; Israel et al., 2012; Muratore et al., 2014; Nierode et al., 2016; Yagi et al., 2011).

The primary advantage of *in vitro* models is that they can be maintained in a completely controlled environment, which allows the study of specific cellular and molecular pathways at the high spatiotemporal resolution, in shortened experimental timescales. Moreover, the iPSC-neurons constitute a potential tool for studies of toxicity or for drug screening that may highlight possible candidate molecules for new therapeutics, although caution should be exerted while using these models (reviewed in (Arber et al., 2017)). However, cells growing *in vitro* should not be considered as the exact dissociated replicates of their *in vivo* counterparts.

For instance, the models using human cell-culture present some limitations, including the lack of standardized protocols to generate and maintain the cell lines after reprogramming, the

variability and epigenetic modifications from the donor cells. One of the major drawbacks is that *in vitro* conditions do not represent precisely the brain environment. For instance, the absence of other types of neuronal cells than neurons might have a critical role in the development of the disease (REF).

Overall, the *in vitro* models permit a different kind of studies, but the isolated cells do not reflect the organism physiology given the lack of contacts between cells (Humpel, 2015).

5.3. Ex vivo - Organotypic slices

5.3.1. Background

The first detailed description of organotypic brain slice cultures dates back to the early 1970s (Hauw et al., 1972; Wolf, 1970). Nevertheless, it was in the 1980s, with the implementation of the roller-tube method, that they became an established model to study physiological and developmental aspects of the brain (Gähwiler, 1981). However, this technique does not maintain the cytoarchitecture of the tissue; thereby, this limitation led to the development of the membrane interface-slice culture method one decade later (Stoppini et al., 1991).

This method consists of slices culture at the interface between a culture medium and a CO₂-enriched atmosphere in a petri dish. The explant tissue is placed on a sterile and porous membrane, and through capillary action, the slices receive nutrition from the culture medium. The organotypic slices preserve well the organization of the tissue and maintain the thickness of a few cell layers (Stoppini et al., 1991).

The slices generated through this technique have begun to be widely used in neuroscience due to their numerous advantages (Norberg et al., 2005). In neurodegenerative disorders investigation, the hippocampus is the brain region that is most frequently cultured. As already described before, the hippocampus is classically characterized by neuronal loss and affected by the pathologies associated with the neurodegenerative diseases. Besides the hippocampus, several brain regions have been kept in culture, such as the cortex, cerebellum, and thalamus (Bahr, 1995; Beach et al., 1982).

Technically, it is feasible to generate cultures from donors at different ages; however, the age of the donors largely contributes to the quality and survival of the slices. Hence, the organotypic slice cultures are usually prepared from mice or rats up to postnatal day 12. At this age, the brain is easier to manipulate and dissect, the cytoarchitecture is established, and

neuronal cells have a better morphology, and the chances of survival are increased. (Bahr, 1995; Plenz & Kitai, 1996).

Contrary to the postnatal donors, there is not much work published in adult organotypic slices. The few that exist investigate, mostly, acute processes in very short-living adult slices since the neuronal cells within these slices have a low survival rate (Lossi et al., 2009). Nonetheless, some modifications of the old-fashioned protocols have been made, such as culturing at lower temperatures, different culture mediums, and reduce the thickness of slices, to overcome these limitations. Still, the extensive cell loss seems to be persistent (Daria et al., 2017; Humpel, 2015; Xiang et al., 2000). Some studies also reported the development of cultures from human brain biopsies or post-mortem brains (Eugène et al., 2014; Verwer et al., 2003). Recently, a study demonstrates the ability to preserve the complex neuronal cytoarchitecture as well as electrophysiological properties of human pyramidal neurons in long-term brain slice cultures derived from neurosurgical resections (Schwarz et al., 2019). As an experimental model, these cultures present their limitations, including, the availability of tissue and the post-mortem delay that might compromise the viability of the culture.

5.3.2. Advantages and limitations of organotypic slices cultures

Organotypic slice cultures are a well-established *ex vivo* model which maintain the three-dimensional organization in culture. They preserve the anatomy, almost intact, from the explanted region and can be kept in culture for long periods. Also, neuronal and non-neuronal cells remain in the organotypic slices in a representative brain environment. Various studies described that the different cell populations found *in vivo* are also observed in organotypic slices (Beach et al., 1982; Staal et al., 2011; Stoppini et al., 1991), which offers a significant advantage over dissociated cultures, primary cell lines and iPSCs. This evidence is particularly relevant in the study of most neurodegenerative diseases where the interaction between the different cell types might be implicated in the pathophysiological processes. For instance, these cultures contain glial cells (Daria et al., 2017; del Rio et al., 1991; Hailer et al., 1996) and vascular cells (Humpel, 2015; Hutter-Schmid et al., 2015) in similar proportions as encountered *in vivo*.

Another advantage of the *ex vivo* model is the ability to develop cells and synapses, which mimics the development of the brain *in vivo*. In organotypic hippocampal slice cultures, the neurons are morphologically and functionally comparable to the *in vivo* acute preparations. The neurons maintain their intact neuronal function and typical hippocampal neural circuitry (Finley et al., 2004a). Likewise, the electrical properties and synapses mature in culture are also similar to those found in acute slices (De Simoni et al., 2003; Bahr, 1995).

Lastly, organotypic slice cultures can be easily manipulated offering an opportunity for several applications, such as multi-electrophysiological recordings and stimulations (Dong & Buonomano, 2005; Egert et al., 1998; Jahnsen et al., 1999; Karpiak & Plenz, 2002) gene transfer techniques (Ridoux et al., 1995; Thomas et al., 1998; Murphy & Messer, 2001), or long-term live imaging (Gogolla et al., 2006). They can be used for immunohistochemically stained after PFA fixation and analysed using all typical molecular biology techniques such as westerblot, ELISAs, RT-PCR, HPLC and so forth. Besides, they are a useful platform for drug testing or perfusion of antibodies or other approaches, providing valuable insights on target binding in the brain. Thus, techniques that sometimes can be difficult to perform *in vivo* can be applied in organotypic slices cultures.

Despite the mentioned advantages, this *ex vivo* model has some limitations in the study of neurodegenerative diseases (Table 2). The majority of brain slices models are produced from the transgenic mice models and, therefore, have similar constraints. Usually, these *ex vivo* models overexpress wild-type or human mutant genes which facilitates the development of the disease. Also, the seeding of the slices, with proteinopathic seeds, is used to drive pathology in these models. Both overexpression and seeding constitute a limitation in the sense that they are considered non-physiological, which should be taken into account in the interpretation of the results (Croft et al., 2019) Concerning the use of organotypic slices as a platform for drug testing, it is clear that this model confers significant advantages. However, they are low throughput, and it must be acknowledged that they cannot model blood-brain-barrier. Hippocampal organotypic slices also account with a further limitation which is the axotomy that they suffer during the preparation. Indeed, some of the neurons in cultured slices maintained their axonal connections within the tissue slice; however, others lose regular afferent connection with other regions in the brain. Nevertheless, during the culture, the cut axons can regrow again, create new synaptic connections and restore the network (N. Takahashi et al., 2010).

5.4. Modelling tau pathology in organotypic slices

AD-linked tauopathy is strongly associated with dementia and neuronal loss. The development of *in vitro* models which recapitulates its main pathological features is essential to understand the mechanisms underlying the development of tau pathology or tau-targeting therapies.

The different reports concerning organotypic slices models of tau pathology use mainly slices generated from postnatal tau transgenic mice expressing familial mutations in MAPT or viral approaches to express mutant MAPT.

Organotypic slice cultures prepared from JNPL3 mice show conformationally altered and phosphorylated tau after two weeks *in vitro* (Duff et al., 2002). In the hippocampal slices obtained from the transgenic mice overexpressing P301S mutations (PS19), applying synthetic tau fibrils to the culture induce tau aggregation in hippocampal CA1 neurons ten days after seeding (Stancu et al., 2015). *In vivo*, both JNPL3 (Lewis et al., 2000) and P301S (Yoshiyama et al., 2007) transgenic mice display phosphorylated and conformationally altered tau at five and six months of age, respectively.

These models recapitulate *in vitro* the critical features of tau pathology, highlighting their applicability for investigating disease-associated changes in tau such as tau aggregation, spreading and propagation.

Likewise, organotypic slice cultures from mice expressing pro-aggregant mutation, TauRDΔK, exhibit accumulation of phosphorylated tau and thioflavin-S positive tau after twenty-five days *in vitro*. This model is might a suitable option to screen for anti-aggregation therapeutics (Messing et al., 2013).

Alternative models of the slices from transgenic mice are the *ex vivo* models using viral approaches. In this case, the brain slices are transduced with viral constructions to express mutant MAPT, which reproduce the accumulation of phosphorylated tau and in long-term culture leads to cell loss. This approach allows comparing different MAPT mutations (Croft et al., 2019). Although less explored, organotypic hippocampal slices can offer an opportunity to study functional aspects of the disease. They provide a less complex environment than the whole brain, however, some reports demonstrated that neuronal network within the brain organotypic slices emits *in vivo*-like spontaneous activity (Finley et al., 2004b; Okamoto et al., 2014).

In summary, organotypic slices could be considered as an alternative to some *in vivo* models of AD and other tauopathies research. Also, their usage in neurodegeneration studies reduce the number of animals and avoid the necessity to age multiple animals. Thus, organotypic brain slices cultures are a complex system that comprises several advantages as a model to study neurodegenerative disease as AD and tauopathies. Notably, their use reduces the number of animal experiments and contributes to the practice of the 3Rs, reduce, refine, replace.

Table 2. Some examples of *in vivo*; *in vitro* and *ex vivo* models modelling tau pathology.

Model (Tau protein expressed)	Seed used	Injection age/Seeding	Culture type	Results
<i>In vivo</i>				
TauP301L (P301L)	Synthetic fibrils K18-P301L	3 months		Injection resulted in phosphorylated tau (Peeraer E, Bottelbergs A, 2015).
hTau P301S (P301S)	Brain extracts from hTau P301S 5months mice	2 months		Tau aggregation in hippocampus 6 months post-injection (Clavaguera F, Akatsu H, 2013)
PS19 (P301S)	Synthetic fibrils K18-P301L-myc tagged	3 months		Tau aggregation in synaptically connected regions
<i>In vitro</i>				
PS19 (P301S)	Tau2N4R-P301S-myc; K18-P301L-myc	6 DIV	Primary cultures neurons from P301S	Tau aggregation induced by K18-P301L 18 days after seeding (Guo JL, Lee VMY, 2013)
P301S	Brain lysates from AD patients at Braak stage I-II, III-IV or V-VI		HEK293T	Overall seeding increased with Braak staging of the lysate (Furman JL, Vaquer-Alicea, 2017)
<i>Ex vivo</i>				
PS19 (P301S)	Synthetic fibrils K18-P301L-myc tagged	DIV 3 and DIV6	Slice culture	Tau aggregation 10 days after seeding (Stancu 2015)
Tau(RD) Δ K proaggregant mutation			Slice culture	Tau aggregation 28 DIV (Messing, 2013)
Slices transduced with rAAV2/8-P301L			Slice culture	Tau aggregation at 28 DIV (L. Crof 2019)

Objectives

In this work, we proposed to develop a physiologically relevant *ex vivo* model of AD-linked tauopathy, based on organotypic hippocampal slices, for the investigation at the proper spatiotemporal resolution of progressive impairments in neuronal activity. The work was performed in collaboration with Janssen Pharmaceuticals, as part of a European Consortium, Synaptic Dysfunction in Alzheimer Disease (SyDAD) - European Training Network sponsored by Horizon 2020 Marie Skłodowska Curie.

The main objectives and aims of this project are:

1. Development of an *in vitro* model of AD-linked tau pathology using organotypic slices:

1.1 Establish the optimal conditions to implement the model in organotypic hippocampal slices.

1.2 Characterize the progressive development of tau pathology in cultured P301S organotypic hippocampal slices and compare with *in vivo* phenotype.

1.3 Identify the cell-specificity to tau pathology in cultured P301S organotypic hippocampal slices and compare with *in vivo* phenotype.

2. Characterize the mechanisms underlying the altered neuronal activity.

2.1 Characterize the neuronal activity exhibited at early and late stages of the disease in cultured P301S organotypic hippocampal slices with respect to WT slice cultures

2.2 Investigate whether the GABAergic system might be compromised at late stages of tau pathology.

2.3 Evaluate the neuronal loss in CA3 during the progression of tau pathology

Materials and Methods

1. Animals

Ethical approval

All experiments were approved by the Ethical Committee #50 and the French Ministry for Education and Research (N° saisine éthique: 10137). The animals were obtained and cared for according to the regulations of the University of Bordeaux/CNRS Animal Care and Use Committee.

1.1. Mice TauP301S (PS19 line)

TauP301S transgenic mice (Yoshiyama et al., 2007) and their WT littermates (B6C3) were used in this study. Heterozygous males TauP301S were first purchased from Jackson Laboratory and backcrossed with B6C3 WT mice produced in house (Pole in vivo – IINS, Bordeaux). For a small group of experiments, they were bred to homozygosity (mating system: Heterozygous x Heterozygous). TauP301S expresses mutated human tau, P301S (4R1N) driven by the mouse prion protein promoter (Prnp). The mice were genotyped at P0/P1 the local animal facility (Transcriptomics Platform, Neurocentre Magendie, Bordeaux). The experiments were performed in animals at P6-P7 for organotypic slice cultures and P22, 6, 8 and 10 months for acute slices.

2. Organotypic hippocampal slice culture

In this study, organotypic hippocampal slice cultures were prepared from P301S and WT pups at postnatal day 6 or 7 (P6-P7) using the membrane interface-slice culture method (Stoppini et al., 1991). All the procedures were performed under sterile conditions.

Materials:

Dishes Ø 35mm (Falcon, ref. 353001)

Membrane (Merck Millipore, ref. FHLC04700)

6 well-plates (Falcon, ref. 353046)

Culture inserts (Merck Millipore, ref. PICM03050)

Mcllwain Tissue Chopper, Standard Table, 220V (ref. 10180-220)

Reagents:

Table 1 Composition of cultures media.

Medium	Reagents
Dissection medium	Gey's Balanced Salt Solution (GBSS) (Sigma, ref. G9779), Sodium pyruvate (Sigma, ref. P4562) (10 mM) D-Glucose (45%- 2,5M) (Sigma, ref. G8769) (25 mM) Ascorbic acid (Sigma, ref. A92902) (0,1 mM) Vitamin E ((±)-α-Tocopherol) (Sigma, ref. T3251) (440 µM) Hepes (1 M) (Gibco, ref. 15630-056) (10 mM)
Culture medium	Hanks' Balanced Salt Solution (HBSS) (Gibco, ref 24020) Opti-Mem (Gibco, ref. 31985) D-Glucose (45%- 2,5M) (Sigma, ref. G8769) (25 mM) Sodium pyruvate (Sigma, ref. P4562) (10 mM) Ascorbic acid (Sigma, ref. A92902) (50 µM) Vitamin E ((±)-α-Tocopherol) (Sigma, ref. T3251) (440 µM) Horse serum - heat-inactivated (Gibco, ref. 26050088) 25%
Neurobasal medium	Neurobasal A (Gibco, ref. 12349) Glutamine 200mM (Sigma, ref. G7513) (1 mM) Sodium pyruvate (Sigma, ref. P4562) (10 mM) Ascorbic acid (Sigma, ref. A92902) (50 µM) Vitamin E ((±)-α-Tocopherol) (Sigma, ref. T3251) (440 µM) B-27™ Supplement (50X), serum-free (Gibco, ref. 17504044) 2% Horse serum, heat-inactivated (Gibco, ref. 26050088) 14%

Preparation of organotypic hippocampal slice cultures:

Pups were sacrificed by decapitation and the head flushed with 70% ethanol. Brain extraction was performed by an incision into the skin along the head's midline, and the skin was removed. The skull was cut from the foramen magnum along the midline, following two lateral cuts, starting from the midline towards the sides. The skull was removed and the brain carefully placed in a petri dish with dissection media (A). The two hemispheres were separated, exposing the hippocampus and connecting areas (B). The hippocampus was dissected and placed it the plastic platform on the tissue chopper and chopped into 310 µM thick transverse sections (C). The freshly cut sections were immediately transferred to a dish with cold dissection medium (D). About 8 and 10 hippocampal slices were selected to culture (E). The slices were plated onto the inter-face culture inserts, which were placed in a 6-well plate containing pre-heated culture medium (F). Organotypic slices were incubated at 34°C and

5%CO₂. The culture medium was changed from the bottom of each well every 2 days until the experiment was performed.

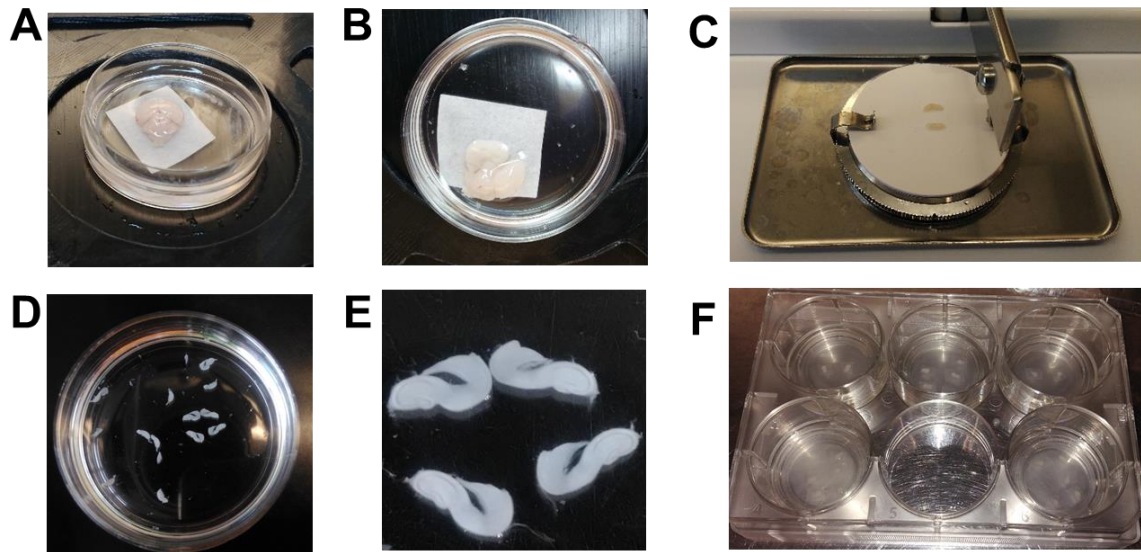


Figure 1. Different steps in the preparation of organotypic hippocampal slice cultures using the membrane interface-slice culture method.

3. Generation of tau synthetic fibrils (Tau seeds K18-P301L)

The tau synthetic fibrils or tau seeds (K18) were generated based on the protocol published by (Guo & Lee, 2011a). Some slight modifications were made, namely concerning the incubation time and equipment to sonicate. The plasmid CN998-MAPT-P301L-myc was kindly provided by Janssen Pharmaceuticals.

Production of tau recombinant protein, K18-P301L-myc

The recombinant tau was purchased from Tebu-bio (Le Perray-en-Yvelines, France). The human tau fragment, construct K18; residues Q244-E372 of 2N4R with the P301L mutation, C-terminally myc-tagged were produced in *Escherichia coli* (bacterial strain, BL21 DE3 codon plus RIL competent cells). The concentration of the delivered protein was ~1.38 mg/mL in 50mM Ammonium acetate (pH 7.0).

Generation of tau seeds K18-P301L-myc

Materials:

Ultracentrifuge Optima MAX XP Beckman Coulter (rotor TLA100.3)

PC centrifuge tubes; 13x51mm (Beckman Coulter, ref. 349622)

Bath sonicator Advantage-Lab - Typ AL 04-02 (U - 220-240 v; P - 90W; F - 50/60 hZ)

Pierce BCA Protein Assay Kit (ThermoFisher Scientific, ref. 23225)

Reagents

Sodium Acetate (Sigma, ref. S2889)

Heparin sodium salt, porcine (MP Biomedicals, ref. 194114) Average molecular weight: ~3000 g/mol; Anti-Xa > 65 IU/mg

DTT (1M) (Invitrogen, ref. P2325)

K18-P301L C-terminal Myc-tagged (concentration ~1.38 ml/mL); MW = 15 kDa (K18-myc)

A solution containing K18-myc (0.6 mg/ml), heparin (40 µM), DTT (2 mM) and sodium acetate (100 mM - pH 7.0) was prepared. The solution was incubated at 37°C for 10 days in the presence of heparin. Following centrifugation 100,000g for 1 h at 4 °C, the pellet was resuspended in PBS. The solution was sonicated in the water bath sonicator for 30 minutes, aliquoted and stored at -80.

The final concentration of K18-myc was measured using the protein quantitation assay, the Pierce BCA Protein Assay Kit. The BCA kit is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colourimetric detection and quantitation of total protein. The method is based on the reduction of cupric ion Cu^{+2} to Cu^{+1} by protein in an alkaline medium. The colourimetric detection of the cuprous cation (Cu^{+1}) occurs using a reagent containing bicinchoninic acid (BCA). The complex exhibits a strong absorbance at 562 nm. Protein concentration was determined with reference to standards of the protein bovine serum albumin (BSA). The absorbance reading of BCA was performed using the plate reader POLARstar Omega (BMG Labtech) at wavelength 562nm.

Note: Tau seeds K18-P301L-HA (K18-HA) and K18-P301L (K18-NoTag) were produced at Janssen Pharmaceutical following the protocol described above.

3.1. ThioflavinT assay

Materials:

Plate reader POLARstar Omega (BMG Labtech)

Black 96 well clear bottom plate (Greiner Bio-one ref. 655090)

Reagents:

Thioflavin T in PBS (1mM) (Sigma, ref. T3516-25G)

K18-HA (~0.5mg/mL)

K18-myc (~0.5mg/mL)

K18-noTag (~0.5mg/mL)

Tau fibrillisation of K18-myc, K18-HA and K18-noTag was confirmed and quantified using Thioflavin T (ThioT) assay. ThioT is a dye which binds the fibrils presenting a β -structure and emits strong fluorescence at approximately 482 nm when excited at 450 nm.

The fluorescence was measured by the plate reader POLARstar Omega with filters combination: excitation 410 nm and emission 485 nm; gain 2000.

Note: This experiment was also performed at Janssen Pharmaceuticals following the same protocol but using different equipment (Fluoroskan Ascent FL, filters combination: excitation 440 nm and emission 480 nm).

3.2. NanoBRET™ Protein: Protein Interaction system

Note: This experiment was performed at Janssen Pharmaceuticals. The NanoLuc (NL) donor and HaloTag (HT) acceptor tags were fused with full-length Tau2N4R with P301L mutation cell. The stable transfection was performed, and the cell line was kept for several passages.

Assay principle

NanoBRET (bioluminescence resonance energy transfer) is a Protein: Protein Interaction proximity-based assay. The protein interactions are detected by measuring energy transfer from a bioluminescent protein donor to a fluorescent protein acceptor. The NanoLuc fusion protein is used as the energy donor and a fluorescent HaloTag fusion protein as the energy acceptor. (Figure 2.2 A). The bioluminescence of NanoLuc is blue-shifted and HaloTag acceptor red-shifted which minimises spectral overlap within the assay, resulting in an improved signal: background ratio (Figure 2.2 B).

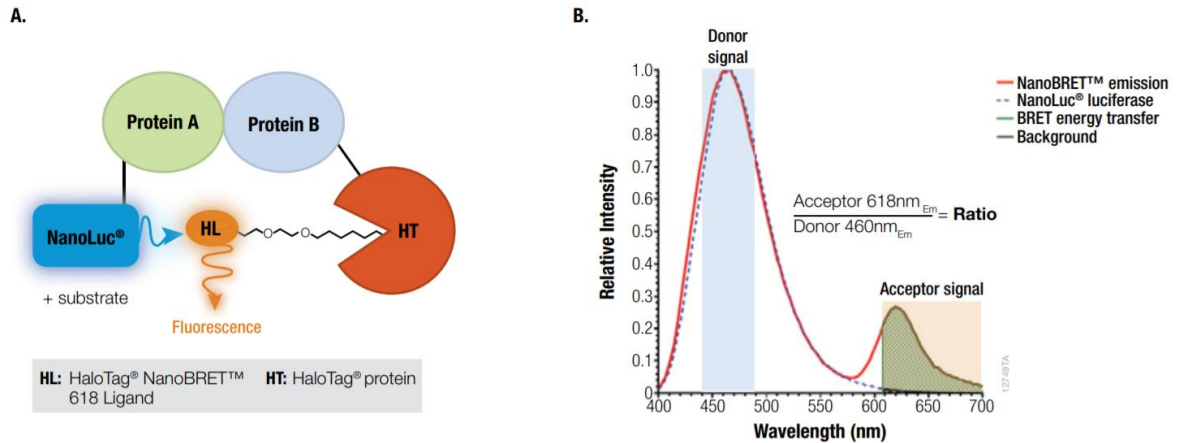


Figure 2. The principal of NanoBRET assay. **Panel A)** Illustration of energy transfer from a NanoLuc-Protein A fusion (energy donor) to a fluorescence labelled HaloTag-Protein B fusion (energy acceptor) upon the interaction of Protein A and Protein B. **Panel B)** Separation of the NanoLuc emission spectrum (460nm) and the fluorescent HaloTag NanoBRET ligand emission spectrum (618nm and the calculation of NanoBRET ratio. (Note: in this assay Protein A and B are both Tau-P301L). (Adapted from the technical manual Promega NanoBRET™ Nano-Glo® Detection System (N1662)).

Materials:

Promega NanoBRET™ Nano-Glo® Detection System (N1662)

NanoBRET™ Nano-Glo® Substrate

HaloTag® NanoBRET™ 618 Ligand

96 Well Solid White Flat Bottom Polystyrene TC-Treated Microplates (Sigma, ref. CLS3917)

EnVision™ Multilabel Reader (PerkinElmer)

Reagents:

Table 2 Composition of cell culture media and reagents.

Cell culture media	Dulbecco's Modified Eagle Medium (DMEM) (Lonza, ref. BE12-917F) Sodium pyruvate (Gibco, ref 11360-039) 1% PenStrep (Gibco, ref. 15140-122) 1% Gentamicin 0.04% Ala-Glutamine 2 mM Blasticidin 5 µg/mL Zeocin 200 µg/mL Hygromycin 200 µg/mL Horse serum 10%
Assay medium	Opti-MEM I Reduced-Serum Medium (Thermofisher Scientific, ref. 11058021) Fetal bovine serum 4%
	Trypsine-EDTA (0,05 %) (Sigma, ref. 25300062)
	Dulbecco's phosphate-buffered saline (DPBS) (Gibco, ref. 14190144)

Cell culture preparation:

The cell culture was incubated with trypsin-EDTA at room temperature until cells lift from the bottom. The culture medium was added to neutralise trypsin, and the cells were collected, resuspended and transferred to a 15ml conical tube. Following centrifugation at 125 × g for 5 minutes, the cell culture medium was discarded, and the cells were resuspended in an equal volume of assay medium. The cell density was estimated and adjusted to 2.2 × 10⁵ cells/ml. The cells were divided into two pools, and add HaloTag® NanoBRET™ 618 Ligand or DMSO vehicle as follows:

- Experimental samples (**+ ligand**): Add HaloTag® NanoBRET™ 618 Ligand (0.1 mM) to a final concentration 100 nM
- No-acceptor controls (**- ligand**): Add DMSO to a final concentration of 0.1% DMSO.

The cells were plated in a 96-well plate (~ 19,800 cells per well). Finally, the cells were seeded with K18 fibrils or vehicle and incubated at 37°C, 5% CO₂ for 72 hours.

Adding NanoBRET Substrate and NanoBRET measurements:

To generate the donor signal, NanoBRET Nano-Glo Substrate was diluted in cultures medium (1:100) and added into each well. The measurement of donor emission (460 nm) and acceptor emission (618 nm) was performed within 10 minutes of substrate addition using a PerkinElmer EnVision Multilabel Reader. The filters combination was: 585 Long Pass filter (acceptor channel) and 450/80 Band Pass filter (donor channel) with an integration time of 0.5 sec per well and channel.

NanoBRET Calculations:

First, the NanoBRET raw ratio was calculated dividing acceptor emission value (618 nm) by the donor emission value (460 nm) for each sample

$$\frac{618\text{nm}_{\text{Em}}}{460\text{nm}_{\text{Em}}} = \text{Raw NanoBRET}^{\text{TM}} \text{ Ratio} = \text{BU}$$

The raw NanoBRETTM units (BU) were converted to milliBRET units (mBU) multiplying each raw BRET value by 1,000.

$$\frac{618\text{nm}_{\text{Em}}}{460\text{nm}_{\text{Em}}} = \text{BU} \times 1,000 = \text{mBU}$$

Finally, the mean NanoBRET ratio for each set of samples: Experimental samples with HaloTag NanoBRET 618 Ligand and no-acceptor control samples was determined. To remove the background and the bleed-through value of the donor, the mean value of a suspension of cells without ligand was subtracted to the experimental mean, giving the corrected NanoBRET ratio.

$$\text{Mean mBU experimental} - \text{Mean mBU no-ligand control} = \text{Mean corrected mBU}$$

2.2.4 Seeding and viral infection of organotypic slices

Virus:

AAV1.hSyn.GCaMP6f.WPRE.SV40 (Titer $1.8E+13$) (Addgene Plasmid #100837):

Adeno-associated virus expressing the protein calcium sensor (GCaMP6f) under the control of neuron specific synapsin promoter. The virus was stored at -80°C and thawed just before use and kept on ice.

AAV9.mDLX.EGFP (Titer $2.2E+13$) (Addgene Plasmid #83900)

Adeno-associated virus expressing the green fluorescence protein (GFP) under the control of the mDlx enhancer element, which is specific to interneurons (Dimidschstein et al., 2016). The virus was stored at -80°C and thawed just before use and kept on ice.

The tau seeds were sonicated in the water bath for 45 minutes before use. The seeding was performed sequentially at 3-4 DIV and 6-7 DIV. Tau seeds ($1\ \mu\text{L}$; $\sim 1\text{mg/mL}$) was gently added on top of hippocampal slices (Stancu et al., 2015).

For the experiments using virus approaches, the slices were infected along with the seeding. The virus was diluted in tau-seeds solution or PBS, $1\ \mu\text{l}$ (1:10/1:15 dilution) was added on a membrane which was then carefully placed on top of the slice. The membranes were removed 14 hours later.

4. Immunohistochemistry

Reagents

Table 3. Composition of the solution used in the immunolabeling

ACSF for staining	Glucose (20 mM) CaCl ₂ (2,3 mM) MgCl ₂ (1,3 mM) NaCl (112 mM) KCl (2,5 mM) Hepes (10 mM) Na pyruvate (5 mM) L-Ascorbic acid (0,05 mM)
Fixation solution	Paraformaldehyde (PFA) (Sigma, ref. 158127) - 4% (v/v) - old animals Paraformaldehyde (PFA), EM Grade, Purified (EMS, ref. 30525-89-4) 4% (v/v) in ACSF - organotypic slices animals
Permeabilisation solution	Triton X-100 (Sigma, ref. T9284) 1% (v/v) in PBS
Washing solution (PBS-Tween)	Tween 20 (Sigma, ref. P9416) 0.1% (v/v) in PBS
Blocking solution	Normal Goat Serum (NGS) (Thermofisher Scientific, ref. 31872) 5% (v/v) in PBS-Tween
Antibody solution	NGS 3.5% (v/v) in PBS-Tween
ProLong™ Diamond Antifade Mountant (Thermofisher Scientific, ref. P36961)	

Table 4. Antibodies used for immunostaining.

Antibody	Specificity	Host	Isotype	Origin (Reference; stock concentration)	Dilution	
					Organotypic slices	Acute slices
AT8	Tau phosphorylated at Ser202 and Thr205	Mouse monoclonal	IgG1	Thermofisher ref. MN1020 (200µg/mL)	1:200	1:200
MC1	Conformation dependent tau antibody	Mouse monoclonal	IgG1	Kind gift from Peter Davies	1:2 (TC supernatant)	
PHF1	Tau phosphorylated at Ser396 and 404	Mouse monoclonal	IgG1	Kind gift from Peter Davies	1:2 (TC supernatant)	
CB28	Calbindin D28K calcium-binding protein	Rabbit polyclonal		SynapticSystem, ref. 214 002 (1mg/mL)	1:600	1:1000
SPO	Synaptoporin-synaptic vesicle protein	Rabbit polyclonal		Merck, ref. ABN78 (1v/1v)	1:500	
NeuN	Neuron-specific nuclear protein	Rabbit polyclonal		Merck, ref. ABN78 (1mg/mL)	1:1000	1:1000
GFAP	Glial Fibrillary Acidic Protein	Chicken polyclonal		Abcam, ref. ab4674	1:1000	1:1000
Iba1	Microglia	Rabbit polyclonal		Wako, ref. 019-19741 (500µg/mL)	1:200	1:500
PV	Parvalbumin - calcium binding protein	Guinea pig polyclonal		SynapticSystem, ref. 195 004 (1v/1v)	1:1000	1:1000

4.1. Old animals

Aged P301S and WT were perfused with 0.9% saline, followed by perfusion of 4% PFA. Brains were fixed in 4% PFA for 24h at 4°C. Next day, brains were transferred to PBS until the brains were sliced as consecutive serial coronal sections (5 series, 50 µm thickness) on a vibratome. For the immunostaining, the slices were first permeabilised with 1% Triton for 2h at RT. Following the block with NGS 5%, the primary antibodies were diluted in antibody solution at concentration mentioned in table 2.4, added to the slices and incubated overnight at 4 °C. Except for the antibody anti-Iba1, the antibodies were incubated together according to the possible combinations taking into account the host species. The slices were rinsed for 10min with washing solution, PBS-Tween 0.1% for 3 times and kept in it. Then, the secondary antibody was diluted in antibody solution at the concentration of 1:500, added to the slices and incubated for 2h at RT. The slices were rinsed for 10 min in PBS-Tween 0.1% for 3 times and incubated with DAPI for 20min The slices were rinsed for 10 min in PBS for 5min and mounted with Prolong Diamond mounting media on glass slides and coverslips for fluorescence microscopy.

4.2. Organotypic slices

Organotypic were fixed for 45min in PFA 4%. For the immunostaining, the slices were first rinsed in for 10min with PBS for 3 times. The permeabilisation was performed with 1% Triton for 2h at RT or overnight 4°C for some antibodies. Following the block with NGS 5%, the primary antibodies were diluted in the antibody solution at concentration mentioned in table 2.4, added to the slices and incubated overnight at 4 °C. Except for the antibody anti-Iba1, the antibodies were incubated together according to the possible combinations taking into account the host species. The slices were rinsed for 10min with washing solution, PBS-Tween 0.1% for 3 times and kept in it. Then, the secondary antibody was diluted in antibody solution at the concentration of 1:500, added to the slices and incubated for 2h at RT. The slices were rinsed for 10 min in PBS-Tween 0.1% for 3 times and incubated with DAPI for 20min The slices were rinsed for 10 min in PBS for 5min and mounted with Prolong Diamond mounting media on glass slides and coverslips for fluorescence microscopy.

4.3. Imaging and image analysis

The organotypic slices and the brain sections of adult animals were imaged on Leica DM6 CFS TCS SP8 upright microscope at Bordeaux Imaging Center (BIC, Bordeaux).

Confocal Z-series stacks using a HCX PL APO 20x 0.7 multi immersion objective (Tile Scan acquisition mode) and HCX PL APO 40x NA 1.3 Oil immersion objective were acquired. The 488nm, 561nm and 633nm laser lines were used, and spectral detection was adjusted for the emission of the Alexa488 (500-550nm), Alexa 594 (600-640nm) and Alexa 647 (660-700nm) fluorochromes, respectively, using PMT detectors.

The image analysis was performed using Fiji software.

AT8 quantification

Confocal Z-series stacks using 40x objective were acquired in 3 different subsequent areas covering either the region CA1 or CA3 of the hippocampus. The analysis was performed in 3- μ m thick stacks using a custom made algorithm. Briefly, the region of interest (CA1 and CA3) was outlined and tau AT8 pathology was analysed by brightness thresholding. In CA3 the AT8 signal was quantified in mossy fiber bundle using a mask for CB28 staining and detecting the overlap with AT8. The area covered by AT8 and CB28 were reported as a percentage of the total area analysed.

MC1 quantification

Confocal Z-series stack of the whole organotypic slice was acquired using 20x objective. The analysis of CA1 and CA3 regions was performed in the 3- μ m thick stack using a custom-made algorithm. Briefly, the region of interest (CA1 and CA3) was outlined, and the area of MC1 immunoreactive signal was analysed by brightness thresholding.

NeuN quantification

Confocal Z-series stacks using 40x objective were acquired in 3 different subsequent areas cover the regions CA3 of the hippocampus. The analysis was performed in 3- μ m thick using a custom-made algorithm (F. P. Cordelières, BIC, Bordeaux). Briefly, the region of interest (CA3) was outlined, and the cells within the region were segmented using Find Maxima plugin. Find maxima determines the local maxima in an image and creates a binary image of the same size of the segmented object per maximum find maxima found. The plugin 3D Objects Counter (Bolte & Cordelières, 2006) was used to count the cells (objects).

mDLx and PV quantification

Confocal Z-series stack using 20x objective was acquired the whole organotypic slice. The analysis of the CA3 region was performed in the 3- μ m thick stack using an automatic method. Briefly, the region of interest (CA3) was outlined, and the cells within the region were segmented and counted using the Find Maxima plugin (explained above).

5. Westernblot

Protein quantification- sample preparation

The samples consisted of 4 slices per condition. A 2% SDS buffer (Laemli buffer) has been tested and chosen as the extraction buffer. Protease and phosphatase inhibitor cocktails (Sigma Aldrich, ref. 539134) were added according to the manufacturer instructions. The brain slices and the extraction buffer were added to a screw-cap tube (pre-cooled) containing lysing matrix beads for extraction using a FastPrep24 (MP Biomedical) apparatus. After sample homogenisation, samples were spun down at 4°C at 10,000 g for 10 minutes. The supernatant was recovered, and proteins dosed using the BCA method (microplate version, ThermoFisher Scientific, ref. 23225). For gel loading, 5% beta-mercaptoethanol and 0.02% bromophenol blue were added freshly. Samples were heated at 90C for 2 min.

Immunoblotting

Proteins were separated by SDS-PAGE using a pre-cast 4-15% gradient gel based on Tris-Glycine buffer (Mini-PROTEAN, BioRad Laboratories). The running buffer was also based on Tris-Glycine and samples were separated using constant voltage (120 V) until the dye front had migrated the entire gel. In each gel, at least one lane was loaded with pre-stained molecular weight standards (PageRuler prestained protein ladder – ThermoFisher Scientific ref. 26619). Sample lanes were loaded with 20 µg of total protein. After SDS-PAGE separation, proteins were transferred to a 0.2 µM nitrocellulose membrane using a Trans-Blot Turbo Transfer System (Biorad Laboratories). Immediately after the transfer, the membrane was rinsed with TBS buffer, and both protein loading and transfer efficiency were verified using reversible total protein fluorescence staining according to the manufacturer instructions (Revert 700 Total Protein stain; Li-Cor Biosciences ref. 926-11010). The fluorescent image of the total protein staining (680 nm channel; Odyssey Fc – Li-Cor Biosciences) was also used for the normalisation of the immunoblotting. Next, the membrane was rinsed in water to remove the fluorescent protein staining and then incubated with the blocking buffer for 1 hour at room temperature (Blocker FL – ThermoFisher Scientific ref. 37565). After blocking, the membrane was incubated with the primary antibody on an orbital shaker at 4°C overnight. Then, the membrane was rinsed three times with TBS + 0.02% Tween-20 (10 min incubations) and incubated for 1 hour at room temperature with the appropriated secondary antibody diluted in blocking buffer.

The primary antibodies used, and their final concentrations are summarised in the table below.

Table 5. Antibodies used for westerblot

Antibody	Origin (Reference; stock concentration)	Phosphorylat ed sites	Host	Isotype	Dilution
PHF1	P Davis	Ser396 and Ser404	Mouse monoclonal	IgG1	1:10
AT8	Thermofisher ref. MN1020 (200µg/mL)	Ser202, Thr205	Mouse monoclonal	IgG1	1:500
AT270	Thermofisher ref. MN1050 (100µg/mL)	Thr181	Mouse monoclonal	IgG1	1:500
AT180	Thermofisher ref. MN1040 (100µg/mL)	Thr231	Mousemo noclonal	IgG1	1:500

A-mouse light chain specific secondary antibody was used at 1: 25 000 dilution (Alexa Fluor® 790 AffiniPure Goat Anti-Mouse IgG, light chain specific, Jackson Immunoresearch).

The membrane was rinsed as above and the immunodetected bands visualised using the 800 nm channel of the Odyssey Fc Imaging System (Li-Cor Biosciences). Data were acquired and quantified using Image Studio software (Li-Cor Biosciences).

6. Detection of neuronal activity

Neuronal activity was analysed by detection of fast changes in intracellular free calcium triggered by neuronal depolarization, using a high-affinity GCaMP calcium sensor. GCaMPs are the most widely used genetically encoded calcium sensors (Akerboom et al., 2012; L. Tian et al., 2009). GCaMP comprises a circularly permuted green fluorescent protein (cpGFP), the calcium-binding protein calmodulin (CaM) and M13 peptide which interact with CaM (Figure 2).

Upon cytosolic calcium increase, calcium binds to CaM which interacts with M13, causing a conformational change of the chromophore and an increase in its brightness (Nakai et al., 2001) Neurons display fast calcium dynamics and low peak calcium accumulations (Sabatini et al., 2002). The GCaMP6f variant, used in this work, has a higher sensitivity and faster fluorescence transients compared to other sensors. The detection of a single action potential calcium signature in the soma and dendritic tree *in vivo* was demonstrated (T.-W. Chen et al., 2013). Thus, the individual spikes within a burst caused fluorescence increases and could be

resolvable if they were separated by an interval on the order of the rise time of GCaMP6f, 50-75ms (T.-W. Chen et al., 2013).

Additionally, the calcium sensor concentration should be compatible with physiological conditions and minimise buffering effects. In this sense, different virus concentrations were tested to guarantee optimal conditions (check 2.2.4).

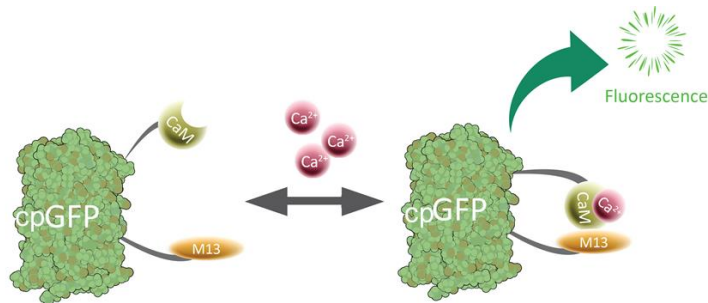


Figure 3. GCaMP structure comprises the circularly permuted green fluorescent protein (cpGFP), the calcium-binding protein calmodulin (CaM), and CaM-interacting M13 peptide. The complex CaM-M13 is in proximity to the chromophore inside the cpGFP. When calcium binds, CaM-M13 cause increased brightness.

Reagents:

Table 6. ACSF composition for calcium imaging

	Linear Formula	ACSF molarity (mM)
D-(+)-Glucose	$C_6H_{12}O_6$	20
Calcium chloride	$CaCl_2$	2.3
Magnesium chloride	$MgCl_2$	1.3
Sodium chloride	$NaCl_2$	100
Potassium chloride	KCl	4.5
Sodium phosphate monobasic	NaH_2PO_4	1.1
Sodium bicarbonate	$NaHCO_3$	26
Sodium pyruvate	$CH_3COCOONa$	5
L-Ascorbic acid	$C_6H_8O_6$	0.05

Drugs:

Bicuculline (Hellobio, HB0896)

Bicuculline is a selective competitive GABAA antagonist. It was used to identify GABAA-mediated responses.

CGP-55845 (Hellobio, HB0960)

CPG is a potent and selective GABAB receptor antagonist that prevents agonist binding.

Diazepam (Roche)

Diazepam (DZP) belongs to the benzodiazepines. DZP enhances GABA receptor function in an allosteric fashion in the sense that binds at a site distinct from the agonist (GABA) binding site.

L-CCG-1 (Tocris, Cat. No. 0333)

L-CCG-1 is an agonist of type 2 mGlu receptors which potently blocks MF transmission. (Nicoll RA 1994-5-6)

Organotypic slices from P301S and WT mice, both seeded and non-seeded, at 13 DIV and 21DIV, expressing GCaMP6f, were placed into the chamber with ACSF at 32°C. The ACSF was constantly oxygenated during the experiment, and the flow rate was kept at high speed (3mL/min) to guarantee the optimal perfusion of the slice. The chamber volume was kept at approximately 1 ml.

The time series were recorded on the CA3 region of the slices, on a 246*246 µm window, using a Leica TCS SP5 upright microscope equipped with an HCX APO L 20x 1.0 NA water immersion objective. The 488nm laser line was used, and emission was collected between 500–530 nm. The laser power was kept below 10% to avoid phototoxicity. PMT gain was kept at 800. Bidirectional line scans, 256 pixels in width, lasting 8 minutes (3200 frames, interval 150ms) were acquired at a line rate of 1 kHz. The pinhole was opened at 3 Airy Units, leading to a xy resolution of 0,48 µm and a z resolution of 0,05 µm.

The calcium imaging recordings were performed in the CA3 region after 10 minutes in ACSF for slice stabilisation. The slices were perfused with Bicuculline (10µM) and CGP (10µM) for 10 minutes and to test the effects of GABA antagonists. The L-CCG-1 (10µM) was applied for 10 minutes to confirm that the neuronal activity observed integrated MF-CA3 transmission.

The setup was washed for at least 30 minutes at the end of each recording, with water and ethanol, guaranteeing the washout of the drugs used.

The experiments were performed blinded to prevent the experimental biases. The recorded slices were chosen based on previously defined criteria: maintenance of tissue cytoarchitecture and no signs of tissue damage (holes in the tissue).

6.1. Analysis

Leica files (time series) were converted to .tiff format and analysed with MATLAB code from the Van Hooser lab (Brandeis University) (<http://github.com/VH-Lab/vhlab-TwoPhoton-matlab> and <http://github.com/VH-Lab/vhlab-calciumactivity-matlab>). 9-pixels diameter circular regions of interest (ROIs) were defined on a projection of the standard deviation of the fluorescence intensity of 2000 frames of the record (Results-Figure 7.1.). Between 16 and 22 cells per time series were selected by hand based on the following criteria: the position of the cell in the CA3 cell layer, shape (pyramidal), no visual sign of damages (visible nucleus, no blebs). ROIs were added on top of each cell, and the average fluorescence in the ROI was calculated for each frame. Absolute intensity (x) was transformed to $\Delta F/F$ by scaling to the baseline (defined as the 5th percentile of the entire time series) by the formula $\frac{x - \text{baseline}}{\text{baseline}}$. Periods of activity were detected on a per-cell basis. First, a slope higher than $+0.7 \Delta(\Delta F/F)/\text{frame}$ of the trace when smoothed with a spline (Matlab function: fit; fitype smoothingspline, SmoothingParam 0.99) indicated the beginning of an event. Second, the next local maximum would be used to determine the peak of the activity. Third, the cessation of activity was defined as the time when the trace dropped back to 30% of the local peak. A refractory period of 30 frames (4.5 s) was applied after each peak, where no other peak could be detected.

Several parameters were used to characterise the periods of activity: the average number of cells active in a given frame as a percentage of all cells (seen in Figure 7.2.), the average period of activity per cell (s), the number of firing events per cell, and the duration of each cellular event (s). The percentage of cells displaying any activity was also calculated for each record.

A correlation coefficient is also given for slices exhibiting 100% of active cells. Basically, when a population-wide event occurred that induced a slope of $+0.15 \Delta(\Delta F/F)/\text{frame}$ in the mean trace of all cells, smoothed with a fit as described above, we considered this to be a period of firing and calculated the cross correlation of spikes between cells during this period, using the MATLAB function: crosscoef. Crosscoef calculates the cross correlation between each pair of cells. The average correlation across all cell pairs across all periods of firing was used as a summary statistic. A coefficient close to 1 reflects a highly similar activity between cells.

6.2. Statistical Analysis

Statistical analysis was performed with Prism8 (GraphPad Software, USA) using two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test and nonparametric Mann–Whitney test. Data were expressed as mean \pm SEM and differences were considered significant when p values <0.05 .

Results

1. Synthetic tau fibrils K18 induces tau pathology in organotypic hippocampal slices of transgenic P301S mice.

1.1. The tau mutation P301S is not sufficient to induce tau pathology in organotypic hippocampal slices.

The transgenic mouse P301S expressing the mutated human form of tau (4R1N) with the mutation P301S is widely used as an experimental model for AD and other tauopathies. This model has been shown to display seeding activity, which means that extracellular misfolded tau fibrils (tau seeds) it can be uptaken by the cells and recruit large amounts of soluble tau which assembled into filamentous inclusions (reviewed in (Michel Goedert et al., 2010; Colin et al., 2020)). Thus, tau seeds extracted from tau transgenic mice (Sanders et al., 2014) or synthetic tau fibrils (Guo & Lee, 2011b) have been demonstrated to induce tau-aggregation *in vitro* and *in vivo* (Guo & Lee, 2014; Iba et al., 2013; Sanders et al., 2014; Stancu et al., 2015). Additionally, histopathological analysis has been widely used to quantify tau deposition in post-mortem diagnosis of AD and the related tauopathies (Bradley T. Hyman et al., 2012; Montine et al., 2012). The AT8 antibody recognises AD-derived tau and specifically hyperphosphorylated tau species the at Ser202/Thr205 residues (Biernat et al., 1992) which make it idyllic for AD diagnostics. In this work, we used AT8 to determine tau pathology detecting the progressive accumulation of aggregated hyperphosphorylated tau in slices.

To implement an *in vitro* model of tauopathy, we generated slices from the P301S transgenic mice. We evaluate whether the expression of endogenous mutated tau was sufficient to induce tau pathology *in vitro*. First, we confirmed, through western blot performed in brain extracts from P22 animals with the anti-human tau HT7 antibody, that the mutated tau was being expressed in both heterozygous (n=3 animals) and homozygous (n=3 animals) P301S but not in WT (n=3 animals) mice (Figure 1B). The western blot also shows that mutated tau is present at higher concentration in homozygous compared to heterozygous. Next, we investigate the presence of hyperphosphorylated tau in organotypic hippocampal slices cultures from both P301S and WT genotypes. To investigate the development of tau pathology in slices in the absence of tau seeds, we added PBS consecutively at DIV3/4 and DIV6/7 on the slices. After 21 DIV neither the slices from heterozygous (n=11 slices), homozygous (n=10 slices) P301S and WT (n=7 slices) mice showed an accumulation of aggregated hyperphosphorylated tau, as demonstrated by the lack of AT8 staining (Figure 1A). These results confirmed that the

expression of endogenous mutated tau, even at high levels as in homozygous animals, is not sufficient to induce the aggregation of hyperphosphorylated tau after 21 DIV.

1.2. K18 propensity to generate fibrils and the efficiency to induce aggregation in cell cultures.

Tau synthetic fibrils generated from the fibrillisation of recombinant tau fragment containing the P301L mutation (K18-P301L) have been used in several studies to induce tau pathology not only *in vivo* (M. Albert et al., 2019; Peeraer et al., 2015) but also in cell cultures (Strang et al., 2018) and organotypic slices (Stancu et al., 2015).

We first compared the ability of K18-P301L seeds bearing different tags, K18-HA, K18-myc and K18-noTag, to generate tau fibrils in the presence of heparin. Thioflavin T (ThioT) assay demonstrated that all types of seed could fibrillise. However, if compared to K18-myc (100%; n=3 repeats), K18-HA seemed to generate half of the amount of fibrils (56,29%; n=3 repeats) while K18-noTag generated twice the amount (209,46%; n=3 repeats) (Figure 2A).

To evaluate the efficiency of K18-myc and K18-HA to induce tau aggregation in cells, we applied the tau seeds to HEK293 transfected with mutated tau P301L using the NanoBRET assay. The bioluminescence analysis demonstrated that, in line with the results of ThioT, K18-HA seems to induce half of the tau aggregation (49,27%; n=4 repeats) compared to K18-myc (100%; n=4 repeats) (Figure 2B).

1.3. K18 efficiency to induce tau pathology depends on the tag of the seeds and the endogenous level of mutated tau in organotypic slices.

To establish optimal conditions for inducing tau pathology in organotypic slices, we used different combinations of tau seeds and organotypic slices generated from homozygous and heterozygous P301S mice and WT mice as a control.

For all combinations, we performed a consecutive addition of tau-seeds at DIV3/4 and DIV6/7 and revealed tau aggregation at 13 and 21 DIV using AT8 staining.

We first test K18-HA in slices from homozygous P301S, and we observed accumulation of hyperphosphorylated tau at 21 DIV (n= 14 slices) but not at 13 DIV (n= 7 slices). On the other hand, in slices from heterozygous P301S, we did not detect any AT8 signal either at 13 DIV (n= 4 slices) or 21 DIV (n= 5 slices). On the other hand, K18-myc induced a robust accumulation of hyperphosphorylated tau in slices from heterozygous P301S at 21 DIV (n= 11 slices), unlike K18-HA, while no AT8 signal was also detected at 13 DIV (n= 11 slices). Finally,

K18-noTag triggered a substantial accumulation of hyperphosphorylated tau at 13 DIV (n= 5 slices) which further increase at 21 DIV (n= 3 slices) (Figure 1C). Noteworthy, we observed that in the combinations where the tau pathology was induced, most of the hippocampal regions presented an accumulation of hyperphosphorylated tau at 21 days.

None of the seeds induced tau pathology in control slices from WT neither at 13 DIV (K18-HA, n= 4 slices (data not shown); K18-myc n= 7 slices; K18-noTag, n= 6 slices) nor at 21 DIV (K18-HA, n= 5 slices (data not shown); K18-myc n= 7 slices; K18-noTag, n= 5 slices) (Figure 1C).

Altogether, these observations indicate that synthetic tau seeds (K18-P301L) induce accumulation of hyperphosphorylated tau in organotypic hippocampal slices, as previously shown (Stancu et al., 2015). Even though K18-HA and K18-noTag can induce tau pathology in slices from homozygous and heterozygous P301S, respectively, we consider the use of K18-myc in slices from heterozygous P301s more suitable to establish the model. Firstly, the homozygous P301S are poor breeders and display a low survival rate. Secondly, K18-noTag induced the accumulation of hyperphosphorylated tau in all hippocampal regions at 13 DIV. Hence, the pathology would develop in slices between 6 and 13 DIV, when some features (DG-CA3 synaptic transmission) are still immature. Thus, we consider K18-myc as inducer and slices from heterozygous P301S as recipient as optimal conditions to establish a reliable and reproducible organotypic slice model of tauopathy.

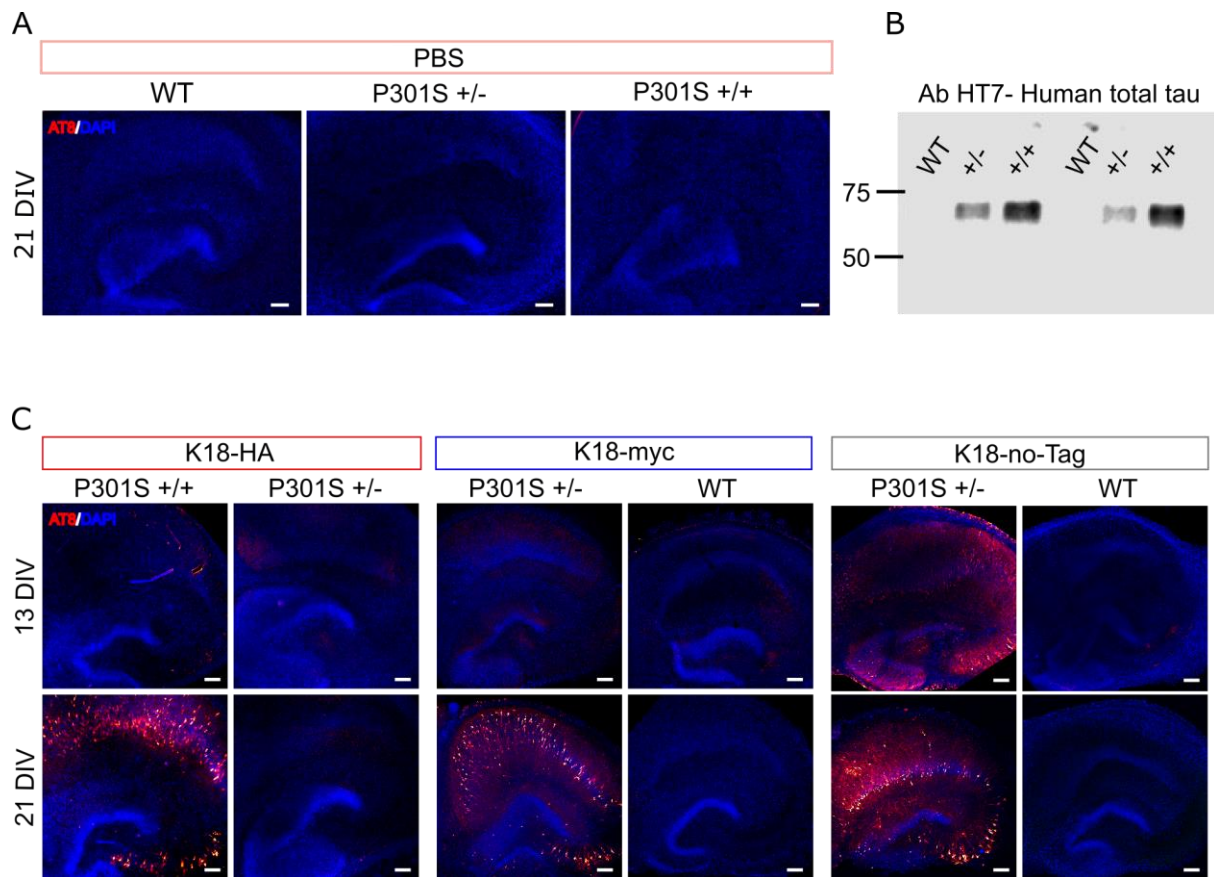


Figure 1. The efficiency of synthetic tau fibrils K18 to induce tau pathology in organotypic hippocampal slices depends on the tag of the seeds and the endogenous level of mutated tau. A: The expression of P301S mutations is not sufficient to induce tau pathology at 21 DIV. AT8 (Ser202/Thr205) staining was not detected in slices from WT, P301S homozygous (P301S +/+) or P301S heterozygous (P301S +/-) animals non-seeded after 21 DIV. B: Representative Western blots with HT7 (human tau antibody) of hippocampus homogenates from WT, P301S homozygous (+/+) or P301S and heterozygous (+/-) mice at age P22. C: Tau pathology is induced by K18-HA in slices from P301s +/+ at 21DIV but not in slices from P301S +/- . K18-myc induces tau pathology in P301S +/- at 21DIV and K18-noTag induces tau pathology in slices from P301S +/- at 13 DIV. Accumulation of phosphorylated tau is revealed by AT8 immunostaining against phosphorylated tau at the residues Ser202/Thr205. (Confocal images, Tifescan acquired 20x, scale bar: 100 μ m)

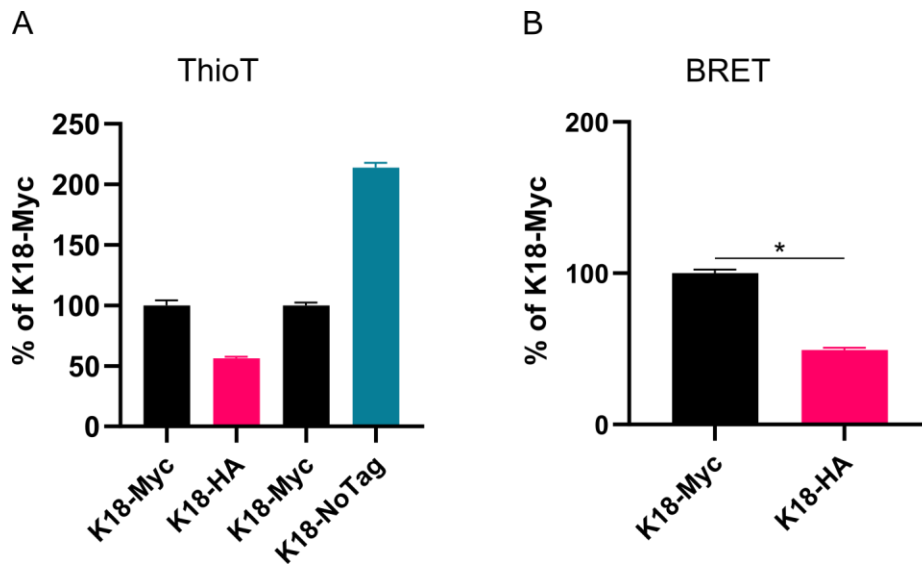


Figure 2. K18 propensity to generate fibrils and efficiency to induce aggregation in cell cultures depending on the tag. A: Compared with K18-Myc tagged seeds, K18-noTag exhibit more fibrils and K18-HA less fibrils generated in the presence of heparin. B: K18-Myc is more efficient to induce tau aggregation in cells. Detection of tau aggregates in HEK cells after 72 hours after seeding. The graphs represent the mean \pm SEM. The differences between conditions were assessed by a Mann Whitney test, * $p < 0.05$.

2. Tau pathology develops progressively in the hippocampus *in vivo* and *in vitro* in the P301S mice.

2.1. Development of tau pathology *in vitro* and *in vivo*.

The P301S mouse model has been mainly used *in vivo* either to characterise the development of tau pathology (Yoshiyama et al., 2007) or to evaluate the seeding activity (Kaufman et al., 2016a). Furthermore, *in vitro* studies have demonstrated that seeding induces tau aggregation in CA1 region of organotypic slices (Stancu et al., 2015). However, there is no mention of the progression of the pathology nor of other hippocampal regions affected

In this work, we characterised the progression of tau pathology in organotypic slices and compared it with the development of the pathology *in vivo* in the P301S model.

We evaluated the tau pathology by the percentage of area positive to AT8 staining, over time. In fixed slices of aging transgenic mice P301S we observed a progressive accumulation of hyperphosphorylated tau in CA1 neurons between 6 and 10 months (Age; Mean \pm SEM, % AT8 positive area per total area; 6months; $2,385 \pm 0,937\%$, n=6 slices; 8months; $6,472 \pm 0,6306\%$; n=7 slices; 10months; $8,171 \pm 0,923\%$; n=5 slices), with a significant increase between 6 and 8 months ($p=0,014$) but not between 8 and 10 months ($p=0,202$). In CA3 region we also observed a progressive appearance of the tau pathology (Age; Mean \pm SEM, % AT8 positive area per total area; 6months; $1,277 \pm 0,510\%$, n=6 slices; 8months; $3,432 \pm 0,400\%$; n=7 slices; 10months; $4,582 \pm 0,956\%$; n=5 slices), with a significant increase between 6 and 8 months ($p=0,014$) but not between 8 and 10 months ($p=0,876$). Regarding the the rate of progression of tau pathology, we found similar increase in both CA1 and CA3 between 6 and 8 months (CA1 63,16%; CA3 62,78%) and between 8 months and 10 months (CA1 20,80%; CA3 25,11%) (Figure 3A).

In organotypic slices we found the same progressive accumulation of hyperphosphorylated tau in CA1 region between 13 DIV and 21 DIV (DIV; Mean \pm SEM, % AT8 positive area per total area; 13 DIV; $2,388 \pm 0,580\%$, n=9 slices; 19 DIV; $4,852 \pm 0,428\%$; n=11 slices; 21 DIV; $6,82 \pm 0,651\%$; n=12 slices), with a significant increase between 13 DIV and 19 DIV ($p=0,0057$) and 19 DIV and 21 DIV ($p=0,439$). We observed the same development of the pathology in CA3 (DIV; Mean \pm SEM, % AT8 positive area per total area; 13 DIV; $0,855 \pm 0,135\%$, n=9 slices; 19 DIV; $1,545 \pm 0,219\%$; n=10 slices; 21 DIV; $3,847 \pm 0,454\%$; n=10 slices), with a significant increase between 13 DIV and 19 DIV ($p=0,028$) and 19 DIV and 21 DIV ($p=0,0002$). Remarkably, concerning the rate of progression of the tau pathology, there is a similar increase (CA1 49,6%; CA3 44,7%) in CA1 and CA3 between 13 DIV and 19 DIV. However, between 19

DIV and 21 DIV this increase seems to be more accentuated in CA3 (CA1 20,2% CA3 59,8%) (Figure 3B).

In both controls, WT aged mice and organotypic slices from P301S non seeded we did not observe the accumulation of aggregated hyperphosphorylated tau in CA3 neurons (WT, Age; Mean \pm SEM, % AT8 positive area per total area; 6months; $0,166 \pm 0,027\%$, n=5 slices; 8months; $0,159 \pm 0,021\%$; n=6 slices; 10months; $0,175 \pm 0,046\%$; n=5 slices; P301S non seeded, DIV; Mean \pm SEM, % AT8 positive area per total area; 13DIV; $0,141 \pm 0,057\%$, n=3 slices; 19 DIV; $0,240 \pm 0,049\%$; n=5 slices; 21 DIV; $0,119 \pm 0,025\%$; n=5 slices). We did not found differences either in WT between 6 and 8 months ($p > 0,999$) and between 8 and 10 months ($p=0,7922$), or in organotypic slices non seeded between 13 DIV and 19 DIV ($p=0,536$) or 19 DIV and 21 DIV ($p=0,079$) (Figure S1).

Here we demonstrated that tau pathology occurs progressively in organotypic slices as *in vivo* in the hippocampal region of P301S mice. Additionally, in organotypic slices, there seems to be a worsening of tau pathology first in CA1 than in CA3 neurons

2.2. Reduction of the mossy fiber bundle correlates with the progression of tau pathology.

The granule cells from the dentate gyrus extend their axons, the mossy fibers (MF), to contact the pyramidal cells in the CA3 area of the hippocampus. This excitatory synapse has been implicated in the memory storage and retrieval processes. Their loss might play a part in memory deficits present in AD. A recent study reported that hyperphosphorylated tau (AT8 staining) accumulates in MF in AD post-mortem brain samples (Christensen et al., 2019a). Moreover, the P301S mice model displayed a reduced MF bundle at 6 months (Yoshiyama et al., 2007). Also, organotypic slices generated from transgenic mice expressing a different FTDL-associated mutation ($\Delta K280$ pro-aggregant tau) showed some structural changes in MF (Messing et al., 2013). Thus, here we investigated whether the MF are affected in our *in vitro* model. In both acute slices of aged transgenic mice P301S and organotypic slices, we assess the appearance of tau pathology in MF, determined by the percentage of area occupied by AT8 within the area occupied by CB28 (calbindin 18 is a specific marker of the mossy fiber bundle).

In vivo, we found an accumulation in MF that is more pronounced between 6 and 8 months than between 8 and 10 months (Age; Mean \pm SEM, % AT8 positive area per CB28 area; 6months; $3,217 \pm 1,165\%$, n=6 slices; 8months; $9,537 \pm 1,486\%$; n=7 slices; 10months; $9,688$

$\pm 0,927\%$; n=5 slices) with significant differences between 6 and 8 months ($p=0,0082$) but not between 8 and 10 months ($p=0,4318$) (Figure 4A).

The analysis of MF in organotypic slices revealed the same tendency as *in vivo*: the accumulation of hyperphosphorylated tau seems to be more pronounced between 13 DIV and 19 DIV than between 19 DIV and 21 DIV (DIV; Mean \pm SEM, % AT8 positive area per CB28 area; 13DIV; $2,121 \pm 0,670\%$, n=9 slices; 19 DIV; $6,389 \pm 1,300\%$; n=10 slices; 21 DIV; $7,662 \pm 1,863\%$; n=10 slices) with significant differences between 13 DIV and 19 DIV ($p=0,0057$) but not between 19 DIV and 21 DIV ($p=0,8534$) (Figure 4B).

In both controls, WT aged mice and non seeded organotypic slices from P301S mice, we did not observe the accumulation of hyperphosphorylated tau in the MF bundle (WT, Age; Mean \pm SEM, % AT8 positive area per total area; 6months; $0,211 \pm 0,065\%$, n=5 slices; 8months; $0,166 \pm 0,050\%$; n=6 slices; 10months; $0,117 \pm 0,052\%$; n=5 slices; P301S non seeded, DIV; Mean \pm SEM, % AT8 positive area per total area; 13DIV; $0,456 \pm 0,0184\%$, n=3 slices; 19 DIV; $0,508 \pm 0,129\%$; n=5 slices; 21 DIV; $0,6901 \pm 0,075\%$; n=5 slices). We did not found differences either in WT between 6 and 8 months ($p=0,5368$) or 8 and 10 months ($p=0,9307$) neither in organotypic slices non seeded between 13 DIV and 19 DIV ($p=0,7321$) or 19 DIV and 21 DIV ($p=0,1349$) (Figure S1).

To assess the MF loss, we calculate the area of CB28 positive staining. As CB28 is not present in immature MF, we performed another set of experiments using anti- synaptoporin (SPO), another marker of MF projections which is not specific from mature or immature fibers (Grabs et al., 1994; Grosse et al., 1998).

In vivo, we observed a significant loss of MF between 6 and 8 months using CB28 staining(Age; Mean \pm SEM, % CB28 area; 6months; $31,22 \pm 2,074\%$, n=6 slices; 8months; $15,73 \pm 1,792\%$; n=7 slices; 10months; $20,41 \pm 2,863\%$; n=5 slices) with significant differences between 6 and 8 months ($p=0,0023$) but not between 8 and 10 months ($p=0,0,202$) (Figure 4A).

We also found axonal degeneration in organotypic slices (DIV; Mean \pm SEM, % CB28 area; 13DIV; $47,52 \pm 2,352\%$, n=9 slices; 19 DIV; $51,81 \pm 3,601\%$; n=10 slices; 21 DIV; $39,94 \pm 1,539\%$; n=10 slices) with no significant differences between 13 DIV and 19 DIV ($p=0,40062$) but showing a significant decreased between 19 DIV and 21 DIV ($p=0,0039$) (Figure 4B).

The analysis of MF in both controls, WT and organotypic slices revealed that in the absence of tau pathology, the MF projections are not affected over time (WT, Age; Mean \pm SEM, % CB28 area; 6months; $30,27 \pm 1,606\%$, n=5 slices; 8months; $29,71 \pm 1,108\%$; n=6 slices; 10months; $30,07 \pm 1,263\%$; n=5 slices; P301S non seeded, DIV; Mean \pm SEM, % CB28 area; 13DIV; $44,44 \pm 1,059\%$, n=3 slices; 19 DIV; $46,4 \pm 1,414\%$; n=5 slices; 21 DIV; $51,42 \pm 4,744\%$;

n=5 slices). We did not find differences either in WT between 6 and 8 months ($p=0,9307$) or 8 and 10 months ($p=0,6623$) neither in organotypic slices non seeded between 13 DIV and 19 DIV ($p=0,3571$) or 19 DIV and 21 DIV ($p=0,7857$) (Figure S1).

The quantification of SPO retrieved similar results, with a decrease in MF between 13 DIV and 21 DIV (P301S seeded, DIV; Mean \pm SEM, % SPO area; 13DIV; $43,6 \pm 2,126\%$, n=6 slices; 21 DIV; $32,84 \pm 1,86\%$; n=6 slices; $p=0,0087$). On the other hand, we did not find any changes in MF in organotypic slices which not develop tau pathology (P301S non seeded; DIV; Mean \pm SEM, % SPO area; 13DIV; $48,36 \pm 1,281\%$, n=6 slices; 21 DIV; $49,25 \pm 1,943\%$; n=6 slices; $p=0,5079$) (Figure 4C)

Our results showed that the accumulation of phosphorylated tau in MF occurs along with the axonal degeneration both *in vitro* and *in vivo*. Interestingly, in aged animals, both events seem to occur in parallel. However, in organotypic slices, the accumulation of hyperphosphorylated tau preceded the loss of MF projections. We did not find ageing-associated axonal degeneration in the absence of tau pathology

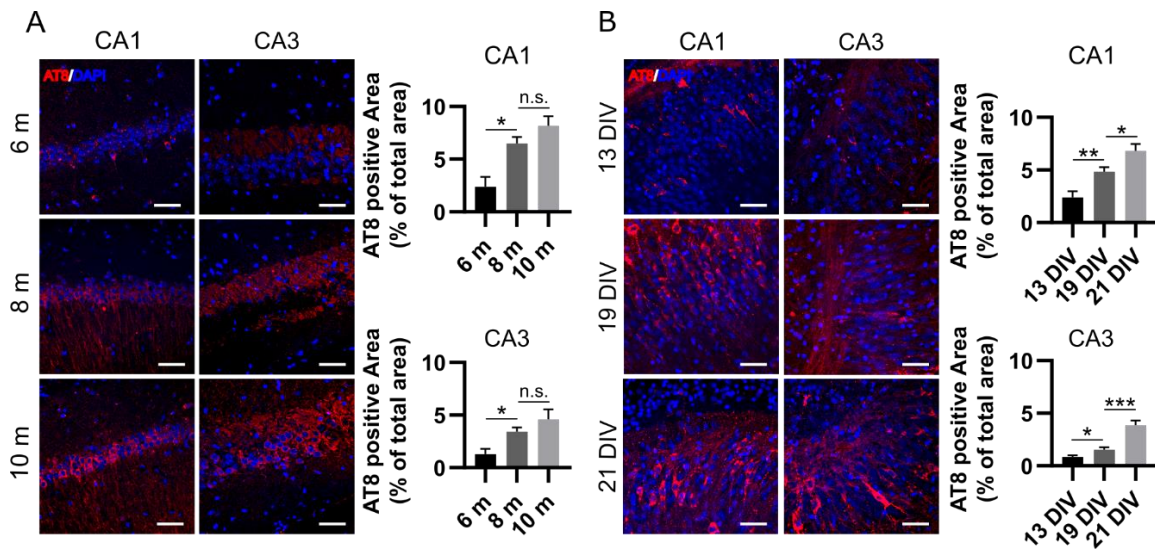


Figure 3. The pathology develops progressively in the hippocampus, affecting CA1 before CA3 neurons. **A-B**: Progressive accumulation of hyperphosphorylated tau in P301S *in vivo* between 6 and 10 months (A) and in P301S seeded slices between 13 DIV and 21 DIV (B) in the CA1 region and the CA3 region. Tau pathology revealed by immunostaining by AT8 (Ser202/Thr205).

A: Graphs represent the percentage of area covered by AT8 staining in CA1 (6 mo= 2,385%, 8 mo = 6,472%; 10 mo= 8,171%; and CA3 (6 mo= 1,277%, 8 mo = 3,432%; 10 mo= 4,582%) cell bodies *in vivo*.

B: Graphs represent the percentage of area covered by AT8 staining in CA1 (13 DIV=2,388%; 19 DIV=4,852%; 21 DIV=6,82%; slices) and in CA3 (13 DIV=0,855%; 19 DIV=1,545%; 21 DIV=3,847%;) cell bodies *in vitro*. Confocal images, acquired 40x, scale bar: 50 μ m. *In vivo* CA1 and CA3 n= slices: 6 mo n=6; 8 mo n=7; 10 mo n=5. *In vitro* n= slices: CA1: 13 DIV=9; 19 DIV=11; 21 DIV=12 and CA3: 13 DIV=9; 19 DIV=10; 21 DIV=10. The graphs represent the mean \pm SEM; the differences between conditions were assessed by Mann Whitney test,

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

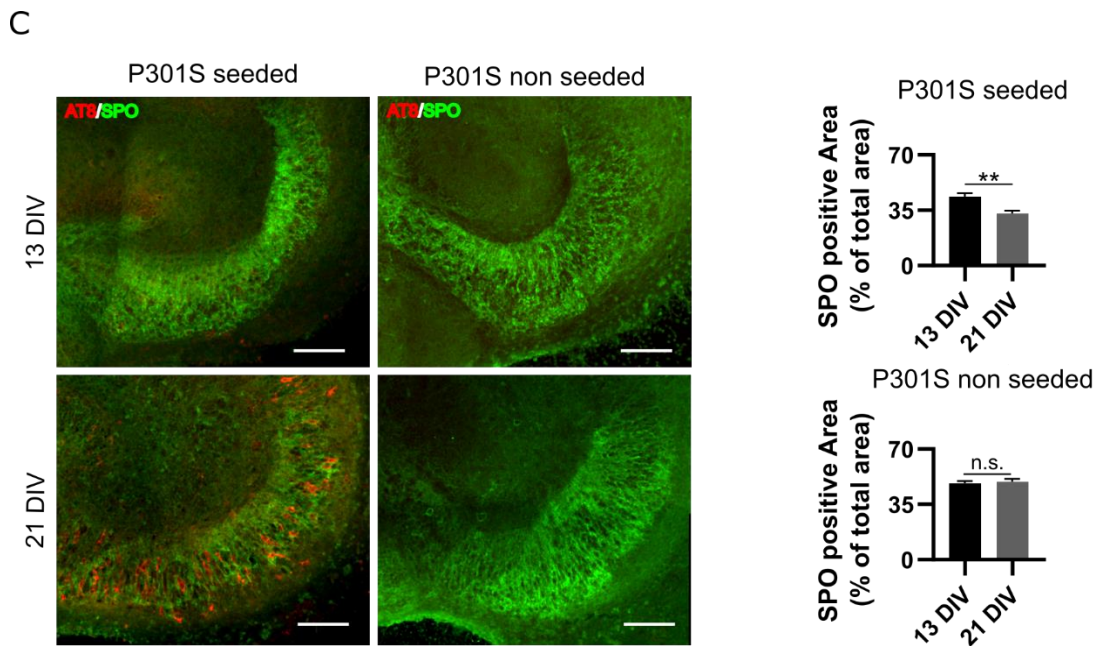
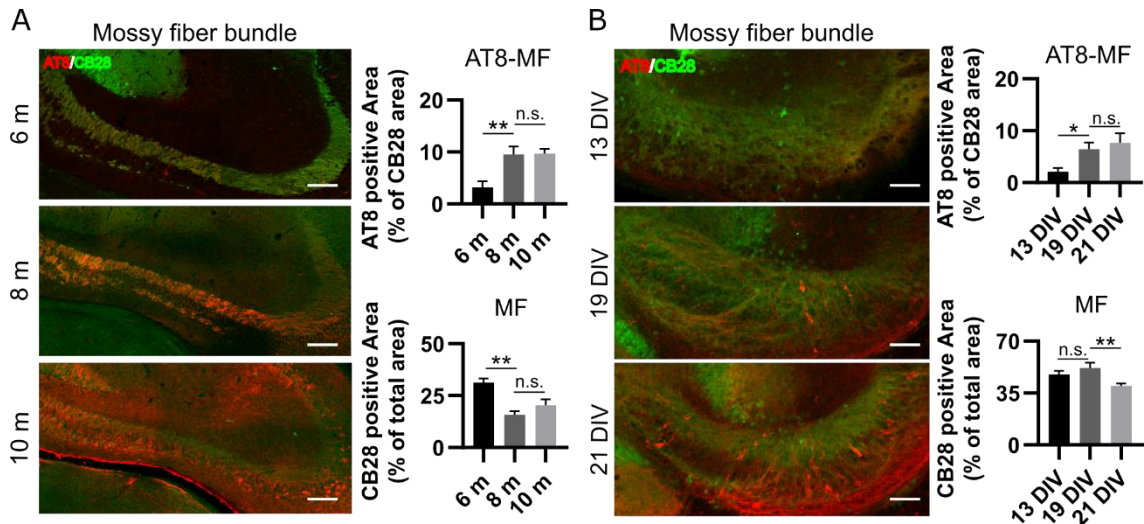


Figure 4. The progressive of the tau pathology correlates with the attrition of the mossy fibers bundles.

Progressive accumulation of hyperphosphorylated tau in P301S mice between 6 and 10 months (A) and P301S seeded slices between 13 DIV and 21 DIV (B) in the mossy fiber bundle (MF). Tau pathology revealed by immunostaining by AT8 (Ser202/Thr205) and immunostaining against calbindin (CB28) to mark the mossy fiber bundle. Loss of MF assessed by the staining with CB28 *in vivo* and *in vitro* (B) and synaptopodin (SPO), another marker of MF projection which is not specific for mature fibers *in vitro* (C).

A: Graphs represent the percentage of area covered by AT8 staining in MF marked by CB28, accumulation of AT8 in MF (6 mo= 3,217 %, 8 mo = 9,537%; 10 mo= 9,688%; and the percentage of area covered by CB28 staining, MF bundle (6 mo= 31,22%, 8 mo =15,73%; 10 mo= 20,41%) *in vivo*.

B: Graphs represent the percentage of area covered by AT8 staining in MF marked by CB28, accumulation of AT8 in MF (13 DIV=2,121%; 19 DIV=6,389%; 21 DIV=7,662 %) and the percentage of area covered by CB28 staining, MF bundle (13 DIV=47,52 %; 19 DIV=51,81 %; 21 DIV=39,94 %) *in vitro*.

C: Graphs represent the percentage of area covered by SPO staining, MF bundle (P301S seeded, 13DIV= 43,6% and 21 DIV= 32,84% and P301S non seeded, 13DIV= 48,36% and 21 DIV= 49,25%) *in vitro*.

Confocal images, acquired 20x, scale bar: 100 μ m. AT8 and CB28: *In vivo* n= slices: 6 mo n=6; 8 mo n=7; 10 mo n=5. *In vitro* n= slices: 13 DIV=9; 19 DIV=10; 21 DIV=10. SPO: *In vitro* n= slices: 13 DIV=6 and 21 DIV=6). The graphs represent the mean \pm SEM; the differences between conditions were assessed by a Mann Whitney test, * p<0.05, ** p<0.01, ***<0.001.

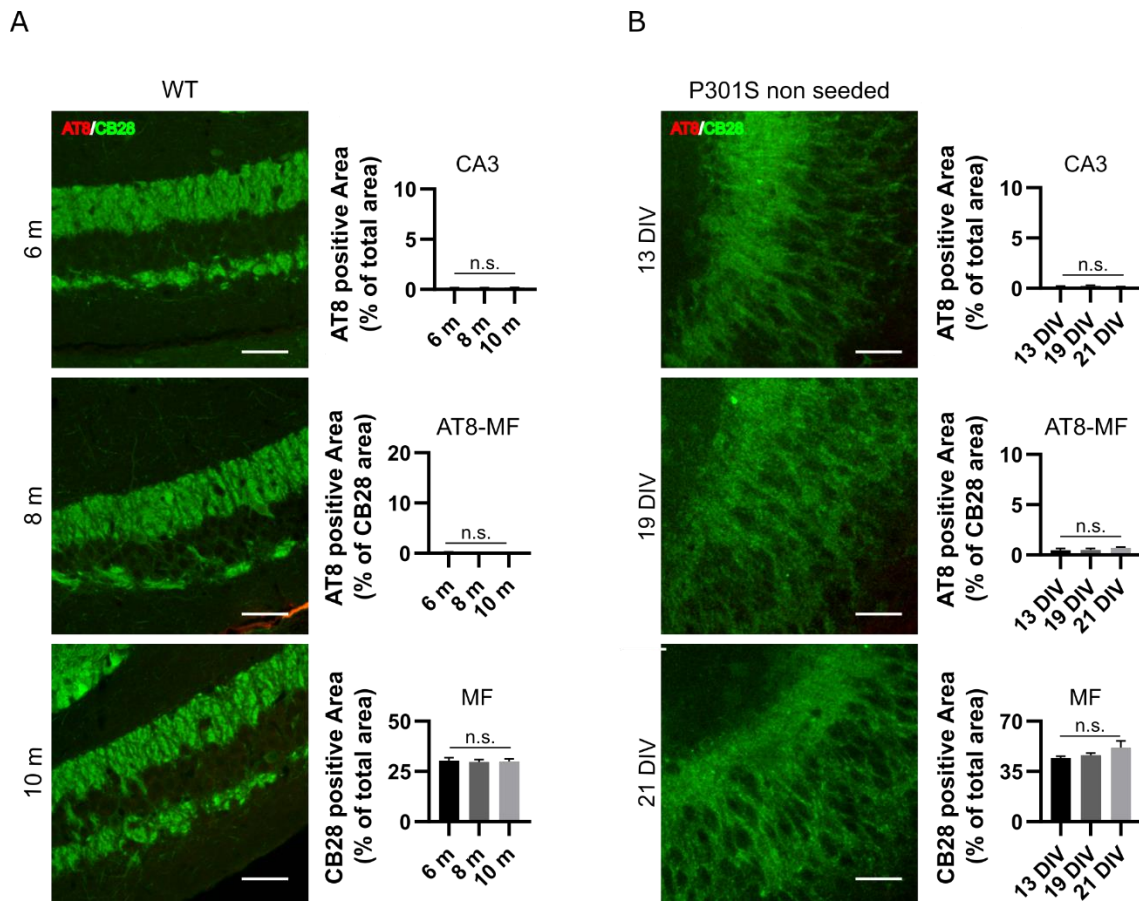


Figure S1. The mutation P301S per se is not sufficient to cause the loss of MF neither *in vivo* nor *in vitro*.

The double staining with AT8 and CB28 revealed no detection of AT8 signal and no loss of MF either *in vivo* (WT) or *in vitro* (P301S non-seeded slices).

Confocal images, acquired 40x, scale bar: 50 μ m. AT8 and CB28: *In vivo* n= slices: WT 6 mo n=5; 8 mo n=6; 10 mo n=5. *In vitro* n= slices: P301S non seeded 13 DIV=5; 19 DIV=5; 21 DIV=5). The graphs represent the mean \pm SEM; the differences between conditions were assessed by Mann Whitney test; no differences were found.

3. K18-myc seeding induces the development of different pathological forms of tau in organotypic hippocampal slices of P301S mice.

3.1. Misfolded tau is present at early stages of tau pathology.

The MC1 antibody recognises two epitopes at the N terminal and within the repeat domain of tau, which is only available in simultaneous when tau protein adopts a disease-associated conformation. MC1 stains pathological conformers of tau not only as one of the earliest detectable events in the brain of AD patients but also with higher specificity than other common markers (Weaver et al., 2000)

In previous studies, the immunohistological analysis of P301S brain sections revealed a general absence of MC1 staining in young (before 3 months) and non-transgenic mice (Holmes et al., 2014). Also, the analysis of post-mortem sampled from the human subject at Braak stage 0 reported a lack of MC1 staining (Weaver et al., 2000).

In this work we performed immunohistochemical analysis using MC1 and PHF1 antibodies (kindly provided by Peter Davies, The Feinstein Institute for Medical Research, Manhasset, NY) to assess whether the K18-myc induces the generation of other pathological forms of tau than AT8. We first analysed the presence of misfolded tau (MC1) in CA1 region comparing WT and P301S seeded slices. In WT seeded we did not detect the presence of MC1 either at 13 DIV or 21 DIV (WT seeded; DIV; Mean \pm SEM, % MC1 area; 13DIV; $0,69 \pm 0,102\%$, n=5 slices; 21 DIV; $0,62 \pm 0,09\%$; n=6 slices; $p > 0,9999$). On the other hand, the P301S seeded slices showed that the area occupied by MC1 is significantly larger at 13 DIV than at 21 DIV (P301S seeded; DIV; Mean \pm SEM, % MC1 area; 13DIV; $4,54 \pm 0,162\%$, n=5 slices; 21 DIV; $3,07 \pm 0,322\%$; n=7 slices; $p=0,0008$). Furthermore, no MC1 signal was detected in the CA3 region of WT seeded slices (WT seeded; DIV; Mean \pm SEM, % MC1 area; 13DIV; $0,69 \pm 0,122\%$, n=5 slices; 21 DIV; $0,75 \pm 0,123\%$; n=6 slices; $p > 0,9999$). In the P301S seeded slices we confirmed the presence of MC1 in CA3 but we did not find differences in the area occupied between 13DIV and 21DIV (P301S seeded; DIV; Mean \pm SEM, % MC1 area; 13DIV; $4,01 \pm 0,402\%$, n=5 slices; 21 DIV; $3,62 \pm 0,410\%$; n=7 slices; $p=0,9472$) (Figure 5A).

The analysis of P301S non seeded slices as controls showed that the seeding with K18-myc is necessary to generate altered conformational form of tau in both CA1 (P301S non seeded; DIV; Mean \pm SEM, % MC1 area; 13DIV; $0,35 \pm 0,083\%$, n=6 slices; 21 DIV; $0,43 \pm 0,044\%$; n=3 slices; $p=0,7143$) and CA3 regions (P301S non seeded; DIV; Mean \pm SEM, % MC1 area; 13DIV; $0,29 \pm 0,045\%$, n=6 slices; 21 DIV; $0,40 \pm 0,024\%$; n=3 slices; $p=0,0952$) (Figure S2).

Additionally, the qualitative inspection of PHF1 indicates the presence of this phosphorylated form more accentuated at 21 DIV than at 13DIV (Figure 5B).

3.2. Phosphorylation of different tau sites associated with tau pathology.

Hyperphosphorylated tau species were revealed in organotypic slices extracts from P301S mice by immunoblotting with anti phospho tau Ser396/Ser404 (PHF1), anti phospho tau Thr181 (AT270), and AT8 antibodies. No bands were detected in slices from WT mice, seeded or non-seeded, demonstrating that seeds alone are not enough to induce a tauopathy even after 3 weeks *in vitro* (Figure 5C). Due to the limited amount of material (4 organotypic slices per animal have to be pooled to allow extraction and detection of phosphorylated tau), we could not perform classical protocols of sequential extraction of tau species with different solubility using buffers of increasing stringency. Instead, we used directly an extraction buffer containing 2% SDS (Yoshiyama et al., 2007). Hence, our experimental conditions only allowed the detection of SDS soluble species, while SDS insoluble tau aggregates have been described in AD brains (Ksieszack-Reding et al., 1994). Indeed, we could not detect any signal with AT100 antibody, which only labels insoluble tau ((Allen et al., 2002b), data not shown).

Phosphorylated tau could be detected as early as at 13 DIV with PHF1, AT270 and AT8 antibodies, in non-seeded slices from P301S mice. Seeded slices at the same DIV exhibited stronger staining, indicating that the seeding increased tau hyperphosphorylation. No differences were detected between 13 and 21 DIV in non-seeded slices, and between seeded and non-seeded slices at 21 DIV (Figure 5C). Interestingly, all antibodies detected bands of higher and lower molecular weight, as has already been described in AD brain under similar extraction conditions. Of note, immunostaining of organotypic slices using AT8 or PHF1 antibodies failed to show any tau aggregates in non-seeded slices and slices at 13 DIV. However, a strong increase in tau aggregates was detected in seeded slices at 21DIV (Figure 5B). These aggregates are probably not solubilised by our extraction protocols, and not detected by immunoblotting. A switch of phospho tau between Sarkosyl soluble and Sarkosyl insoluble fractions have been described in brain extract from seeded P301S mice 6 months after injection (Stancu et al., 2015). Hence, the combination of both techniques (Western Blot and immunostaining) appears necessary to follow the evolution of soluble tau and tau aggregates in our model.

Of note, one particular band around 50 kDa appeared to be significantly affected by the seeding protocol, both at 13DIV and 21 DIV (Figure 5C). Further investigations are needed to determine the nature of this band.

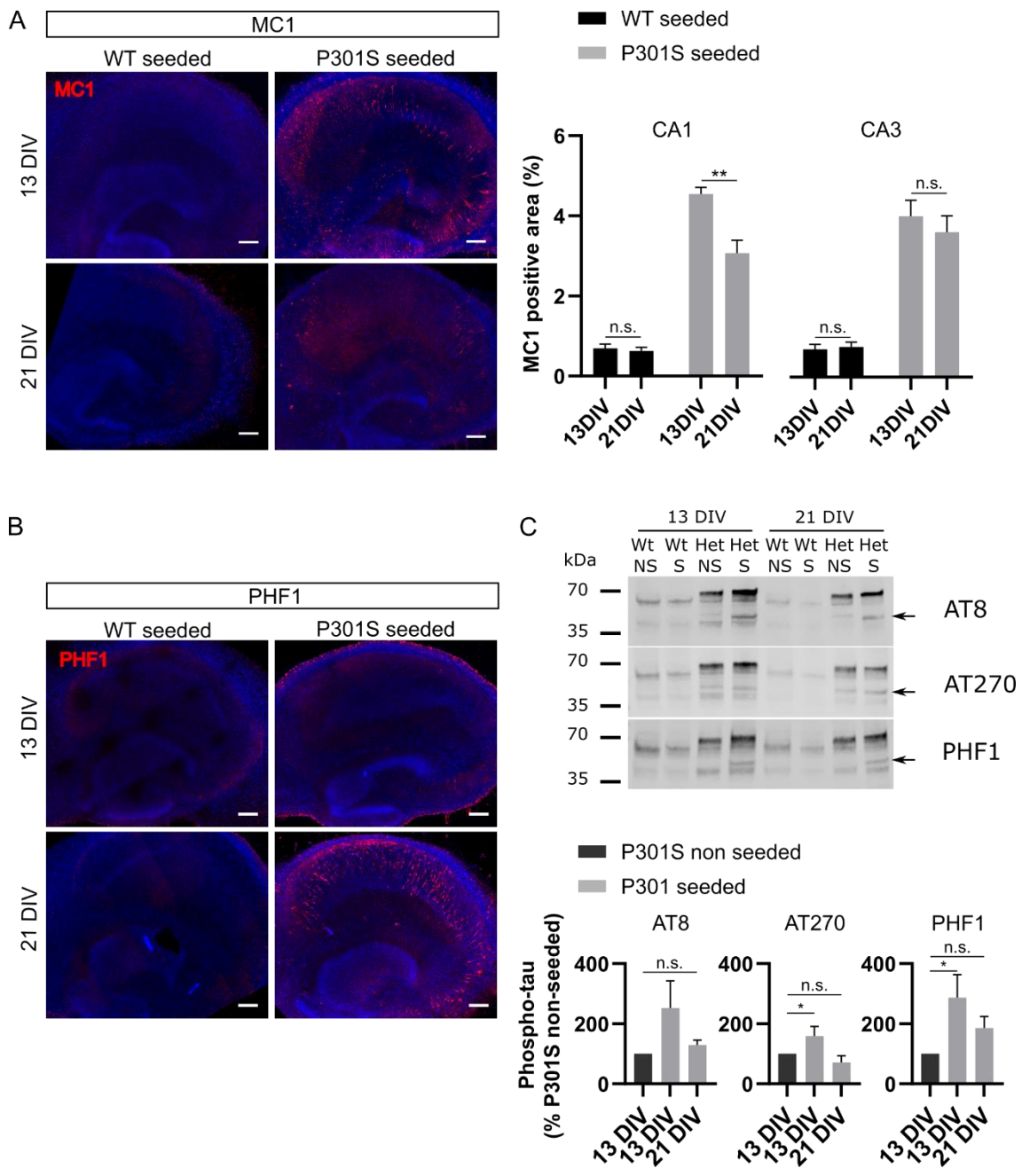


Figure 5. The tau pathology induced by tau seeds generates different pathological forms of tau.

A: The presence of altered conformational tau was revealed by immunostaining with MC1 antibody. The signal appears stronger at 13DIV than at 21DIV, with a significant decrease in CA1 and a slight decrease in the CA3 region.

Graphs represent the percentage of area covered by MC1 staining in CA1 and CA3 regions. (CA1 - WT seeded 13DIV: 0,69%, and 21 DIV: 0,62%; P301S seeded; 13DIV: 4,54% and 21 DIV: 3,07%. CA3 - WT seeded 13DIV; 0,69% and 21 DIV:0,75%; P301S seeded; 13DIV: 4,01% and 21 DIV: 3,62%).

B: Hyperphosphorylated tau at the residues Ser396/Ser404 was revealed by PHF1 immunostaining. The presence of PHF1 is more accentuated at 21 DIV than at 13DIV.

C: Western blot confirmed the presence of different phosphorylated tau species (AT8, AT270 and PHF1) in organotypic slices from P301S mice at 13DIV and 21 DIV. Graphs represent the band intensity as a percentage of P301S non-seeded slices at 13DIV. The values were normalised by total protein. Significant differences were found between seeded and non-seeded slices at 13 DIV.

Confocal images, acquired 20x, scale bar: 100 μ m. MC1: *In vitro* n= slices: WT seeded: 13 DIV: 5 and 21 DIV=6; P301S seeded 13 DIV: 5 and 21 DIV=7. Westernblot: AT270 and PHF1: N= 4 animals (4 slices per animal) per condition. AT8: 3 animals per conditions. The graphs represent the mean \pm SEM; the differences between conditions were assessed by Sidak's multiple comparisons test, $p^{**}<0.01$ for immunostaining, and Mann Withney for Western Blot, $p^{*}<0.05$

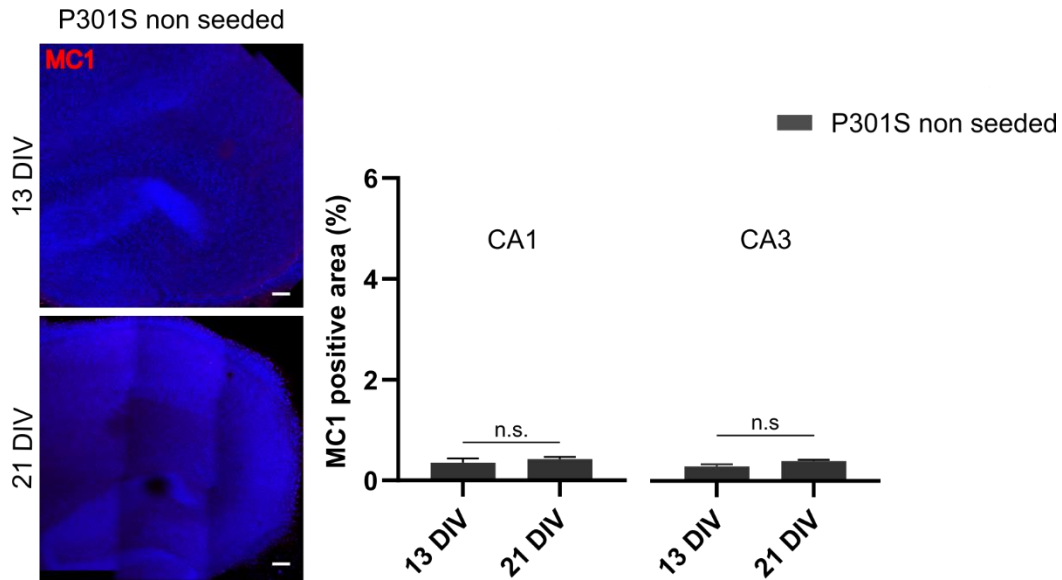


Figure S2. The mutation P301S per se is not sufficient to generate the appearance of misfolded tau (MC1) in organotypic hippocampal slices after 21 DIV. Confocal images of P301S non-seeded slices with MC1 immunostaining

Confocal images, acquired 20x, scale bar: 100 μ m. MC1: *In vitro* n= slices: P301S non seeded: 13 DIV: 6 and 21 DIV=3). The graphs represent the mean \pm SEM. No differences were found.

4. Tau pathology affects specific cell types in P301S mice *in vitro* and *in vivo*.

Different studies have addressed the cell-specific vulnerability in tau pathology. For instance, PV interneurons seem to accumulate MC1 (altered conformational marker) in the hippocampus of a tauopathy model expressing the P301L mutation (Levenga et al., 2013) but not in the EC of a transgenic model with the same mutation but restricted to EC-II (Fu et al., 2019). On the other hand, a study demonstrated that AT8+ hyperphosphorylated tau accumulates in the pyramidal cell layer of CA1 but not in PV+ cells (Soler et al., 2017)

Thus, here, to identify which type of cell accumulate pathological form of tau in the transgenic mouse P301S *in vivo* and our *in vitro* model, we performed sequential staining with antibodies against hyperphosphorylated tau (AT8), neurons (NeuN), subpopulation of interneurons (PV), astrocytes (GFAP) and microglia (Iba1).

We visual inspected acute slices from aged P301S and organotypic P301S seeded slices and the qualitative evaluation demonstrated an accumulation of hyperphosphorylated tau in NeuN+ positive cells but not in PV+, GFAP+ or Iba+ cells in the pyramidal layer of CA3.

This piece of evidence suggests that interneurons, astrocytes and microglia are less prone to accumulate the pathological form of tau, hyperphosphorylated at Ser202/Thr205 residues (AT8) both *in vitro* at 21DIV and *in vivo*, 10 months.

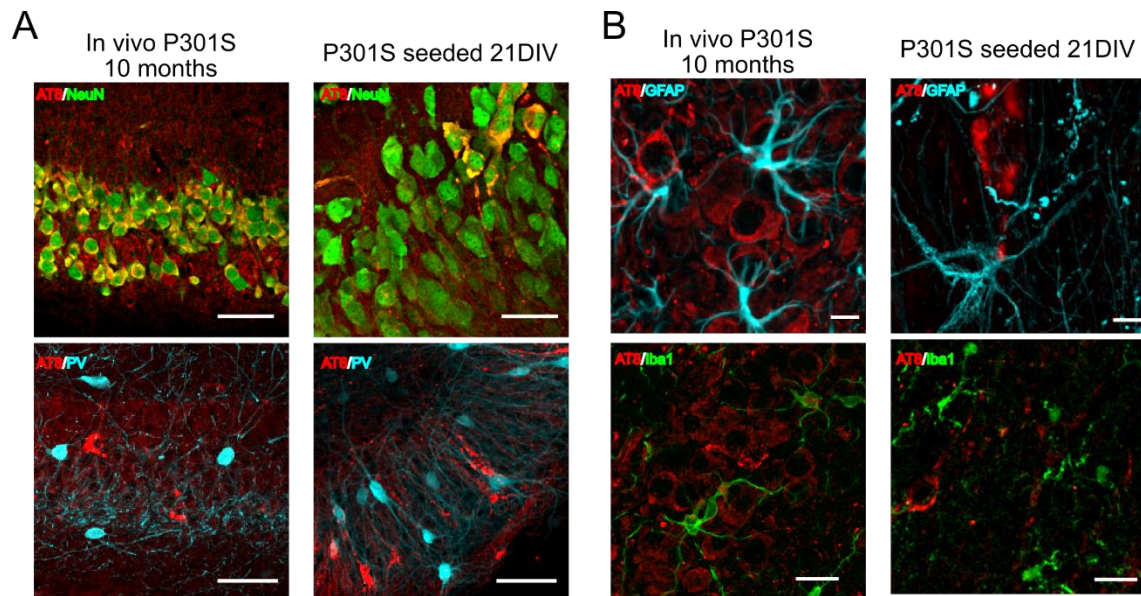


Figure 6. Cell-specific vulnerability for tau pathology in P301S *in vivo* and *in vitro*

A: Confocal images of AT8 positive staining co-localized with the neuronal marker (NeuN) (top), but not with PV-positive GABAergic interneurons (PV) (bottom) in both acute slices from P301S mice at 10 months (top) and P301S seeded organotypic slices at 21 DIV. Confocal images, acquired 40x, scale bar: 50 μm

B: Confocal images of double staining with AT8 and a marker for astrocytes (GFAP) or microglia (Iba1). No co-localisation is observed. Confocal images acquired 63x, Scale bar: 10 μm .

5. Neuronal activity is altered in the CA3 region in P301S seeded slices at 21DIV.

5.1. The neuronal activity in the CA3 hippocampal region is characterised by sparse activity in P301S seeded slices at 13 DIV.

Patients with AD and FTD with the mutations P301S often show seizures and hippocampal hyperexcitability (Sperfeld et al., 1999). Also, a few studies in animal models of tauopathy reported spontaneous epileptic activity and decreased seizure threshold (García-Cabrero et al., 2013). The genetic ablation of tau reduced epileptic seizure susceptibility by reversing aberrant network activity (Roberson et al., 2007). However, the exact mechanism by which hyperphosphorylated tau contributes to aberrant network activity is still elusive.

Hence, after the histopathological characterisation of our *in vitro* tau pathology model, we investigated whether the neuronal activity was altered in pathological conditions. To characterise the neuronal activity during the progression of the tau pathology in the CA3 region, we performed calcium imaging experiments at 13 DIV and 21 DIV. We analysed the WT, and P301S seeded slices to assess the changes associated with tau pathology and WT, and P301S non seeded slices as a control to evaluate whether the mutation per se had any impact on the neuronal activity.

The characterisation of the neuronal activity is based on the calculation of following parameters: 1) the percentage of active cells during the time of recording (% of active cells); 2) the average number of cells active per peak as a percentage of all cells (Mean % of active cells per peak); 3) the average period of activity per cell (s), 4) the duration of each cellular event (s) and 5) the number of firing events per cell (N).

We first calculated the parameters mentioned above at 13 DIV in the absence of accumulation of phosphorylated tau. We did not find any significant differences among all the parameters in our results: **1)** The percentage of active cells during the recording, (13 DIV; Mean \pm SEM, % of active cells; number of slices; WT non seeded; $26,82 \pm 6,075\%$, n=7 slices; WT seeded; $16,10 \pm 4,534\%$, n=11 slices; P301S non seeded; $12,89 \pm 4,893\%$, n=7 slices; P301S seeded; $14,59 \pm 3,079\%$, n=10 slices; p=0,29); **2)** the average number of cells active per peak as a percentage of all cells, 13 DIV; Mean \pm SEM, Mean % of active cells per peak; number of slices; WT non seeded; $8,07 \pm 1,585\%$, n=7 slices; WT seeded; $3,74 \pm 0,914\%$, n=11 slices; P301S non seeded; $4,10 \pm 1,566\%$, n=7 slices; P301S seeded; $7,54 \pm 1,280\%$, n=10 slices; p=0,15); **3)** the average period of activity per cell (s), 13 DIV; Mean \pm SEM, AVG period of activity per cell (s); number of slices; WT non seeded; $5,87 \pm 1,653$ s, n=7 slices; WT seeded; $5,22 \pm 1,475$ s,

n=11 slices; P301S non seeded; $2,24 \pm 1,109$ s, n=7 slices; P301S seeded; $7,89 \pm 2,230$ s, n=10 slices; p=0,18); **4**) the duration of each event (s), 13 DIV; Mean \pm SEM, AVG event length per cell (s); number of slices; WT non seeded; $1,89 \pm 0,263$ s, n=7 slices; WT seeded; $1,12 \pm 0,278$ s, n=11 slices; P301S non seeded; $1,19 \pm 0,495$ s, n=7 slices; P301S seeded; $1,83 \pm 0,414$ s, n=10 slices; p=0,53); **5**) the number of events per cell (N), 13 DIV; Mean \pm SEM, AVG number of events per cell; number of slices; WT non seeded; $3,11 \pm 0,740$, n=7 slices; WT seeded; $2,90 \pm 0,756$, n=11 slices; P301S non seeded; $1,12 \pm 0,588$, n=7 slices; P301S seeded; $4,02 \pm 0,920$, n=10 slices; p=0,18) (Figure 7).

These data demonstrated for all the conditions that in the absence of tau pathology, the slices showed sparse activity and that the mutation per se does not affect the neuronal activity at 13 DIV.

5.2. The neuronal activity in the CA3 hippocampal region is characterised by hyperactivity and hypersynchrony in P301S seeded slices at 21DIV.

We performed the same analysis in slices at 21 DIV. In this scenario, we identified two populations in P301S slices seeded: one that did not show events where 100% of the cells are firing (the total number of active cells is not 100%), further denominated as Non-Population Event (NPE); and another one that showed at least one event where 100% of the cells are firing the Population Event (PE) (Figure 7.1 A-B).

The P301S seeded PE are significant different from the other conditions regarding the parameters: **1**) The percentage of active cells of active cells during the recording, (21 DIV; Mean \pm SEM, % of active cells; number of slices; WT non seeded; $15,48 \pm 4,083\%$, n=12 slices; WT seeded; $9,02 \pm 1,890\%$, n=12 slices; P301S non seeded; $12,76 \pm 3,466\%$, n=14 slices; P301S seeded NPE; $18,60 \pm 8,024\%$, n=5 slices; P301S seeded PE; $93,25 \pm 4,312\%$, n=9 slices p=0,0001); **2**) the average number of cells active per peak as a percentage of all cells, 21 DIV; Mean \pm SEM, Mean % of active cells per peak; number of slices; WT non seeded; $5,35 \pm 0,929\%$, n=12 slices; WT seeded; $5,227 \pm 0,585\%$, n=12 slices; P301S non seeded; $4,07 \pm 0,935\%$, n=7 slices; P301S seeded NPE; $8,81 \pm 3,719\%$, n=5 slices; P301S seeded PE; $55,74 \pm 7,814\%$, n=9 slices p=0,0001); **3**) the average period of activity per cell (s), 21 DIV; Mean \pm SEM, AVG period of activity per cell (s); number of slices; WT non seeded; $8,44 \pm 3,417$ s, n=12 slices; WT seeded; $8,77 \pm 2,265$ s, n=12 slices; P301S non seeded; $8,48 \pm 3,450$ s, n=14 slices; P301S seeded NPE; $6,7968 \pm 2,59105$ s, n=5 slices; P301S seeded PE; $68,01 \pm 31,36$ s, n=9 slices p=0,008); **4**) the duration of each event (s), 21 DIV; Mean \pm SEM, AVG event length per cell (s); number of slices; WT non seeded; $1,80 \pm 0,339$ s, n=12 slices; WT seeded;

3,60 ± 1,015 s, n=12 slices; P301S non seeded; 1,74 ± 0,609 s, n=14 slices; P301S seeded NPE; 2,27 ± 0,963 s, n=5 slices; P301S seeded PE; 18,12 ± 7,583 s, n=9 slices p=0,005). However, we did not find any differences among all the conditions in regard to the number of events. **5)** the number of firing events per cell (N), 21 DIV; Mean ± SEM, AVG number of events per cell; number of slices; WT non seeded; 4,12 ± 1,470, n=12 slices; WT seeded; 2,76 ± 0,692, n=12 slices; P301S non seeded; 3,61 ± 1,028, n=14 slices; P301S seeded NPE; 2,46 ± 0,941, n=5 slices; P301S seeded PE; 5,66 ± 1,619, n=9 slices; p=0,46) (Figure 7.2).

For the Population event, the plot of the number of events and the average event lengths showed two patterns of activity: multiple short-duration events (pattern 1) and few long-duration events (pattern 2) (Figure 7.1B).

We also calculate the cross coefficient, which defines the correlation across all cell pairs across all periods of firing. This parameter indicates the level of similarity of the neuronal activity between cells, with the coefficient close to 1 indicating high similarity. We calculated the correlation coefficient (Correlation coefficient= 0.781 ± 0.0812), which indicate that the cell pairs firing in the same period displayed similar kinetic of activity.

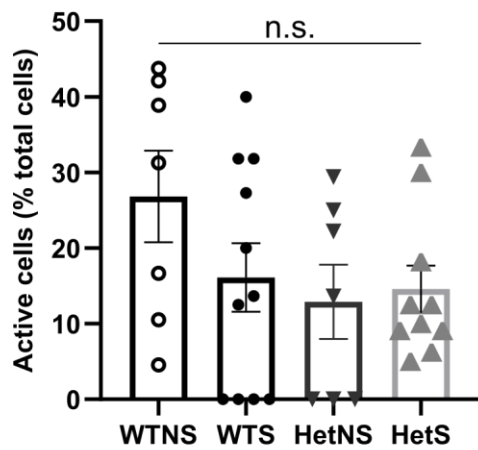
Interestingly, our preliminary results at 19DIV showed that a similar activity is observed in WT, while the P301S seeded slices showed either no activity or hyperactivity: **1)** The percentage of active cells during the recording, 19DIV; % of active cells; WT seeded; slice 1= 23,7%, slice 2= 3,6%; P301S seeded; slice 1= 0%, slice 2= 63,%; slice 3= 0%; **2)** the average number of cells active per peak as a percentage of all cells, 19DIV; % of active cells per peak; WT seeded; slice 1= 18,75%, slice 2= 5,56%; P301S seeded; slice 1= 0%, slice 2= 100%; slice 3= 0%; **3)** the average period of activity per cell (s), 19DIV; AVG period of activity per cell (s); WT seeded; slice 1= 23,8 s, slice 2= 3,6 s; P301S seeded; slice 1= 0 s, slice 2= 63,8 s; slice 3= 0 s; **4)** the duration of each cellular event (s), 19DIV; AVG event length per cell (s); WT seeded; slice 1= 3,25 s, slice 2= 1,8 s; P301S seeded; slice 1= 0 s, slice 2= 8,38 s; slice 3= 0 s; **5)** the number of firing events per cell (N), 19DIV; WT seeded; slice 1= 7,3 , slice 2= 2 s; P301S seeded; slice 1= 0 , slice 2= 7,6 , slice 3= 0) (Figure S3).

Lastly, we used L-CCG-1, an agonist of type 2 mGlu receptors which blocks MF transmission (ref). Our preliminary results showed a reduction in the percentage of active cells as well as the average number of cells active per peak and the average period of activity per cell. **1)** DIV 21; % of active cells; number of cells; P301S seeded PE, 100%, n=1 cell; P301S seeded PE + LCCG-1, 19%, n=1 cell); **2)** the average number of cells active per peak as a percentage of all cells, 21 DIV; Mean % of active cells per peak; number of cells; P301S seeded PE; 65,1%, n=1 cells; P301S seeded PE+ L-CCG-1; 7,1, n=1 cell; **3)** the average period of activity per cell (s), 21 DIV; AVG period of activity per cell (s); number of cells; P301S seeded PE; 308,1 s, n=1 cell; P301S seeded PE + L-CCG-1; 3,5 s, n=1 cell). These data demonstrated that the

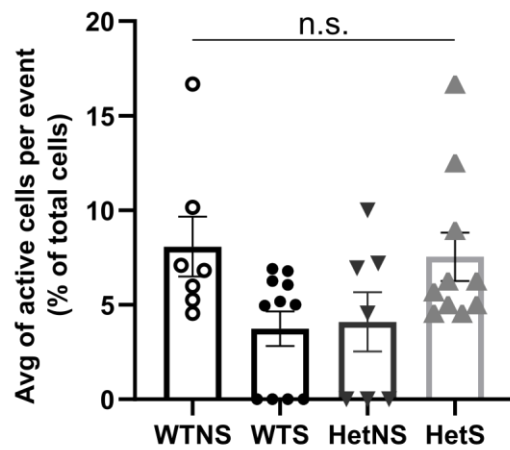
recorded activity reflects the sum of excitatory and inhibitory (see below) inputs to the CA3 region rather than spontaneous discharged of CA3 cells (Figure 7.3).

Neuronal activity at 13 DIV

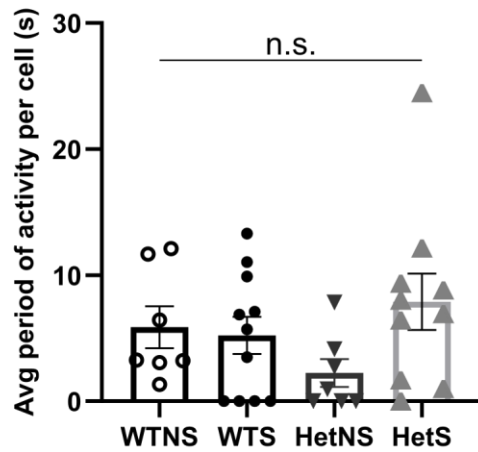
1) Percentage of active cells per recording



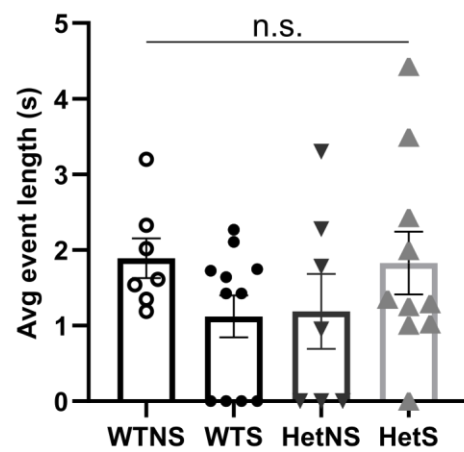
2) Average number of active cells per event



3) Average period of activity per cell



4) Average event length



5) Number of events per cell

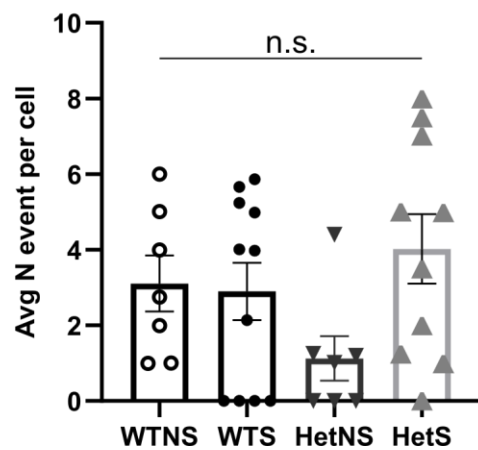
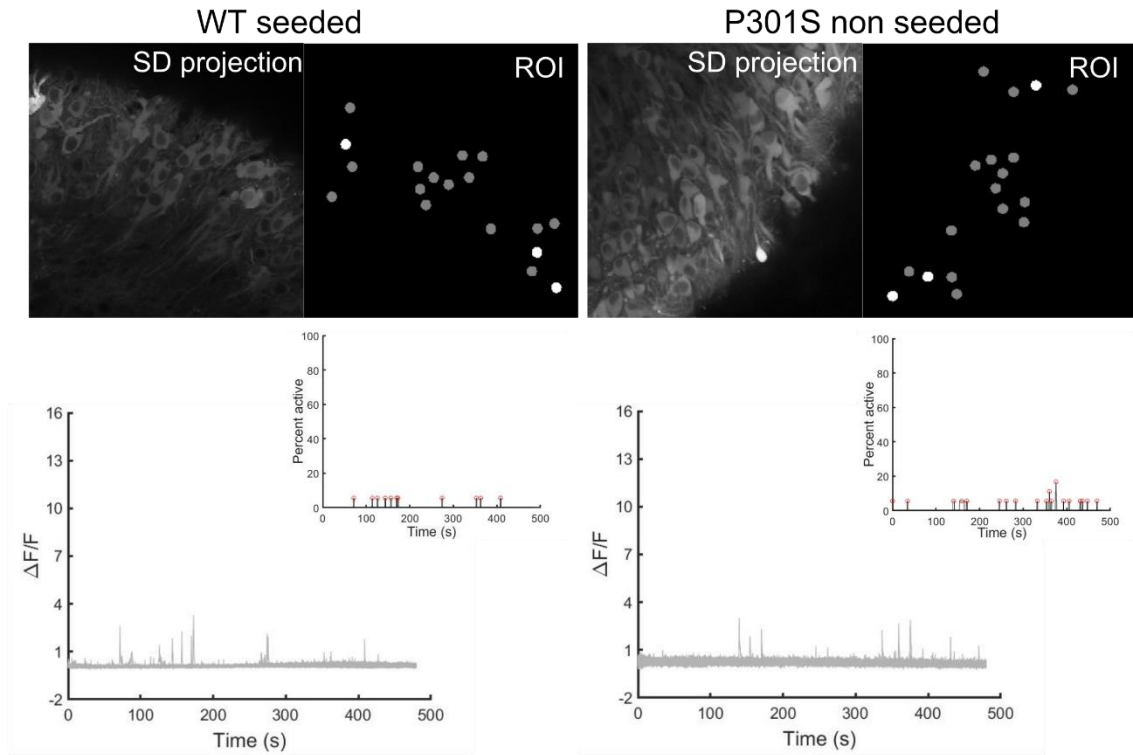


Figure 7. Neuronal activity in the CA3 hippocampal region at 13DIV is characterised by sparse activity in the absence of tau pathology.

Parameters analysed on the characterisation of neuronal activity: **1)** The percentage of active cells during the recording, (% of active cells; WT non-seeded; $26,82 \pm 6,075\%$; WT seeded; $16,10 \pm 4,534\%$; P301S non-seeded; $12,89 \pm 4,893\%$; P301S seeded; $14,59 \pm 3,079\%$; **2)** the average number of cells active per event as a percentage of all cells, WT non-seeded; $8,07 \pm 1,585\%$; WT seeded; $3,74 \pm 0,914\%$; P301S non-seeded; $4,10 \pm 1,566\%$; P301S seeded; $7,54 \pm 1,280\%$; **3)** the average period of activity per cell (s), WT non-seeded; $5,87 \pm 1,653$ s; WT seeded; $5,22 \pm 1,475$ s; P301S non-seeded; $2,24 \pm 1,109$ s; P301S seeded; $7,89 \pm 2,230$ s; **4)** the duration of each cellular event (s), WT non-seeded; $1,89 \pm 0,263$ s; WT seeded; $1,12 \pm 0,278$ s; P301S non-seeded; $1,19 \pm 0,495$ s; P301S seeded; $1,83 \pm 0,414$ s; **5)** the number of firing events per cell (N), WT non-seeded; $3,11 \pm 0,740$; WT seeded; $2,90 \pm 0,756$; P301S non-seeded; $1,12 \pm 0,588$; P301S seeded; $4,02 \pm 0,920$. No differences were found among the conditions, WT seeded, WT non-seeded, P301S seeded and P301S non-seeded. Each symbol represents one slice (WT non-seeded n=7, WT seeded n=11, P301S non-seeded n=7 and P301S seeded n= 10). The graphs represent the mean \pm SEM; the differences between conditions were assessed by Kruskal Wallis ANOVA.

WTNS (WT non-seeded), WTS (WT seeded); HetNS (P301S non-seeded); HetS (P301S seeded)

A



B

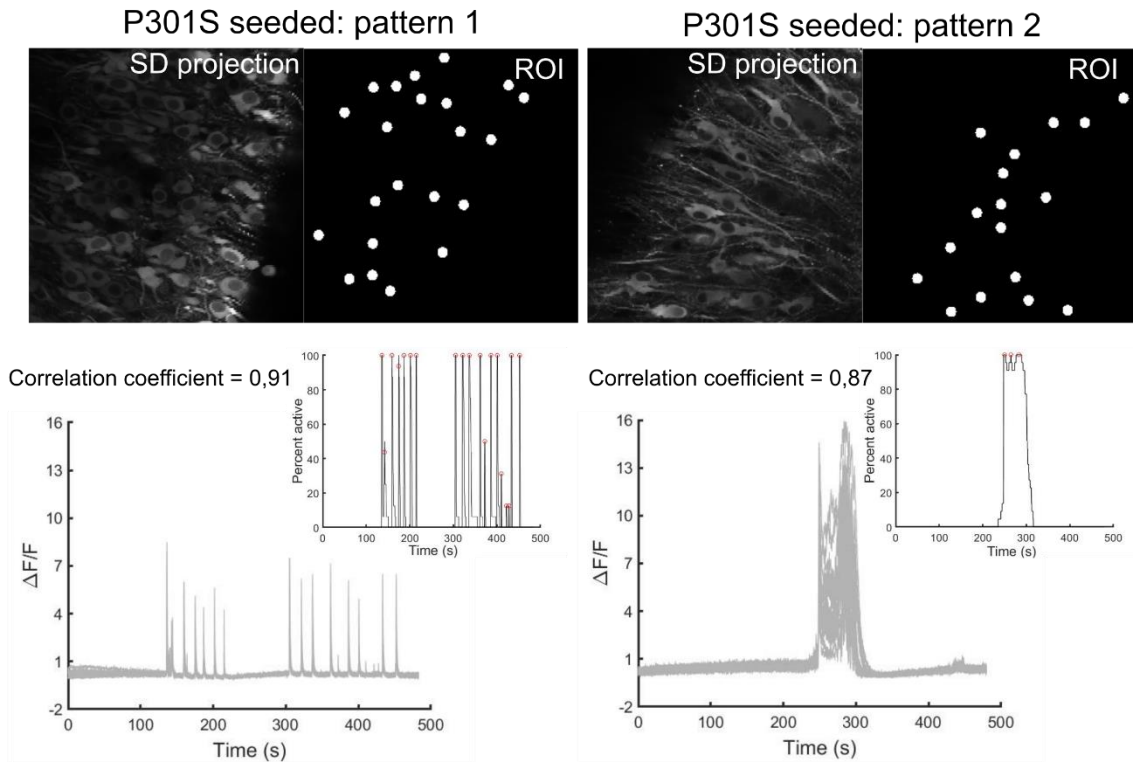
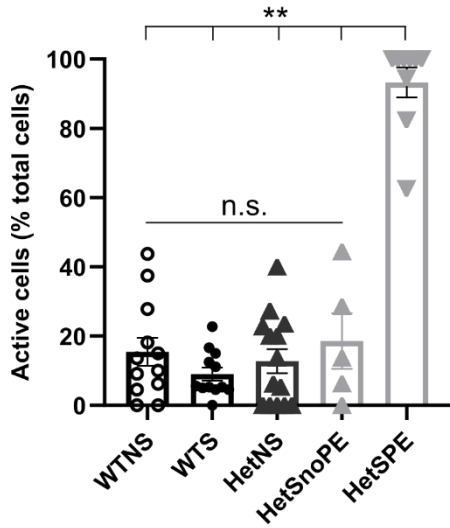


Figure 7.1. Illustration of the analysis of neuronal activity analysis recorded in the CA3 hippocampal region at 21 DIV. A: Top: Projection of the standard deviation of fluorescence intensity on the first 2000 slices (SD Projection) and the activity maps represented by the regions of interest (ROIs) defined in the SD Projection from WT seeded and P301S non-seeded slices. (9-pixels diameter circular ROIs; Grey ROI: inactive cells. White ROI: active cells). Bottom: Representative traces of spontaneous Ca²⁺ transients in the ROI ROI indicated in the top panel ($\Delta F/F$ by scaling to the baseline) and the average number of cells active in a given frame as a percentage of all cells.

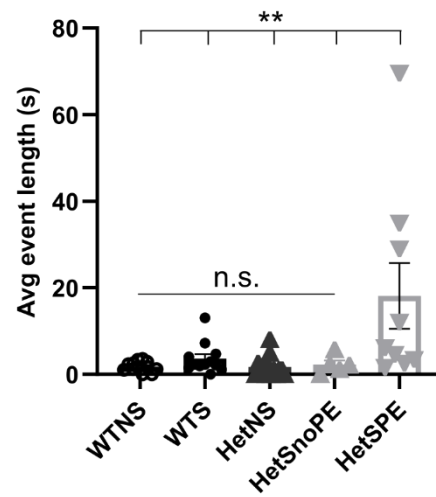
B: Projection of the standard deviation of fluorescence intensity on the first 2000 slices (SD Projection) and the activity maps represented by the regions of interest (ROIs) defined in the SD Projection from P301s seeded displaying two different patterns of activity: pattern 1 characterised by multiple events of short-duration and pattern 2 characterised by fa ew long-duration events (9-pixels diameter circular ROIs; Grey ROI: inactive cells. White ROI: active cells). Bottom: Representative traces of spontaneous Ca²⁺ transients in the ROI indicated in the top panel ($\Delta F/F$ by scaling to the baseline) and the average number of cells active in a given frame as a percentage of all cells. For those slices which exhibit 100% of active cells, a correlation coefficient (CorrCoef) was calculated. Pattern 1: CrossCoef = 0,91 and Pattern 2: CrossCoef = 0,87. A coefficient close to 1 reflects a highly similar activity between cells.

Neuronal activity at 21 DIV

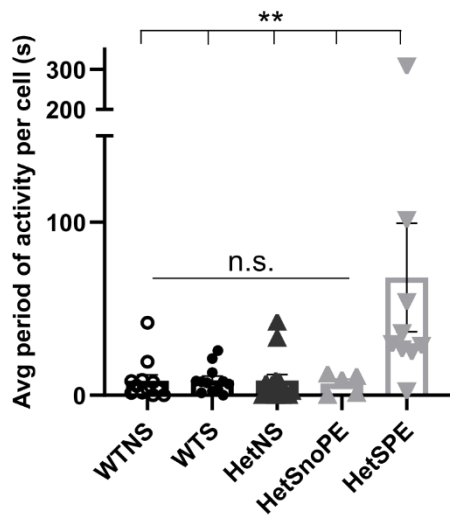
1) Percentage of active cells per recording



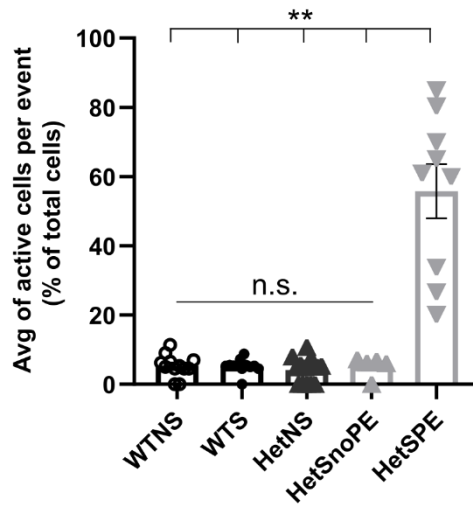
2) Average number of active cells per event



3) Average period of activity per cell



4) Average event length



5) Number of events per cell

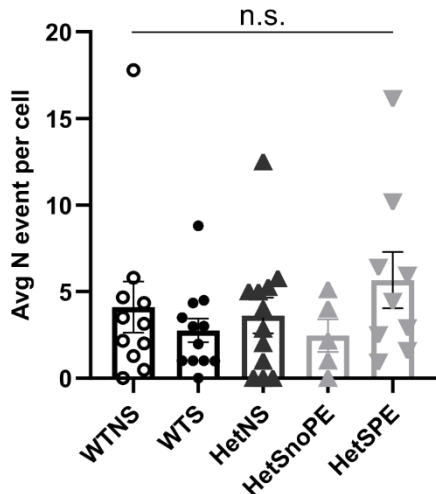
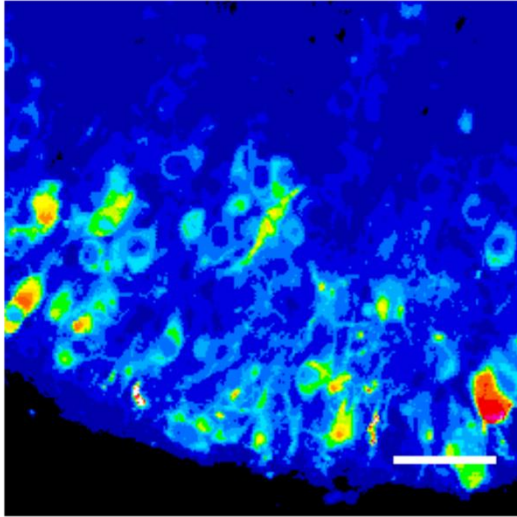


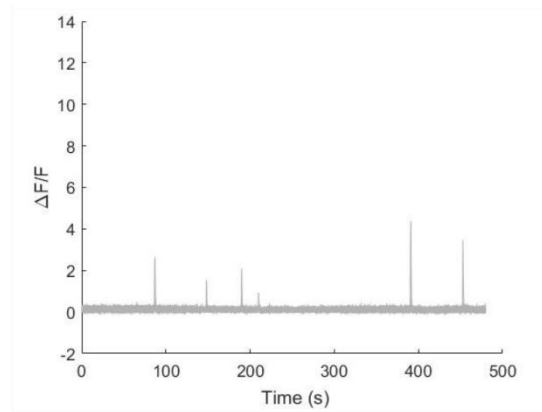
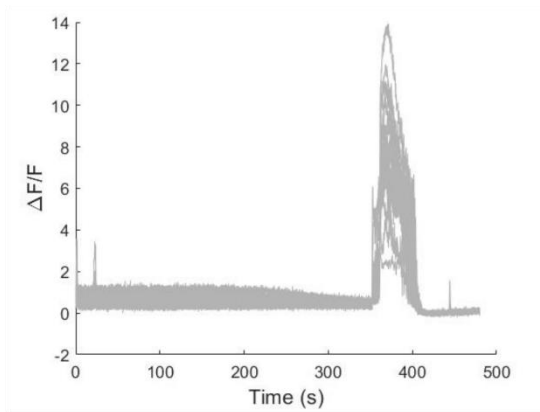
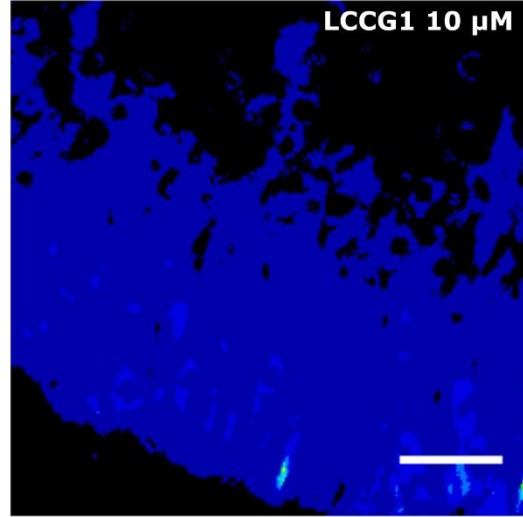
Figure 7.2. Neuronal activity in CA3 hippocampal region at 21 DIV is characterised by hyperactivity and hypersynchrony in P301S seeded slices along with tau pathology. Two population were identified: No Population Event (NPE) which did not exhibit any event where 100% cells are active and Population Event (PE) which exhibit at least one event where 100% of the cells are active. Parameters analysed on the characterisation of neuronal activity: **1)** The percentage of active cells of active cells during the recording, WT non-seeded; $15,48 \pm 4,083\%$; WT seeded; $9,02 \pm 1,890\%$; P301S non-seeded; $12,76 \pm 3,466\%$; P301S seeded NPE; $18,60 \pm 8,024\%$; P301S seeded PE; $93,25 \pm 4,312\%$; **2)** the average number of cells active per peak as a percentage of all cells; WT non seeded; $5,35 \pm 0,929\%$; WT seeded; $5,227 \pm 0,585\%$; P301S non-seeded; $4,07 \pm 0,935\%$; P301S seeded NPE; $8,81 \pm 3,719\%$; P301S seeded PE; $55,74 \pm 7,814\%$; **3)** the average period of activity per cell (s), WT non-seeded; $8,44 \pm 3,417$ s; WT seeded; $8,77 \pm 2,265$ s; P301S non-seeded; $8,48 \pm 3,450$ s; P301S seeded NPE; $6,7968 \pm 2,59105$ s; P301S seeded PE; $68,01 \pm 31,36$ s; **4)** the duration of each cellular event (s); WT non-seeded; $1,80 \pm 0,339$ s; WT seeded; $3,60 \pm 1,015$ s; P301S non-seeded; $1,74 \pm 0,609$ s; P301S seeded NPE; $2,27 \pm 0,963$ s; P301S seeded PE; $18,12 \pm 7,583$ s; **5)** the number of firing events per cell (N); WT non seeded; $4,12 \pm 1,470$; WT seeded; $2,76 \pm 0,692$; P301S non-seeded; $3,61 \pm 1,028$; P301S seeded NPE; $2,46 \pm 0,941$; P301S seeded PE; $5,66 \pm 1,619$. Each symbol represents one slice (WT non-seeded n=12, WT seeded n=12, P301S non-seeded n=14 and P301S seeded NPE n= 5 and P301S seeded PE n=9). The graphs represent the mean \pm SEM; the differences between conditions were assessed by Kruskal Wallis ANOVA and pair-wise comparison using Mann Whitney tests.

WTNS (WT non-seeded), WTS (WT seeded); HetNS (P301S non-seeded); HetS (P301S seeded)

P301S seeded



P301S seeded+LCCG-1

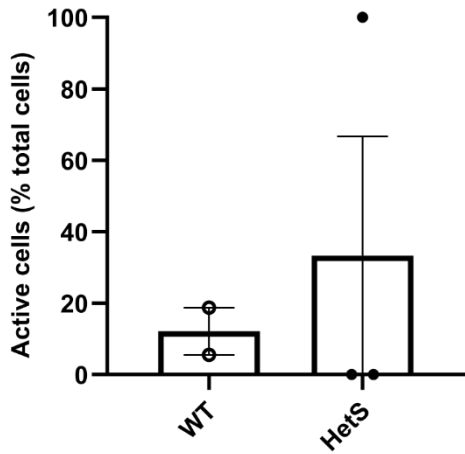


	Het seeded	Het seeded + 10μM LCCG1
% active cells	100	19
Mean % active cells per event	65.1	7.1
Average period of activity per cell (s)	308.1	3.5

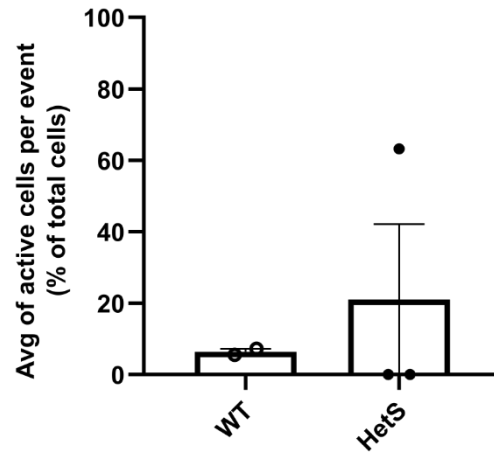
Figure 7.3. Example of neuronal activity recorded in the CA3 hippocampal region at 21 DIV from P301S seeded before and after the inhibition of MF-CA3 transmission with 10 μ M LCCG-1. Top: Projection of the standard deviation of the fluorescence intensity during the time of the record, highlighting a higher proportion of active cells in P301S seeded (% of active cells = 100%; Mean % of active cells per event = 65,1 %; Average of period of activity per cell = 308,1 s) and a decrease of the activity after LCCG-1 treatment seeded (% of active cells = 19%; Mean % of active cells per event = 7,1 %; Average of period of activity per cell = 3,5 s). Bottom: Representative traces of spontaneous Ca²⁺ transients in the slice ($\Delta F/F$ by scaling to the baseline). Scale bar: 30 μ m. Right: 8 min records of calcium signals in CA3 pyramidal cells

Neuronal activity at 19 DIV

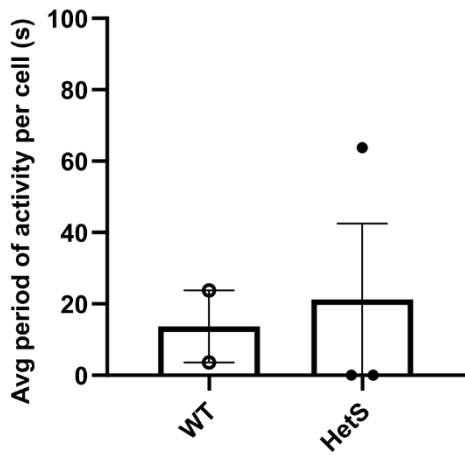
1) Percentage of active cells per recording



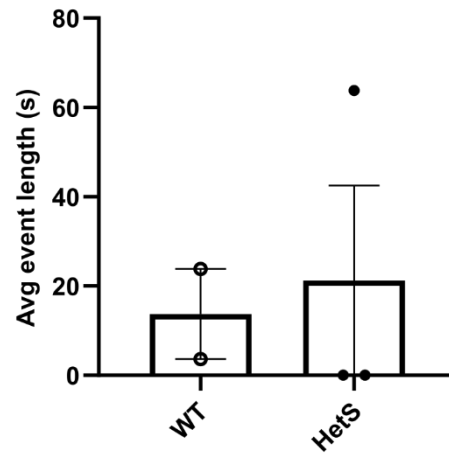
2) Average number of active cells per event



3) Average period of activity per cell



4) Average event length



5) Number of events per cell

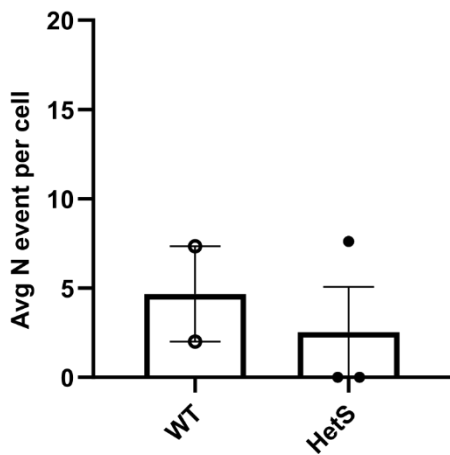


Figure S3. Figure S3. Neuronal activity in the CA3 hippocampal region at 19 DIV is characterised by either no activity or hyperactivity.

The WT slices showed similar activity at 19 DIV than the one observed at 13 DIV and 21 DIV. The P301S seeded slices showed either no activity or hyperactivity (preliminary results): **1)** The percentage of active cells during the recording, 19DIV; % of active cells; WT seeded; slice 1= 23,7%, slice 2= 3,6%; P301S seeded; slice 1= 0%, slice 2= 63,%; slice 3= 0%; **2)** the average number of cells active per peak as a percentage of all cells, 19DIV; % of active cells per peak; WT seeded; slice 1= 18,75%, slice 2= 5,56%; P301S seeded; slice 1= 0%, slice 2= 100%; slice 3= 0%; **3)** the average period of activity per cell (s), 19DIV; AVG period of activity per cell (s); WT seeded; slice 1= 23,8 s, slice 2= 3,6 s; P301S seeded; slice 1= 0 s, slice 2= 63,8 s; slice 3= 0 s; **4)** the duration of each cellular event (s), 19DIV; AVG event length per cell (s); WT seeded; slice 1= 3,25 s, slice 2= 1,8 s; P301S seeded; slice 1= 0 s, slice 2= 8,38 s; slice 3= 0 s; **5)** the number of firing events per cell (N), 19DIV; WT seeded; slice 1= 7,3 , slice 2= 2 s; P301S seeded; slice 1= 0 , slice 2= 7,6 , slice 3= 0). Each symbol represents one slice (WT seeded n=2, and P301S seeded n= 3). The graphs represent the mean \pm SEM.

WTNS (WT non-seeded), WTS (WT seeded); HetNS (P301S non-seeded); HetS (P301S seeded)

6. Pharmacological inhibition of GABA receptors in WT induce a pathological pattern of neuronal activity

6.1. Inhibition GABA A and GABAB receptors

Several studies pointed toward a GABAergic dysfunction as a cause for hyperactivity and hypersynchrony observed in patients and mouse models of AD (Palop et al., 2007; Palop & Mucke, 2010).

Here we first used GABAB antagonist in WT seeded slices, and we observed an hyperactivity and hypersynchrony with longer events after the treatment than those observed in slices displaying tau pathology (Before CGP 55845: % of active cells = 4,54%; Mean % of active cells per event = 4,54 %; Average of period of activity per cell = 22,66s; Average event length = 4,02s; Average number of events per cell = 5,6); after CGP 55845 treatment (% of active cells = 100%; Mean % of active cells per event = 382,03 %; Average of period of activity per cell = 32,7; Average of number of events per active cell=11,7) (Figure 8) .

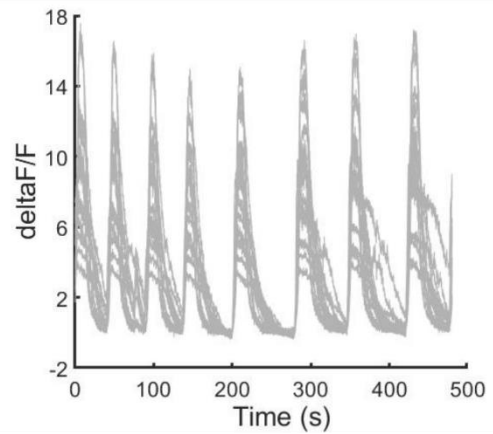
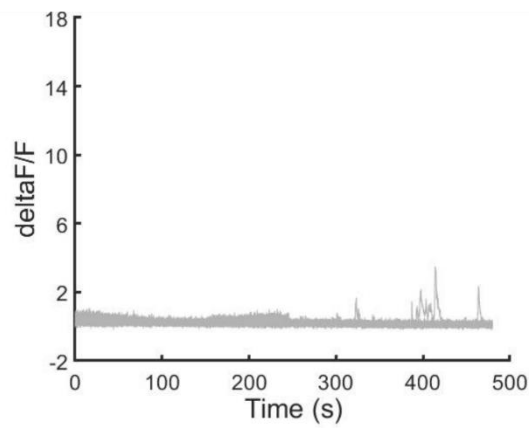
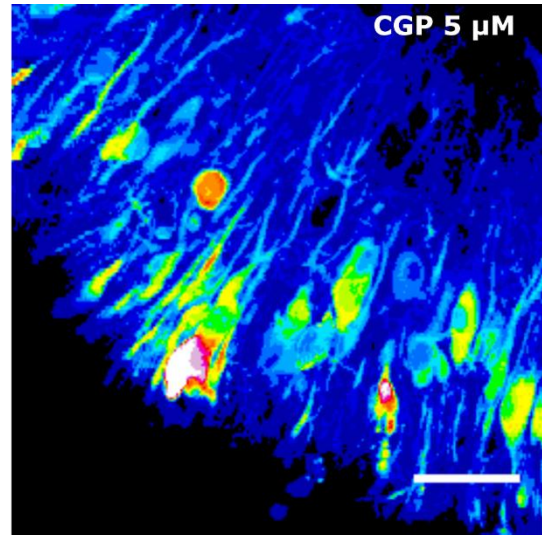
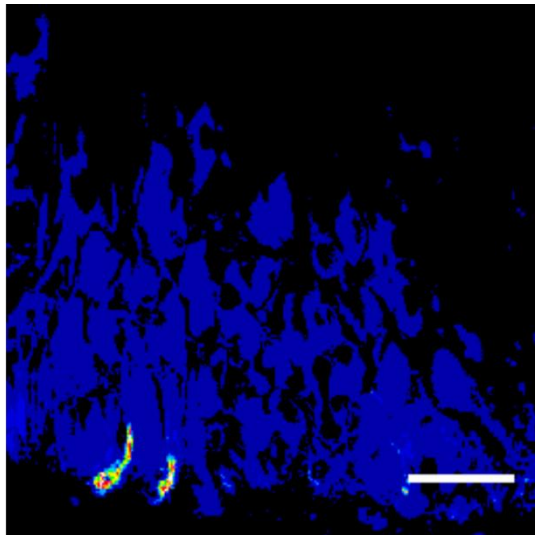
On the other hand, applying, GABAA antagonist, bicuculline in WT seeded and P301S non seeded slices, the neuronal activity displayed similar to this one perceived in P301S seeded pattern 1, characterised by multiple short-duration events (Example P301S non seeded: Before treatment: (% of active cells = 36,36%; Mean % of active cells per event = 5,8 %; Average of period of activity per cell = 15,3s; Average event length = 2,7s; Average number of events per cell = 5,6) and After bicuculline: (% of active cells = 100%; Mean % of active cells per event = 95,95 %; Average of period of activity per cell = 107,9; Average event length = 5,35s; Average of number of events per active cell=20,15s)) (Figure 8.1 middle panel).

Finally, the combination of both, antagonists of GABAA and GABAB receptors in WT seeded and P301S non-seeded slices results in a similar to pattern 2 of activity found in P301S seeded slices observed in CA3 (Example P301S non seeded after bicuculline+CGP: (% of active cells = 100%; Mean % of active cells per event = 70 %; Average of the period of activity per cell = 69,8s; Average event length = 19,1s; Average of the number of events per active cell=3,6). (Figure 8.1, bottom panel).

These preliminary results suggest that the pathological-associated altered activity is may due to the impairment of GABAergic transmission

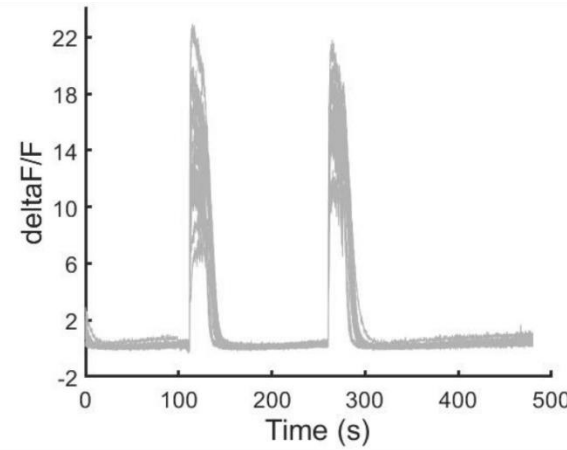
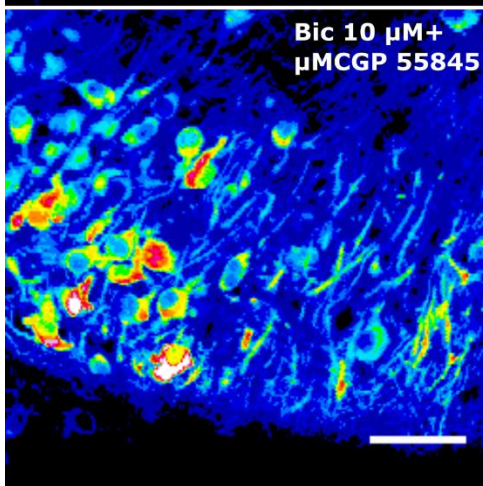
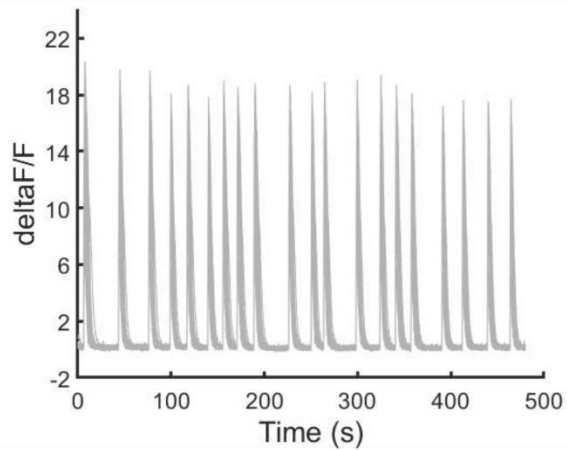
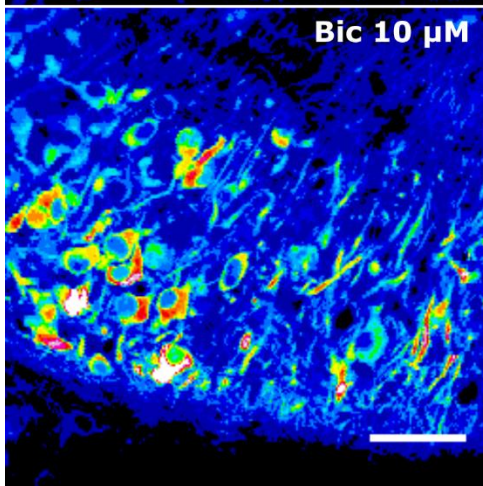
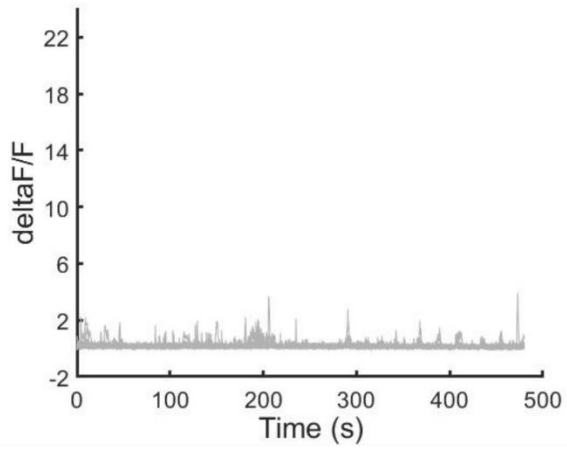
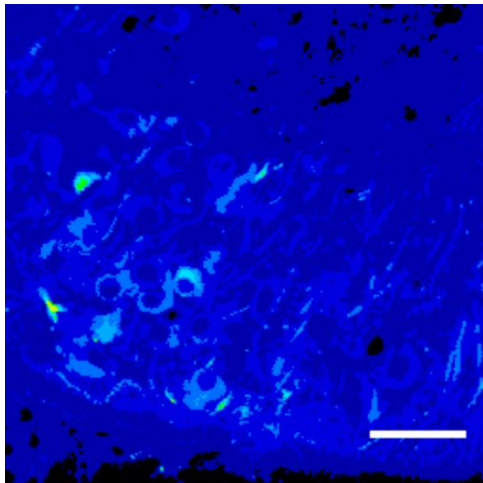
We did not find any effect of GABAergic inhibitors in P301S seeded (Figure 8.2).

Remarkably, preliminary results using Diazepam, which is a positive allosteric modulator of GABA receptors, showed a rescue of the sparse activity. **1)** DIV 21; % of active cells; number of cells; P301S seeded PE, 100%, n=1 cell; P301S seeded PE + DZP, 6,25%, n=1 cell); **2)** the average number of cells active per peak as a percentage of all cells, 21 DIV; Mean % of active cells per peak; number of cells; P301S seeded PE; 26,6%, n=1 cells; P301S seeded PE + DZP; 6,5%, n=1 cell; **3)** the average period of activity per cell (s), 21 DIV; AVG period of activity per cell (s); number of cells; P301S seeded PE; 29,8 s, n=1 cell; P301S seeded PE + DZP; 1,2 s, n=1 cell) (Figure 9).



	Wt seeded	Wt seeded + 5 μM CGP 55845
% active cells	4.54	100
Mean % active cells per event	4.54	89.8
Average period of activity per cell (s)	22.66	382.03
Average event length (s)	4.02	32.7
Average number of events per active cell	5.6	11.7

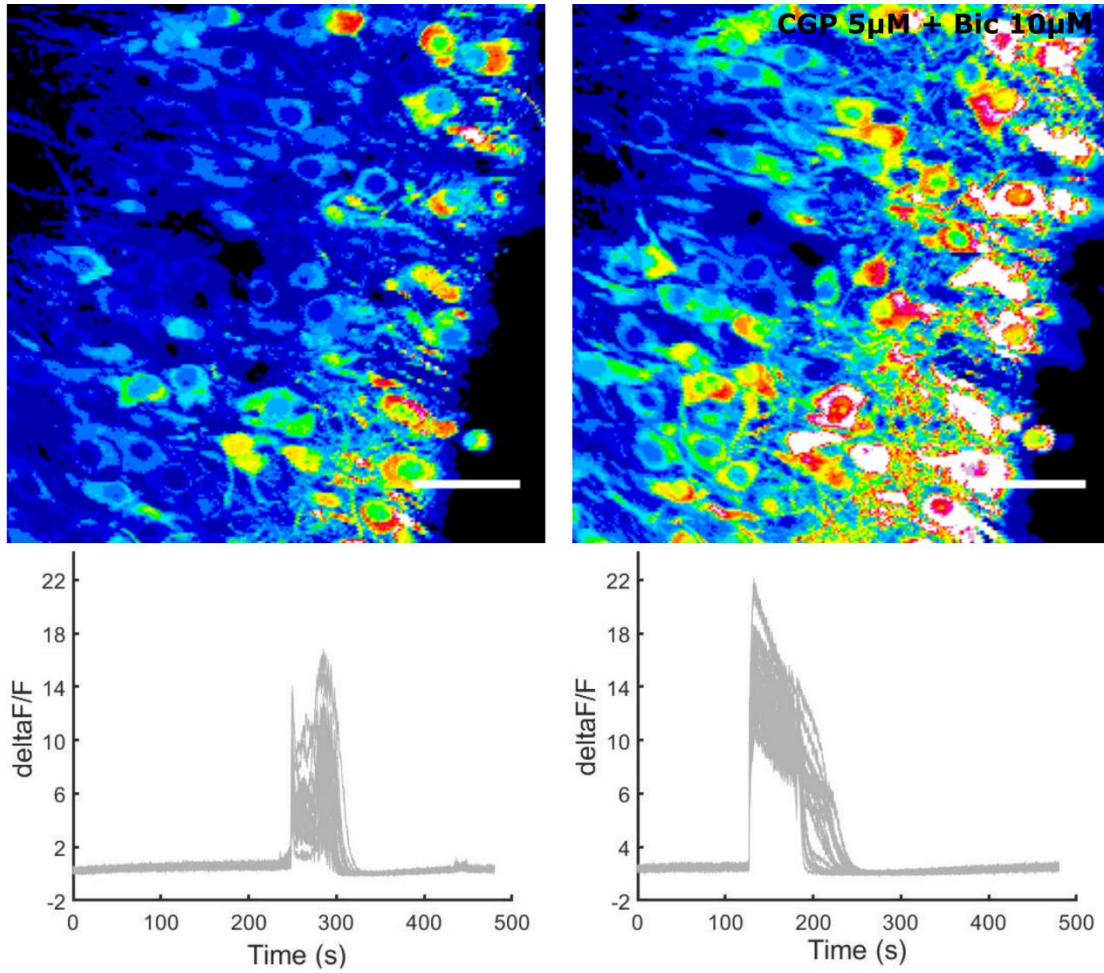
Figure 8. Example of neuronal activity recorded in the CA3 hippocampal region at 21 DIV from WT seeded before and after the inhibition of GABAB receptor (5 μ M CGP). Top: Projection of the standard deviation of the fluorescence intensity during the time of the record, showing lower proportion of active cells in WT seeded (% of active cells = 4,54%; Mean % of active cells per event = 4,54 %; Average of period of activity per cell = 22,66s; Average event length = 4,02s; Average number of events per cell = 5,6) and an increase of the proportion of cells after CGP 55845 treatment (% of active cells = 100%; Mean % of active cells per event = 382,03 %; Average of period of activity per cell = 32,7; Average of number of events per active cell=11,7). Bottom: Representative traces of spontaneous Ca²⁺ transients in the slice ($\Delta F/F$ by scaling to the baseline). Scale bar: 30 μ m. Right: 8 minutes of records of calcium signals in CA3 pyramidal cells.



	Het NS	Het NS + 10 μM Bicuculline	Het NS + 10 μM Bicuculline + 5 μM CGP 55845
% active cells	36.36	100	100
Mean % active cells per event	5.8	95.95	70
Average period of activity per cell (s)	15.3	107.9	69.8
Average event length (s)	2.7	5.35	19.1
Average number of events per active cell	5.6	20.15	3.6

Figure 8.1. Example of neuronal activity recorded in the CA3 hippocampal region at 21 DIV from P301S non seeded before and after the inhibition of GABAA receptor (10 μ M bicuculline) and GABAA and GABAB simultaneously (10 μ M bicuculline + 5 μ M CGP). Top: Projection of the standard deviation of the fluorescence intensity during the time of the record, showing the lower proportion of active cells in P301S non seeded before any treatment (% of active cells = 36,36%; Mean % of active cells per event = 5,8 %; Average of the period of activity per cell = 15,3s; Average event length = 2,7s; Average number of events per cell = 5,6). Middle: increase of the proportion of cells after bicuculline treatment (% of active cells = 100%; Mean % of active cells per event = 95,95 %; Average of period of activity per cell = 107,9; Average event length = 5,35s; Average of number of events per active cell=20,15s) similar to patten1 found in P301S seeded (multiple short-duration events). Bottom: increase of the proportion of cells after bicuculline+CGP treatment (% of active cells = 100%; Mean % of active cells per event = 70 %; Average of period of activity per cell = 69,8s; Average event length = 19,1s; Average of number of events per active cell=3,6) similar to patten 2 found in P301S seeded (few long-duration evens).

The traces represent of spontaneous Ca²⁺ transients in the slice ($\Delta F/F$ by scaling to the baseline). Scale bar: 30 μ m. Right: 8 min records of calcium signals in CA3 pyramidal cells

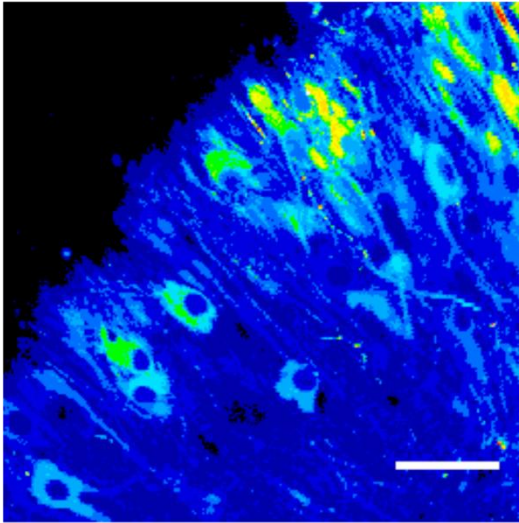


	Het Seeded	Het Seeded + 10µM Bicuculline + 5 µM CGP 55845
% active cells	100	100
Mean % active cells per event	76.1	72.7
Average period of activity per cell (s)	120.3	241.9
Average event length (s)	25	50
Average number of events per active cell	3.05	4.8

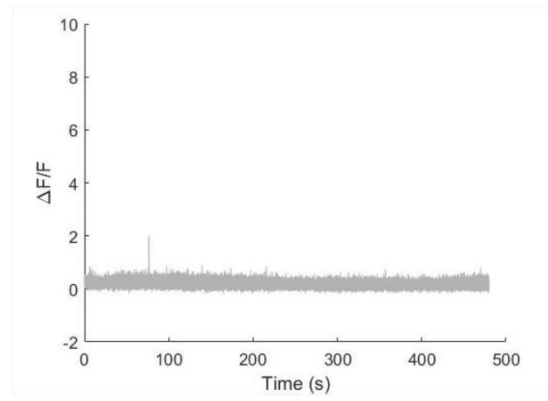
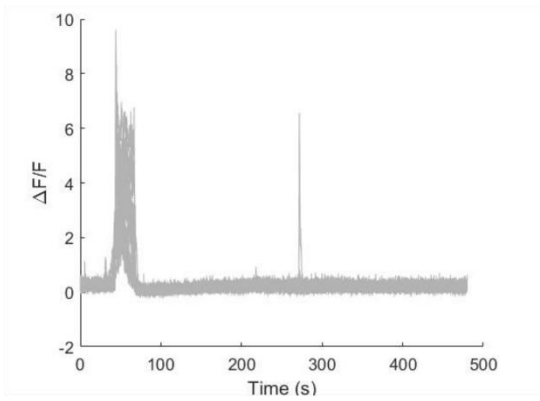
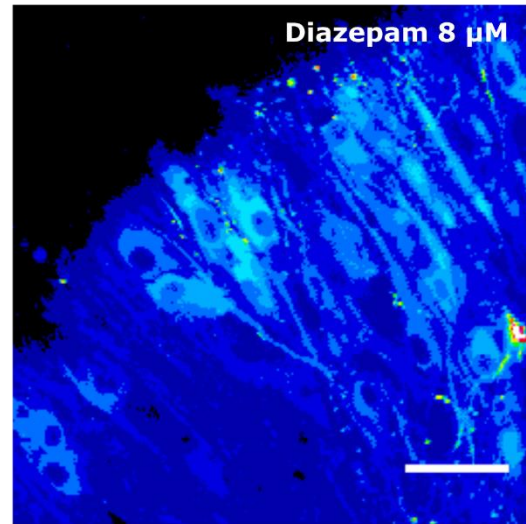
Figure 8.2. Example of neuronal activity recorded in the CA3 hippocampal region at 21 DIV from P301S seeded before and after the inhibition of GABAA and GABAB (10 μ M bicuculine + 5 μ M CGP). Top: Projection of the standard deviation of the fluorescence intensity during the time of the record, showing higher proportion of active cells in P301S seeded before any treatment (% of active cells = 100%; Mean % of active cells per event = 76,1 %; Average of period of activity per cell = 120,3s; Average event length = 25s; Average number of events per cell = 3,05) and after GABAA and GABAB inhibition (% of active cells = 100%; Mean % of active cells per event = 72,7 %; Average of period of activity per cell = 241,9s; Average event length = 50s; Average number of events per cell = 4,8) .

Bottom: Representative traces of spontaneous Ca²⁺ transients in the slice ($\Delta F/F$ by scaling to the baseline). Scale bar: 30 μ m. Right: 8 minutes of records of calcium signals in CA3 pyramidal cells.

P301S seeded



P301S seeded+Diazepam



	Het seeded	Het seeded + 8μM diazepam
% active cells	100	6.25
Mean % active cells per event	26.6	6.25
Average period of activity per cell (s)	29.8	1.2

Figure 9. Example of neuronal activity recorded in the CA3 hippocampal region at 21 DIV from P301S seeded before and after the modulation of GABAergic receptors with 8 μ M Diazepam. Top: Projection of the standard deviation of the fluorescence intensity during the time of the record, highlighting a higher proportion of active cells in P301S seeded slices (% of active cells = 100%; Mean % of active cells per event = 26,6 %; Average of the period of activity per cell = 29,8 s) and a decrease of activity after Diazepam treatment (% of active cells = 6,25%; Mean % of active cells per event = 6,25%; Average of the period of activity per cell = 1,2 s). Bottom: Representative traces of spontaneous Ca²⁺ transients in the slice ($\Delta F/F$ by scaling to the baseline). Scale bar: 30 μ m. Right: 8 min records of calcium signals in CA3 pyramidal cells

7. The progression of the tau pathology and altered activity correlates with neuronal loss in CA3 hippocampal in P301S seeded slices at 21DIV.

7.1. Neuronal loss in the CA3.

Neurodegeneration is a common and recognised hallmark of AD and other tauopathies, which correlates with the progression of the tau pathology and the cognitive deficits in patients. To assess the neuronal loss, we used the anti-NeuN (neuronal nuclear protein). The NeuN protein localises in nuclei and perinuclear cytoplasm of the neurons, and it has been actively used in immunohistochemical experiments to identify neurons. We assessed the number of NeuN positive cells in CA3 in the organotypic slices P301S seeded in comparison to WT seeded at 13 DIV and 21 DIV. We found a significant decrease between WT seeded and P301S seeded at 21 DIV but not at 13 DIV (DIV; Mean \pm SEM, number of NeuN⁺ cells per 10⁻² mm³; WT seeded; 13DIV; 2573,14 \pm 56,223, n=9 slices; P301S seeded; 13 DIV; 2539,025 \pm 124,516; n=8 slices; p=0,9506; WT seeded; 21 DIV; 2720,82 \pm 54,813, n=6 slices; P301S seeded; 21 DIV; 2320,32 \pm 104,701; n=6 slices; p=0,0185).

These results confirmed a neuronal loss in CA3, which correlates with the progression of tau pathology *in vitro*.

7.2. Decrease in PV⁺ interneurons in CA3.

The impairment of GABAergic interneurons seems to play an important role in network dysfunction in AD (Kurudenkandy et al., 2014; Marczyński, 1998). Previous studies demonstrated a loss of GABAergic interneurons induced by tau pathology in the CA1 hippocampal region, which likely compromises the hippocampal GABAergic function (Levenga et al., 2013).

Hence, we first investigated the impact of the tau pathology in the interneurons in CA3. In this sense, we used an AAV vector expressing EGFP and driven by an interneuron specific enhancer (mDlx promotor). The counting of EGFP⁺ interneurons in the CA3 region did not show any differences between slices P301S seeded in comparison to WT seeded at 21 DIV. (Mean \pm SEM, number of EGFP⁺ interneurons per 10⁻² mm³; WT seeded; 170,464 \pm 20,665, n=5 slices; P301S seeded; 136,446 \pm 16,506; n=7 slices; p=0,202). We observed a similar result in the DG at 21 DIV (Mean \pm SEM, number of EGFP⁺ interneurons per 10⁻² mm³; WT seeded; 177,200 \pm 14,326, n=5 slices; P301S seeded; 143,767 \pm 7,229; n=7 slices; p=0,0732).

Even though there is no significant decrease, we observed a tendency for a reduced number of interneurons in CA3. Thus, we analysed one of the major interneuronal populations, which expresses Parvalbumin (PV⁺). The PV⁺ cells have been implicated in the impairment of E/I balance in AD, with functional alteration and loss of interneurons (ref). Therefore, we stained for parvalbumin to investigate whether there is selective neurodegeneration of PV⁺ interneurons.

Our results indicate a significant decreased in PV⁺ interneurons in slices P301S seeded in comparison to WT seeded at 21 DIV in CA3 (Mean \pm SEM, number of PV⁺ interneurons per 10^{-2} mm³; WT seeded; 88,279 \pm 8,093; n=8 slices; P301S seeded; 52,389 \pm 5,275; n=8 slices; p=0,0047) but not in DG (Mean \pm SEM, number of PV⁺ interneurons per 10^{-2} mm³; WT seeded; 54,099 \pm 6,940; n=8 slices; P301S seeded; 44,139 \pm 6,740; n=8 slices; p=0,2786).

Altogether, our results revealed that there is a decrease in PV⁺ interneurons, which correlates with the accumulation of phosphorylated tau. The hyperactivity and hypersynchrony observed in P301S seeded slices may result from the interneuronal loss explicitly found in the CA3 region.

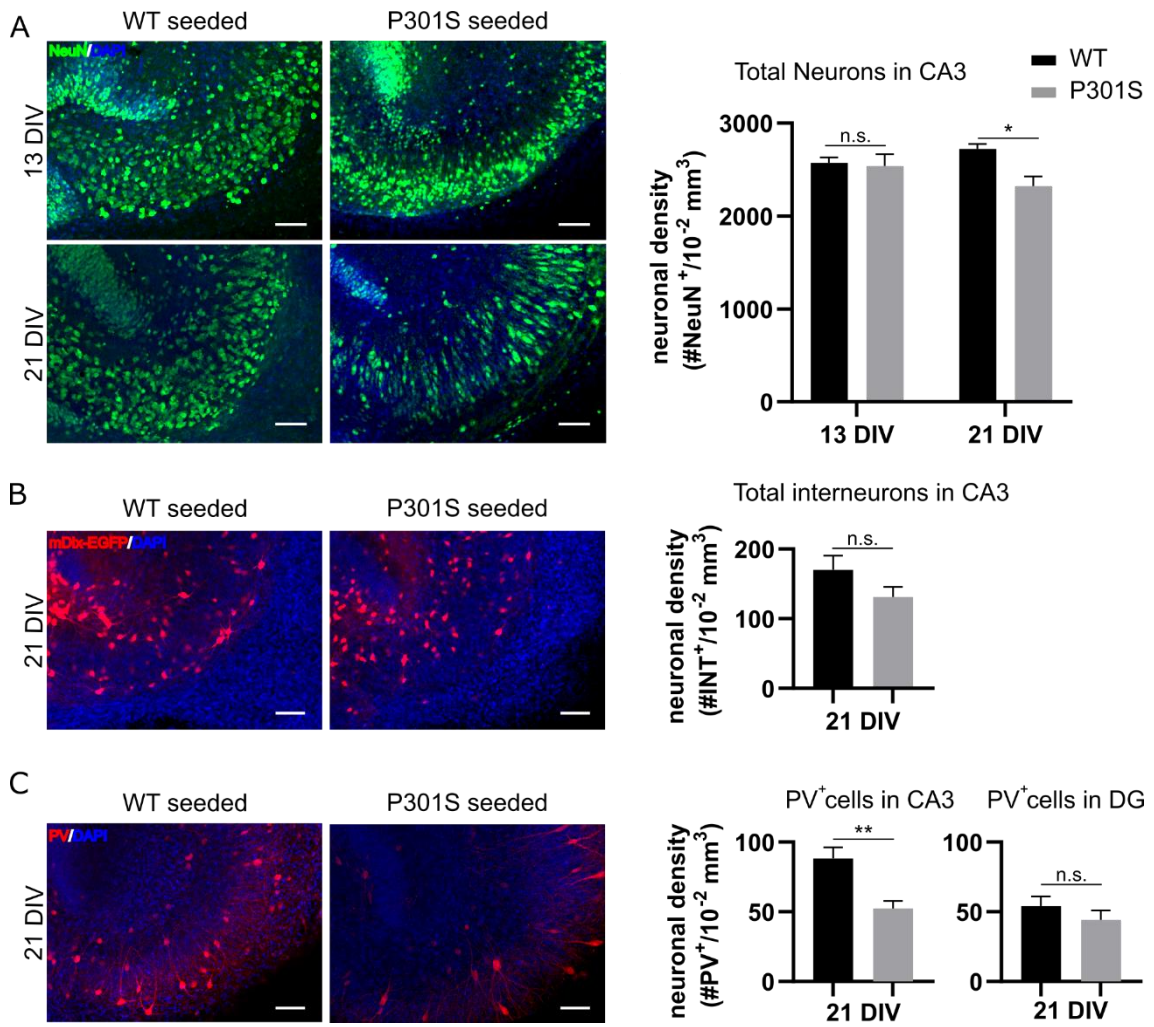


Figure 10. Neurodegeneration in the CA3 region in P301S seeded slices at 21 DIV correlates with tau pathology. A: Quantification of the number of neurons (NeuN positive cells) in the CA3 region in WT and P301S seeded slices at 13 DIV and 21 DIV. B: Expression of EGFP in interneurons in slices infected with AVV-mDlx virus and quantification of the number of interneurons in the CA3 region in WT and P301S seeded slices at 21 DIV. C: Immunostaining and quantification of the number of parvalbumin-positive interneurons (PV⁺) in CA3 and DG region in WT and P301S seeded slices at 21 DIV. Scale bar:100 μ m The graphs represent the mean \pm SEM; the differences between WT and P301S seeded were assessed by Sidak's multiple comparisons test, NeuN⁺ 13 DIV: WT seeded (n=9 slices), P301S seeded (n=8 slices); 21 DIV: WT seeded (n=6 slices), P301S seeded (n=5 slices) and by Mann Whitney test, Total interneurons, 21 DIV: WT seeded (n=5 slices), P301S seeded (n=7 slices) and PV⁺ WT seeded (n=8 slices), P301S seeded (n=8 slices) * p<0.05, ** p<0.01.

Discussion

1. Development of an *in vitro* model of AD-linked tau pathology using organotypic slices:

1.1. Choice of the seeds and the transgenic mice

The primary common histopathological hallmark of AD and other tauopathies is the abnormal accumulation of pathological tau. The progression of tau pathology strongly correlates with cognitive impairment (H. Braak & Braak, 1991), highlighting the critical role of tau in the pathogenesis. The epileptiform activity has also been observed in AD and FTD patients. However, the mechanisms linking the development of tauopathy with the impairment of brain function remain elusive. Animal models expressing FTD-like tau mutations have been used to study tau pathology. To date, there is no tau mutation identified in AD, so other tau mutations are used in AD research which mimics the AD-like progression of the disease. This is the case of the transgenic mice expressing the P301S mutation (Yoshiyama et al., 2007). In this work, we took advantage of the seeding activity exhibited by the P301S transgenic mice to develop an *in vitro* model of tau pathology using organotypic slices.

Organotypic hippocampal slices cultures preserve the anatomy and the physiological synaptic contacts and maintain the different neuronal and non-neuronal cell types representing the brain environment (Stoppini et al., 1991). Additionally, they are easy to manipulate which offers the opportunity to perform several techniques, such as gene transfer, long-term live imaging, immune histochemistry and routine methods in biochemistry such as western blot (reviewed in (Humpel, 2015)).

As demonstrated before, it is possible to induce tau aggregation in organotypic slices from P301S transgenic mice by the addition of synthetic tau fibrils (Stancu et al., 2015).

We confirmed first that the expression of mutated tau (P301S) is not sufficient to induce accumulation of phosphorylated tau in slices after 21 DIV. Then, to establish the optimal conditions to induce progressive tau pathology in slices, we tested K18 tau seeds with different tags, K18-HA, K18-myc and K18-noTag. All of them were able to fibrilize in the presence of heparin and subsequently to induce tau aggregation in cell cultures. However, K18-HA seems to have less efficiency in generating fibrils *in vitro* and in inducing aggregation in cells comparing with K18-myc. When we extended our study to organotypic slices, K18-HA induced robust aggregation only in slices with high expression levels of endogenous mutated tau (slices from homozygous P301S) at 21 DIV.

As we used the same concentration of K18 seeds in the organotypic slices (~1mg/mL, the molecular weight of the seeds being similar: HA= 1.1 kDa, myc= 1.2kDa), we can speculate that the tag interferes with the heparin-induced changes in conformations, affecting the fibrillization process. This is not surprising as HA or myc tags have been shown to affect the bioactivity or the biodistribution of the recombinant tagged protein. Our data also strongly suggest that the seeding efficiency in cells or slices is related to the fibrillization state of the seeds, as measured with ThioT. Further investigations are required to address these questions, which are beyond the scope of the present study.

Even though the K18-HA can induce tau pathology in slices from homozygous P301S, the combination was not kept for the rest of the study due to the difficulties linked to the use of homozygous P301S. Indeed, heterozygous females P301S are poor breeders, and homozygous P301S have a low survival rate. Hence, we considered K18-myc and K18-noTag seeds, that induce tau pathology in slices from heterozygous P301S. Remarkably, at 13 DIV, we detected a high level of tau aggregates in slices seeded with K18-noTag, which is too early if we want to follow the development of the disease in a somehow mature circuit. Indeed, organotypic slices are prepared from brains of animals at postnatal days (P6-P7) as they are more resistant to the mechanical trauma from the culture preparation, which is vital to achieving viable and healthy cultures routinely. However, they reach maturity when cultured for up two weeks, as shown by a distribution of giant MF terminals similar to that found *in vivo* (Dailey et al., 1994), Pouvreau, personal communication). Hence, we kept the combination heterozygous slices/K18-myc seeds, which causes an accumulation of hyperphosphorylated tau between 13 and 21 DIV.

In conclusion, we demonstrated that the ability to induce tau pathology in slices depends on the type of seeds and the expression levels of the mutant tau. We considered the use of K18-myc in organotypic slices from heterozygous as optimal conditions for our purpose

1.2. Development of the tauopathy in ageing mice vs in organotypic slices:

Next, we characterised the pathological features of our *in vitro* model and compared them with the development of the disease *in vivo*. The development of tau pathology in P301S was first fully described between one and twelve months by Yoshiyama et al. (2007). In the hippocampus, they reported an accumulation of hyperphosphorylated tau (PHF1 staining) at six months. The degeneration of CA3 mossy fibers occurred between three and six months. Also, a progressive decrease of synaptophysin, a synaptic vesicle protein widely used to determine synaptic density, was observed along with MF degeneration.

Moreover, P301S mice exhibited a neuronal loss in the hippocampus at eight months (Yoshiyama et al., 2007). Thus, in this work we analysed the hippocampus of P301S aged mice at six, eight and ten months and our tau seeding model of organotypic hippocampal slices at 13 DIV, 19 DIV and 21 DIV. We quantified the accumulation of hyperphosphorylated tau (AT8) in CA1 and CA3, and we found that in both, *in vivo* and *in vitro*, tau pathology appeared progressively in the hippocampus.

In vivo, results showed a similar pattern of increase of hyperphosphorylated tau in the CA1 and CA3 region, which was more pronounced between six and eight months than between eight and ten months. In organotypic slices, CA1 and CA3 were similarly affected between 13 DIV and 19 DIV while the increase in AT8 positive aggregates was more accentuated in CA3 between 19 DIV and 21 DIV (CA1 20,2% CA3 59,8%). This data suggests that CA1 reached the maximal of tau burden before CA3, at 19 DIV. The same effect was observed in MF, which showed a significant increase between 13 DIV and 19 DIV but not between 19 DIV and 21 DIV. Remarkably, we did not find an accumulation of hyperphosphorylated tau in the soma of the granule cells of the DG. These findings are in line with the recent evidence provided by AD human tissue analysis where tau pathology occurs in the axons but not in the cell body of DG granule cells (Christensen et al., 2019b). We also demonstrated MF degeneration in our model and *in vivo*. The differences in the progression of the disease in the hippocampus between ageing animals and organotypic slices might be explained by the fact that the seeding was performed in a non-specific site which makes all regions exposed to K18 seeds (inducer) at the same time. These results suggest that the spreading of pathological tau species along the connected area and the cell-specific vulnerability, two hypotheses used to explain the progressive accumulation of tau aggregates in the different region of the hippocampus *in vivo*, may co-exist and underlie together the development of the disease.

1.3. Different pathological forms of tau in tauopathies

Extensive studies attempted to identify which toxic tau species underlie the pathogenic events during the development of AD and other tauopathies. The misfolded and hyperphosphorylated tau are tightly linked with tau pathology. The altered conformational tau (revealed by the MC1 antibody) was identified as an early species in AD.

There is a 3 fold increase in tau phosphorylation in AD. Several tau phosphorylation sites were characterised and hypothesized to be pathologically relevant (Tenreiro et al., 2014). These phosphorylated sites are associated with tau aggregation. Indeed, as hyperphosphorylation affects the ability of tau to bind to microtubules causing the pre-tangles and NFTs formation (reviewed in (Buée et al., 2000b; Šimić et al., 2016)). Additionally, some of these sites such as

Ser202/Thr205 (AT8); Ser396/Ser404 (PHF1); Thr 231 (AT180); Thr181 (AT270) among others, are known to be abnormally phosphorylated in tau aggregates during the progression of AD but are not phosphorylated in healthy brains (Biernat et al., 1992; Bramblett et al., 1993; M. Goedert et al., 1993; Neddens et al., 2018). The temporal and spatial phosphorylation pattern of tau residue Ser202/Thr205 (AT8) has already been well characterised since it is widely used to determine the staging of AD (H. Braak & Braak, 1991).

Here, we found that our *in vitro* model exhibits different pathological forms of tau associated with AD and other tauopathies. Through immunohistochemistry, we identified pathological conformers of tau (MC1) and disease-associated phosphorylated species (PHF1, AT8). Moreover, we performed a biochemical study to detect phosphorylated tau at the residues Thr181 (AT270) besides the residues Ser202/Ser205 (AT8) and Ser396/Ser404 (PHF1).

Our immunohistochemical analysis suggests that the altered conformational (MC1) may precede the aggregation of hyperphosphorylated tau as detected by PHF1 and AT8. However, western blot analysis showed that hyperphosphorylated tau is present in SDS soluble fraction even in non-seeded slices.

Further investigations are needed to characterise the apparition of different tau species during the development of the pathology

1.4. Cell-specific vulnerability in tauopathy:

Identifying which cells are more vulnerable to tau pathology has been a challenge since the initial analysis performed in post-mortem human AD brains. Several studies have addressed this question; however, the results are still controversial. For instance, the misfolded tau (MC1 staining) is present in interneurons (PV⁺ and SST⁺) in the hippocampus of a tauopathy model expressing the P301L mutations (Levenga et al., 2013). Conversely, in the EC of a transgenic model with the same mutation but restricted to EC-II, the same population of interneurons did not exhibit MC1 signal (Fu et al., 2019). Also, a study using the transgenic model VLW, expressing three mutations related to FTDP-17: G272V (V), P301L (L) and R406W (W) showed accumulation of tau phosphorylated at residues Thr231 (AT180) in hippocampal PV interneurons but not at residues Ser202/Thr205 (AT8) (Dávila-Bouziguet et al., 2019; Soler et al., 2017).

The contradictory results found across these studies could be due to the different transgenic models used, to the brain regions analysed and the age of the animals or even to the pathological forms of tau investigated.

To our knowledge, whether P301S mutation can lead to an accumulation of hyperphosphorylated tau in interneurons was still unknown. Thus, in this study, we investigated the accumulation of hyperphosphorylated tau (AT8) *in vivo* and *in vitro*.

We did not find any accumulation of hyperphosphorylated tau aggregates (AT8) neither in astrocytes, microglia or PV interneurons.

In line with previous observations in CA1 (Soler et al., 2017), we observed AT8 staining in CA3 pyramidal cells but not in PV+ cells which suggests that the excitatory neurons are more prone to accumulate hyperphosphorylated tau (AT8) than the inhibitory ones. Nevertheless, we cannot exclude the hypothesis that in our model, interneurons could gather different toxic species of tau, as mentioned above.

In summary, we established a reliable and reproducible *in vitro* model of tauopathy, which recapitulates in three weeks the pathogenesis in transgenic mice between six and ten months. This model allowed us to characterise the temporo-regional appearance of various pathological forms of tau, their cell specificity, and to correlate the tau pathology with the progressive alteration of the hippocampus anatomy, such as excitatory or inhibitory neuronal loss, and attrition of the mossy fiber bundle. Our next step was to investigate the potential alteration of hippocampal neuronal activity and to correlate them with the histopathological hallmarks of the disease.

2. Characterisation of the mechanisms underlying the altered neuronal activity.

Network hypersynchrony often results in seizures or epileptiform discharges and are observed in AD and FTD mice models and AD and FTD patients (de Waal et al., 2012; Goutagny et al., 2013; Irizarry et al., 2012; Lozsadi & Lerner, 2006; Palop et al., 2007; Palop & Mucke, 2016; Vogt et al., 2011). Additionally, AD patients show hippocampal hyperexcitability even at early stages, and the altered activity in CA3 can predict the development of AD (Dickerson et al., 2005; R. Sperling, 2007; S. L. Miller et al., 2008). However, the link between hippocampal hyperactivity and the development of the disease is still misunderstood.

The respective roles of amyloid and tau pathology in the alteration of the network activity have been discussed. Several transgenic mouse models expressing FAD-associated mutations (APP and PS1) linked to the amyloid pathology display network hyperactivity and hypersynchrony (reviewed in (Palop & Mucke, 2016)). On the other hand, the ablation of tau in AD mouse lines prevents seizures (DeVos et al., 2013) highlighting the critical role of tau in the regulation of network activity. So far, the number of studies in mouse models of tauopathy is limited. However, the findings seem to point toward the involvement of tau in abnormal activity

in pathological conditions. For instance, P301L transgenic mice display spontaneous seizures at five months of age, before tau aggregates are present (García-Cabrero et al., 2013). More recently, two *in vivo* studies using transgenic mice which express the P301S mutation addressed the potential contribution of tau pathology to the generation of seizures. The study using the transgenic mice Tau58/4 found that the tau pathology is sufficient to enhance the seizure susceptibility to the convulsant drug pentylentetrazole (PTZ) (Van Erum et al., 2020). The second study showed that the THY-Tau22 mouse model displays subclinical seizures and increased PTZ-induced seizure susceptibility (Gomez-Murcia et al., 2020). In agreement, both studies demonstrated higher seizure susceptibility in transgenic mice associated with tau pathology.

Still, the link between the hippocampal hyperactivity and the development of tau pathology is poorly understood.

In this work, we used our *in vitro* model of tau pathology, which allows assessing the neuronal activity in preserved hippocampal circuits. We performed calcium imaging in the CA3 region. At 13 DIV, we observed similar neuronal activity between WT and P301S slices, seeded or non-seeded, which at this stage did not exhibit any sign of pathology.

On the other hand, at 21 DIV, we identified two populations in slices which developed the disease (P301S seeded). One small population, which represent 35% of the P301S seeded slices, showed typical neuronal activity, the same found in slices that do not display accumulation of phosphorylated tau: WT non seeded and seeded and P301S non seeded. On the other hand, 65% of the P301S seeded slices displayed altered neuronal activity characterised by hyperactivity and hypersynchrony, with two characteristic patterns: multiple but short duration events (pattern 1) and few long-duration events (pattern 2).

The hypothesis that the imbalance of E/I is an essential mechanism that leads to AD has gained major attention in the last decades (Busche & Konnerth, 2016; Frere & Slutsky, 2018b; Palop et al., 2006; Palop & Mucke, 2016). A body of evidence streaming from the AD mouse models has pointed towards a downregulated GABA interneurons activity as a critical factor in the disease process and a likely mechanism underlying the abnormal network activity (Frere & Slutsky, 2018b; Palop et al., 2006; Palop & Mucke, 2016). Inhibitory synaptic transmission might also be affected.

Also, another hypothesis to explain the increase of hyperexcitability in hippocampal neurons is the altered chloride homeostasis. Under physiological conditions in a mature brain, the chloride flow into the neurons in the direction of the chloride equilibrium potential is mediated by the activation of GABAA receptors, and hyperpolarize the neuron (Ganguly et al., 2001; Misgeld et al., 1986). However, at higher intracellular chloride concentrations, chloride flows in the

opposite direction and depolarises neurons. This overload of chloride is observed in the developing brain (Y. Ben-Ari et al., 1989, 1997; Gao & van den Pol, 2001) and in pathological conditions such as epilepsy (Yehezkel Ben-Ari, 2006; Raimondo et al., 2015). The chloride overload increases the probability of generating seizures and the excitability of the neurons by GABAA receptor-mediated mechanisms (Mahn et al., 2016; Raimondo et al., 2012). A recent study demonstrated that in situations with higher intracellular concentrations of chloride, the AP threshold can significantly change. This may represent another mechanism of cellular and network excitability regulation (Sørensen et al., 2017). Thus, both altered chloride homeostasis and decrease in the AP threshold might contribute to network hyperexcitability in pathological conditions when intracellular concentration of chloride is strikingly higher compared to physiologic conditions.

Hence, to investigate whether GABAergic impairment causes the abnormal neuronal activity associated with tau pathology that we observed, we used GABAA and GABAB antagonists, bicuculline and CGP respectively, in WT slices. Interestingly, we found that the inhibition of GABAA receptor in WT slices and P301S non seeded slices, at 13 DIV and 21 DIV, produces a pattern of activity similar to the pattern 1 described in 21 DIV P30S seeded slices, with multiple but short duration population events. Moreover, the inhibition of GABAA and GABAB in WT slices and P301S non-seeded slices at both age mimics the neuronal activity observed in P301S seeded slices displaying pattern 2: a few long-duration events.

These results show first that excitatory and inhibitory inputs at 13 DIV are similar to the ones at 21 DIV, and that the absence of effect on neuronal activity in P301S seeded slices most likely results from an absence of the pathological factor rather than the immaturity of the circuit. Secondly, our data suggest that an impairment of GABAergic inhibition, involving both receptors, GABAA and GABAB, causes the changes in neuronal activity associated with the tau pathology. Moreover, our preliminary results using Diazepam suggest that there is a rescue of neuronal activity to typical patterns after treatment. Thereby, the hyperactivity and hypersynchrony are likely caused by impairment of GABAergic transmission rather than altered chloride homeostasis. Indeed, diazepam is an allosteric modulator which enhances GABA receptor function. If there were an alteration in chloride homeostasis, the inhibition of GABAergic transmission would increase the hyperexcitability of CA3 neurons, which we did not observe.

In AD patients, the brain regions affected by the disease, such as EC and hippocampus, showed a decrease in the number of PV+ neurons (H. Arai et al., 1987; D. R. Brady & Mufson, 1997; Mikkonen et al., 1999b; Solodkin et al., 1996; H. Takahashi et al., 2010). Likewise, the transgenic mice bearing the mutations APP/PS1, MAPT or the triple mutation APP/PS1/MAPT,

display an interneuronal loss (Levenga et al., 2013; Loreth et al., 2012; Rubio et al., 2012). Interestingly, in the hAPP (J20) mice, the network hypersynchrony results from PV cell dysfunction (Verret et al., 2012) and the reduction of tau prevented excitotoxicity (Roberson et al., 2007). Together this evidence might suggest the involvement of tau in the PV-mediated occurrence of network hyperexcitability. Moreover, the GABAergic interneuron loss (PV⁺ and SST⁺) induced by tau pathology was observed in the P301L transgenic model, thereby demonstrating the implication of tau pathology and GABAergic impairment in the imbalance of neuronal signalling networks (Levenga et al., 2013). Besides, recent studies proposed that the rescue of PV cell numbers either by an enriched environment (Cattaud et al., 2018) or pharmacologically (Q. Zhang et al., 2018) could improve cognitive impairments. Collectively, these findings suggest the loss of PV interneurons as a critical factor involved in the pathogenesis of the disease and the generation of abnormal neuronal activity. Our results showed a reduction in PV⁺ cells in CA3 but not in DG, indicating that the PV⁺ cell loss might contribute to the hyperactivity and hypersynchrony observed along with tau pathology.

To conclude, our work demonstrated that network hyperactivity and hypersynchrony are correlated with the progression of tau pathology and is likely caused by the loss of PV interneurons and/or the impairment of GABAergic transmission involving the dysfunction of GABAA and GABAB receptors.

Conclusions and perspectives

My PhD work was structured around 3 main goals:

1. To develop an *in vitro* model of AD-linked tauopathy that would allow us to study, in a preserved hippocampal circuit with high spatiotemporal resolution, the impairments in hippocampal network activity and synaptic function
2. To use this model to unravel the mechanisms underlying the hyperactivity and hypersynchrony observed *in vivo* in animals models of tauopathy or of AD.
3. To study mitochondrial impairments in the P301S mouse.

Aim 1: Development of an in vitro model of AD-linked tauopathy

To be relevant, the model had to meet the following criteria:

- be related to an area of the brain affected in AD
- keep the synaptic contacts and the variability of brain cells found *in vivo*
- reproduce the main features of the disease progression as observed *in vivo*
- be highly reproducible
- allow the definition of time points highly preserved between preparations (for instance: the appearance of aggregates in the different regions between 13 and 21 DIV, cell death between 19 and 21 DIV.)

We chose to develop our model from organotypic slices as:

- they preserve tissue architecture, main cell types and synaptic connections.
- they are amenable to most techniques used to study neuronal activity and synaptic transmission, such as calcium imaging, electrophysiology, super-resolution imaging, gene transfer and viral strategies, as well as classical techniques of molecular biology and biochemistry.
- they can be kept in culture for months (Gogolla et al., 2006), allowing the experimenter to follow the development of the disease.
- tau aggregation has been demonstrated in organotypic slices following tau seeding (Stancu et al., 2015)

By choosing the appropriate combination of tau seeds and mouse genotype, we have been able to establish a model that progressively develops tau aggregates in a different region of the hippocampus, following a pattern very similar to what is observed in the ageing animal. The tau aggregates appear after 13 DIV, at a stage where the distribution and morphology of giant MF terminals resembles what is observed *in vivo* (Michael E. Dailey 1994, Pouvreau,

personal communication). The pathology then invades the hippocampus between 13 and 21 DIV, following a very precise temporality. We have been able to define highly stable time points where the pathology, in the form of tau aggregates, can be observed in the different regions. Hence, the model can now be used to study the link between tau aggregates or other pathological species and impairment of neuronal activity, synaptic function, axonal transport, mitochondrial morphology, or any other cellular pathways that are involved in tauopathies. The model is also very convenient to study the question of the spreading of tauopathy, for instance, by targeted injection of tau seeds or local expression of different tau mutants using targeted virus injection. Also, the model is highly amenable to test long term pharmacological treatment or therapeutic strategies that can be more complex *in vivo* due to specific limitation such as the crossing of the blood-brain barrier or the animal immune system (this is the case for antibodies targeted against pathological forms of tau, for instance).

In our case, the model was used to reveal and characterise the progressive impairment in CA3 pyramidal cells activity in tauopathy.

Aim 2: Characterisation of the progressive impairment of CA3 neuronal circuits in our *in vitro* model of tauopathy

We have been able to show that the neuronal circuit of the CA3 is progressively affected as the tau aggregates propagate in the hippocampus. The main impairments are:

- a loss of CA3 pyramidal cells and interneurons (PV⁺),
- a loss of neuronal connections (attrition of the MF bundle connecting DG granule cells and CA3 pyramidal cells, as well as DG granule cells and several types of interneurons (review (Rebola et al., 2017))
- an alteration in the activity of CA3 pyramidal cells.

Anatomical features were observed *in vitro* and in the ageing animal, to validate the fact that our observations were following what happens *in vivo*. Although other pathological tau species are present way ahead of the tau aggregates, all the alterations of the CA3 circuit seem to temporally match the appearance of tau aggregates, which begs the question: are tau aggregates responsible for the observed deficit, or are they a consequence of other mechanisms that also triggers neurodegeneration and impairment of neuronal activity. Further investigation will be needed to answer the question. One possibility would be the use of immunotherapy to allow the clearance of pathological forms of tau and the decrease of the insoluble tau fraction (Troquier et al., 2012).

Regarding the CA3 circuit, our data show that the pyramidal cells activity is recorded between 13 and 21 DIV results from a balance between excitatory and inhibitory inputs contacting CA3 cells rather than from spontaneous depolarization of the cells. Indeed, inhibition of DG-CA3

connection with LCCG dramatically decreases the recorded activity. In addition, inhibition of GABAergic transmission causes hyperactivity in WT slices and Het non-seeded slices at 13 and 21 DIV. This also shows that the E/I balance is already established at 13 DIV. Regarding the effect of the tauopathy, hyperactivity and hypersynchrony are found in seeded organotypic slices from P301S mice, but not WT seeded or Het non-seeded slices. This phenotype can, however, be reproduced in these slices by inhibition of either GABAA receptor or of GABAA and GABAB receptors. Besides, inhibition of GABAergic transmission in P301S seeded slices does not worsen the phenotype. This demonstrates that the inhibitory inputs are severely impaired in our model of tauopathy. This could be due to a disruption of GABAergic transmission or the postsynaptic chloride homeostasis (M. Chen et al., 2017). However, the fact that diazepam, a positive allosteric modulator of GABAA receptor rescue our phenotype favours more the hypothesis of an impairment of the synaptic transmission or the interneuron excitability. Impairment of neurotransmitter synthesis has been reported in AD (Le Douce et al., 2020). As the phenotype we observed in ¼ of the slices is reproduced by both inhibition of GABAA and GABAB transmission, we plan to measure the neurotransmitter levels in our organotypic slices. However, preliminary data we just got suggest that GABA synthesis is not affected in our model. The next steps will be to test excitatory and inhibitory inputs, as well as interneurons intrinsic properties, using electrophysiology. In addition, we are expecting results from RNA sequencing that we started a few months ago. These data would allow us to determine whether pathways involved in synaptic function or maintenance of membrane potential are affected in our model.

Aim 3: Characterisation of mitochondrial impairment in P301S mice:

Our data show an impairment of mitochondrial respiration, mitochondria-ER contacts and levels of proteins involved in mitochondria-ER contacts (IP3R, Tom70) in P301S mice before the appearance of tau aggregates (some of these features appear at P22). The role of mitochondria in the regulation of neuronal activity, especially in the control of synaptic function and ionic homeostasis, is being increasingly recognised (Devine & Kittler, 2018). Recent evidence also suggests that mitochondria might control hippocampus firing activity (Styr et al., 2019). Hence, one appealing hypothesis would be that the mitochondrial disorders observed in young animals might participate to, if not cause, the impairment in neuronal activity. Further studies will, of course, be needed to assess this hypothesis, and our *in vitro* model, which allows organelle imaging, electrophysiology and easy metabolites supplementation, will be an excellent platform for testing different pathways.

Annexes

Side project

Introduction

Alzheimer's disease (AD) is characterised by brain deposition of amyloid plaques and tau neurofibrillary tangle. Remarkably, the propagation of tau aggregates is strongly correlated with AD-associated cognitive decline and synaptic loss (H. Braak & Braak, 1991). Mitochondria play an essential role in synaptic function. In presynaptic terminals, mitochondria are responsible for regulating Ca^{2+} homeostasis and producing ATP, both critical elements in neurotransmission. Mitochondrial impairment is one of the earliest features in AD, and both $\text{A}\beta$ and pathological tau can trigger mitochondrial alterations. Various studies point toward mitochondria as a critical target of pathological forms of tau. For instance, the accumulation of hyperphosphorylated tau impairs the regulation of mitochondrial dynamics and altered their distribution (DuBoff et al., 2012; Kopeikina et al., 2011). Moreover, in pathological conditions such as AD, mitochondria transport dynamics (Stoothoff et al., 2009) and functions are impaired (David et al., 2005).

The crucial role of mitochondria in the regulation of Ca^{2+} homeostasis has been the subject of extensive studies. The outer mitochondrial membrane is in contact with a subregion of the endoplasmic reticulum (ER) known as mitochondria-associated ER membranes (MAMs). MAMs are intracellular lipid rafts that, among others, regulate Ca^{2+} homeostasis and metabolism of glucose (Csordás et al., 2006) which are processes that are altered in AD (Ankarcrona et al., 2010; Area-Gomez & Schon, 2017) Hence, mitochondria have become a promising target of new therapeutic strategies.

Objective

This project, in collaboration with the Ankarcrona group (Karolinska Institutet), aimed to unravel impairment in mitochondrial-associated-membranes linked to AD.

1 – Characterisation of mitochondrial respiration *in vivo* in P301S mice hippocampi during the development of the tau pathology (Université de Bordeaux, in collaboration with Anne Devin and Stephane Duvezin Caubet, IBGC)

2 - Study of the impairment in mitochondrial-ER contacts (MERCs) linked to AD (Karolinska Institutet)

Materials and methods

1. Animals

Ethical approval

All experiments were approved by the Ethical Committee #50 and the French Ministry for Education and Research (N° saisine éthique: 10137). The animals were obtained and cared for according to the regulations of the University of Bordeaux/CNRS Animal Care and Use Committee.

1.1 Mice TauP301S (PS19 line)

TauP301S transgenic mice (Yoshiyama et al., 2007) and their WT littermates (B6C3) were used in this study. Heterozygous males TauP301S were first purchased from Jackson Laboratory and backcrossed with B6C3 WT mice produced in house (Pole *in vivo* – IINS, Bordeaux). TauP301S expresses mutated human tau, P301S (4R1N) driven by the mouse prion protein promoter (Prnp). The mice were genotyped at P0/P1 the local animal facility (Transcriptomics Platform, Neurocentre Magendie, Bordeaux). The experiments were performed in animals at P22, 6, 8 and 10 months

2. Assessing Mitochondrial Bioenergetics

Reagents:

Respiration buffer prepared in water:

Sucrose 250 mM

Tris base 20 mM

EGTA 1 mM

Pi (KH₂PO₄) 10 mM, pH 7.2

Material:

- Oroboros Oxygraph-2k System

Oroboros is a high-resolution polygraph for measuring cellular oxygen consumption in a closed-chamber system with very high resolution and sensitivity. Different kinds of biological samples can be analysed, such as intact and permeabilised cells, tissues, or isolated mitochondria. This device is composed of two chambers and uses polarographic oxygen sensors to measure oxygen concentration and calculate oxygen consumption within each chamber. The oxygen consumption is calculated and expressed as picomoles per second per volume (pmol O₂/s*mL). Each high-resolution oxygraph chamber contains a lid with injection ports. The addition of substrate-uncoupler-inhibitor was performed through this injection ports.

2.1 Hippocampal homogenates preparation

The mice were euthanised by cervical dislocation and the head flushed with PBS. Brain extraction was performed by an incision into the skin along the head's midline, and the skin was removed. The skull was cut from the foramen magnum along the midline, following two lateral cuts, starting from the midline towards the sides. The skull was removed and the brain carefully placed in a petri dish with respiration buffer.

The two hemispheres were separated, exposing the hippocampus and connecting areas. One hippocampus was rapidly removed and immediately frozen in liquid nitrogen (for further analysis), and the other was manually homogenised in 200µl of respiration buffer.

2.2 Assessment of Mitochondrial Respiration

Methods:

First, the Oxygraph-2k chambers A and B were rinsed 3 times with MiliQ water, ethanol and MiliQ water. After filling the chambers, A and B with 2.5mL of respiration buffer 0.1% BSA, the air-saturated calibration was performed. Freshly hippocampus homogenate (50ul) was added into the chambers, and the remaining sample was stored at -20 for further analysis (protein concentration). The protocol was initiated after the oxygen flux per volume (pmol O₂/s*mL) stabilised.

The sequential addition of substrates and inhibitors was performed as following:(Figure 1B):

- 20ul Malate (1M) +20ul pyruvate (1M)
- 1ul ADP (1M)
- 15ul oligomycin (1mg/ml)
- CCCP (0,1 mM) – CCCP was continuously added until reached the maximal uncoupled respiration) (Fig. 1)
- 1ul Antimycin
- 20ul Ascorbate (0,5M) +20ul TMPD (0,2M)
- 10ul KCN

At the end of each run, the Oxygraph-2k lids and chambers were washed 3 times with MiliQ water, ethanol and MiliQ water to ensure that all the inhibitors and samples were removed. After each experiment, the protein was estimated by Pierce BCA Protein Assay Kit (ThermoFisher Scientific, ref. 23225). The absorbance reading of BCA was performed using the plate reader POLARstar Omega (BMG Labtech) at wavelength 562. Protein quantification was used to normalise the Oxygen consumption rate (OCR).

Results

1. Mitochondrial respiration is impaired in P301S

The electron transport chain (ETC) is located in the inner mitochondrial membrane, and it is where the oxidative phosphorylation takes place (Brand & Nicholls, 2011; Vacanti et al., 2014). During the oxidative phosphorylation, electrons are transported through the ETC by the electron carriers, coenzyme Q and cytochrome C, which accept electrons from complexes I and II and donate them to complex III, and that transfers electrons from complex III to complex IV, respectively. Several drugs, such as complex substrates and inhibitors, are used to study the specific components of ETC.

Here we analyse the mitochondrial respiratory chain in P301S transgenic mice both, males and females before (P22) and during the progression of tau pathology (6, 8 and 10 months). We performed high-resolution respirometry using an Oxygraph-2k (Oroboros Instruments) to investigate the cellular respiration in hippocampus homogenates under substrate supply.

First, we used the energetic substrates of complex I, pyruvate and malate to assess coupled respiratory chain activity. Our results showed a significant decrease in males P301S at 10 months ($p=0.03$) compared with WT, but we did not find any differences in females. We then added ADP, which stimulates the coupled respiration, and oligomycin, and ATP-synthase specific inhibitor, to evaluate through their ratio (ADP/oligomycin) the respiratory control rate. We did not find differences at any ages between males and females P301S compared with WT. After the inhibition of ATP synthase, we determined the uncoupled rate by adding the uncoupling agent, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). This stage is defined by the maximal oxygen consumption rate, meaning the maximum rate of respiration that the cell can achieve. FCCP stimulates the ETC to function at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, and amino acids) to correspond to this high energy demand. We found significant impairment in uncoupled respiration at 6 ($p=0.03$) and 10 months ($p=0.018$) in males P301S compared to WT. Interestingly, in females, this impairment was found in P22 ($p=0.005$), before the appearance of the tau pathology.

Finally, after the inhibition of the uncoupled respiration at complex III by antimycin A, we evaluated the capacity of cytochrome C oxidase (complex IV). By adding ascorbate (Asc) and TMPD, which is an artificial redox mediator and acts promoting the transfer of electrons from ascorbate to cytochrome C, we assessed the respiration dependent on complex IV subtracting the Asc+TMPD from KCN OCR. In these conditions, we found significant impairment in males at P22 ($p=0.031$) and 10 months ($p=0.005$). On the other hand, in the females, these differences only appeared at 10 months ($p=0.05$) (Figure 1B)

Interestingly, the results from our collaborators suggest that there is an impairment in mitochondria-ER contacts (MERCs) at the early stage of the disease (P22). The number of (MERCs) per cell is increased P301s animals compared to WT in the CA1 region. (Karolinska institute - data not shown). Further investigation is required to unravel how increased MERCs and impaired mitochondria respiration are involved in the pathogenesis of tauopathy in this model.

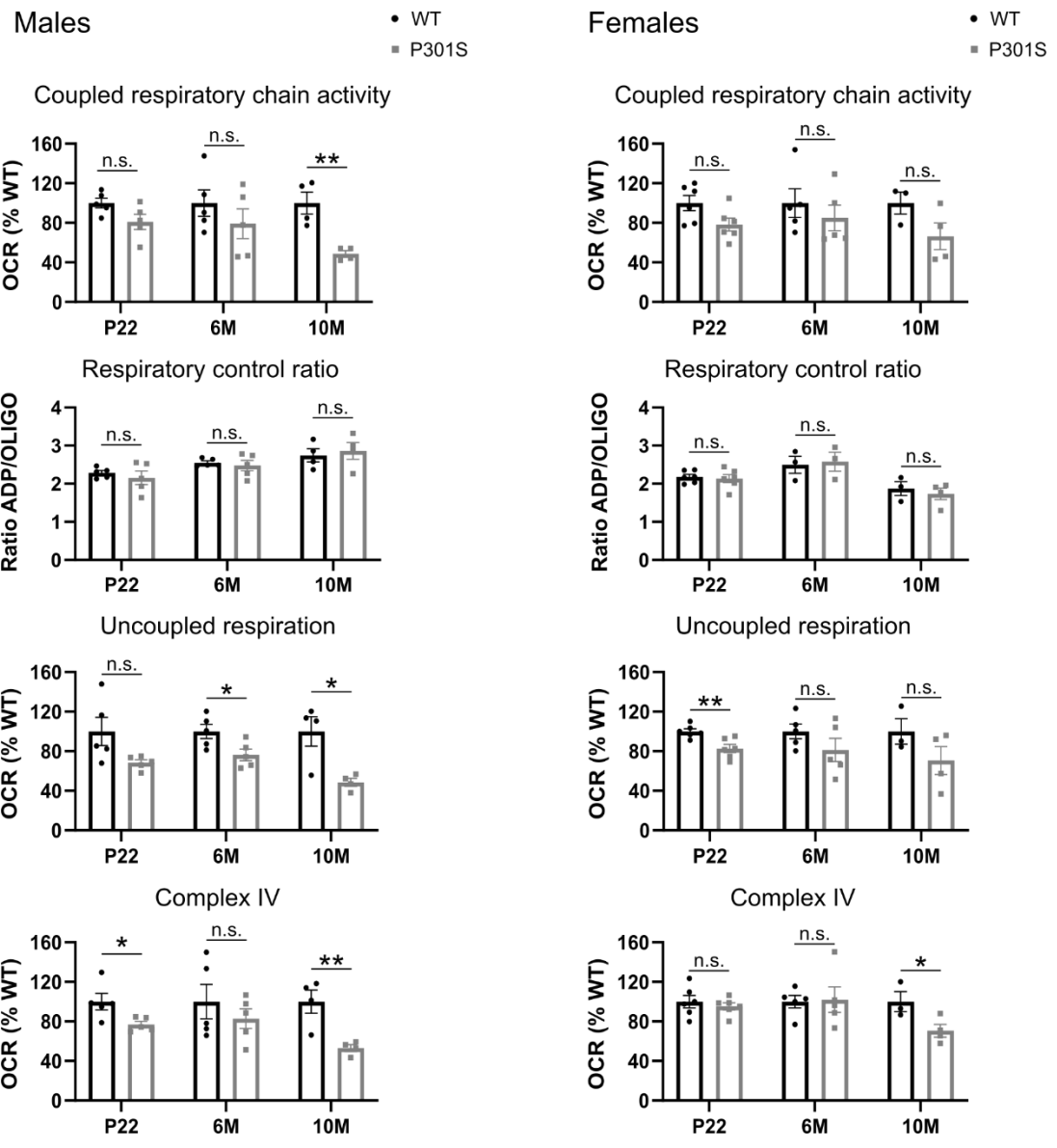
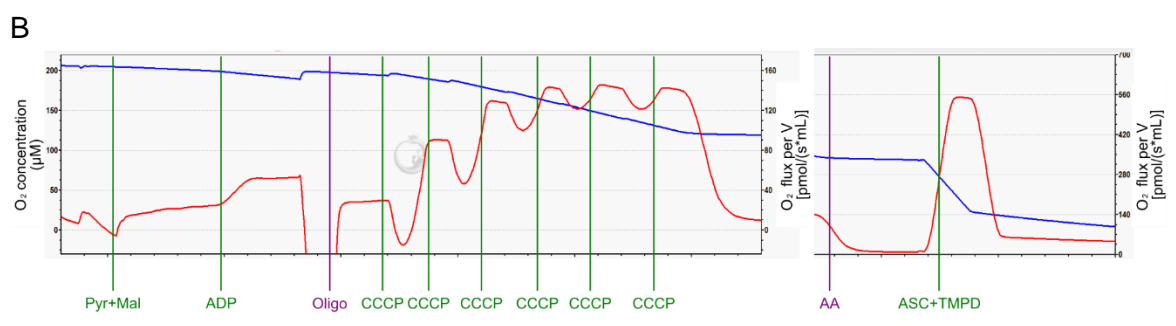
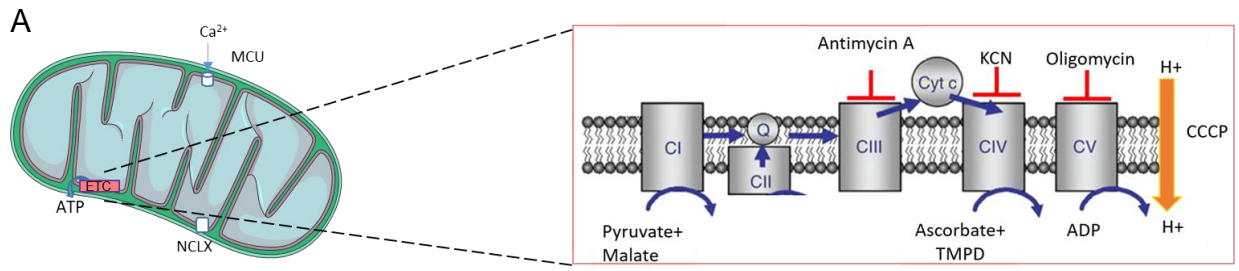


Figure 1. Mitochondrial respiration is affected in P301S males and females before the appearance of tau pathology. A: Illustration of mitochondrial electron transport chain (ETC). ETC is composed of four protein complexes (I-IV) and complex V: ATP synthase. B: Mitochondrial respiration was assessed using an Oroboros high-resolution respirometer through the measurement of mitochondrial oxygen consumption. Representative traces from oroboros measurements (Red curve represents oxygen consumption of the cells (pmol O₂/s*mL) and blue curve the oxygen concentration (μM). The sequential addition of substrates (green lines) and inhibitors (purple lines): addition Pyruvate + Malate (coupled respiratory chain activity), ADP and Oligomycin (respiratory control ratio), adding of CCCP until reaching the ETS capacity (uncoupled respiration), antimycin A (inhibitor of Complex III, residual oxygen consumption); ascorbate and TMPD (Asc+TMPD) (the measurement of complex IV capacity after blockage of complex III). Graphs represent the different parameters measured in the percentage of WT aged-matched in males and females P301S at P22 days, 6 and 10. Males: n= animals; P22 both WT and P301S n= 5; 6 months both WT and P301S n= 5; 10 months WT=3 and P301S=4. Females: Both WT and P301S: P22 n= 6; 6 months n= 4; 10 months, P301S=4. The error bars reflect the mean ± SEM; the differences between conditions were assessed by t test, * p<0.05, ** p<0.01. The OCR = Oxygen consumption rate presented is normalised by protein quantification.

Discussion and perspectives

Several studies indicated that mitochondria dysfunction is an early event in the pathogenesis of AD and other tauopathies. Also, strong evidence suggests that oxidative stress occurs before the onset of symptoms in AD (Cabezas-Opazo et al., 2015a; Gibson & Shi, 2010). Additionally, tau affects mitochondrial dynamics (fusion/fission processes) (DuBoff et al., 2012), impairs mitochondrial transport (Stoothoff et al., 2009) and bioenergetics (David et al., 2005; Dumont et al., 2011).

A study using an AD mouse model (APP^{Swe}/Lon) revealed an elevated number of ER-mitochondria contact points and mitochondrial calcium concentrations, suggesting the involvement of MERCs in AD pathogenesis (Hedskog et al., 2013).

Hence, in this project, we used a transgenic mouse model of tauopathy bearing the P301S mutation to investigate the impact of pathological tau in mitochondrial respiration and MERCs. We demonstrated that the mitochondrial respiration is affected before the appearance of tau pathology and the results from our collaborators suggest that there is an impairment in MERCs at early stages of the disease (P22). This preliminary result indicates that mitochondria might play an essential role in the pathological cascade of AD-linked tauopathy. However, further investigation is required to understand whether these impairments can lead to the appearance of tau pathology.

Mitochondria dysfunction affects neuronal excitability and synaptic transmission and has been implied in several neurodegenerative disorders (Cabezas-Opazo et al., 2015b). Interestingly, mitochondria dysfunction has been identified as one potential cause of epileptic seizures and has been reported for the seizure focus of patients with temporal lobe epilepsy (reviewed in (Folbergrová & Kunz, 2012)). Additionally, the interaction between ER and mitochondria seems to shape intracellular Ca²⁺ signals and modulate synaptic and integrative neuronal activities (Mironov & Symonchuk, 2006). Thus, it would be interesting to investigate the mitochondrial function in the *in vitro* tau pathology model developed. Using a combination of sophisticated techniques, such as calcium sensors, live-imaging, and electrophysiology, we could study, at high spatial and temporal resolution, the progressive impairment of mitochondrial and synaptic function during the development of the pathology.

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