# A viral vector based *in vivo* model of tauopathy to explore therapeutic strategies against tau pathology

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***"To most people, Sky is the limit. To pilots, Sky is home."***
*Anonymous*

***"Fais de ta vie un rêve, et d'un rêve, une réalité."***
*Antoine de Saint-Exupéry*

***To my mom, my dad, my brother and Diane…***

# Abstract

Tauopathies are neurodegenerative diseases whose common pathological feature is the intraneuronal accumulation of tau aggregates. Tau protein has various roles in the neuron, among which stabilization of microtubules (MT), regulation of synaptic activity or axonal transport. Subsequently, tau pathology exerts toxicity through mechanisms that likely involve the loss of normal function or the gain of toxic functions. In tauopathies, tau undergoes post-translational modifications (PTM), which are thought to promote the detachment from the MT, change its subcellular localization, and induce conformational changes that favor its assembly with other tau monomers, forming oligomers and fibrils. Furthermore, both normal and pathological forms of tau can be transferred from neuron to neuron. This process may have an important role in disease progression, by propagating the pathology along synaptically connected brain regions, which reflects the evolution of the symptoms. Therefore, the aggregation of tau protein and its propagation are promising targets for potential treatments. In the present thesis, our objective was to assess the efficacy of two treatments using a mouse model of tauopathy based on local overexpression of the 4R0N human tau (hTau) wild-type (WT) induced by intracerebral injection of an adeno-associated viral (AAV) vector.

In the first part of the thesis, we overexpressed tau in the entorhinal cortex (EC) of WT mice to induce pathology and assess the effects of aggregation inhibitor compounds orally administered. Although we did not observe any effect on markers of tau aggregation, likely because the induced pathology was still at an early stage, the treatment was found to enhance phosphorylation of the Ser202/Thr205 residues in the EC and ipsilateral hippocampus (HPC). Additionally, we measured an increase in the density of microglial cells in the HPC, and significantly decreased levels of total hTau in the cerebrospinal fluid of the treated mice. These findings suggest a potential positive effect of this treatment on the clearance of hTau.

In the second part of the thesis, we unilaterally overexpressed hTau in the mouse CA3 HPC and used the connectivity between both hemispheres to quantify the propagation of tau from neuron-to-neuron to the contralateral HPC. We found neurons labelled for hTau in the contralateral HPC, the number of which was increased in 5xFAD mice carrying the amyloid pathology. Next, to induce an antibody response against pathological tau, we subcutaneously

injected a vaccine against the phospho-Ser396/404 tau epitope. The vaccine induced a potent antibody response in WT mice, but antibody titers remained low in the 5xFAD mice. In the treated WT mice, immunohistochemistry showed a decreased PHF-1 immunoreactivity in the HPC, indicating that the generated antibodies indeed recognized the phospho-Ser396/404 epitope. Remarkably, the vaccine decreased the number of neurons positive for hTau in the contralateral HPC, suggesting an effect of immunotherapy on tau propagation. Furthermore, the vaccine also showed potential neuroprotective effects, by decreasing the level of neurodegeneration in the dentate gyrus of treated mice as a function of the anti-phospho-tau antibody titer.

In this work, using adapted AAV8-based models of local tau overexpression in the mouse brain, we reveal the effects of two treatments on different aspects of tau pathology related to the progression of the symptoms in tauopathies.

Keywords: Tauopathy, Dementia, Alzheimer's Disease, Tau, Vaccine, Misfolding inhibitor, Viral vectors, Mouse model

# Résumé

Les tauopathies sont des maladies neurodégénératives dont la caractéristique commune est la présence dans le cerveau d'inclusions intraneuronales composées de la protéine tau agrégée. Tau est une protéine connue pour son rôle dans la stabilisation des microtubules, mais plusieurs autres rôles dans le neurone lui ont été découverts. Ainsi, le tau pathologique pourrait avoir un effet toxique sur le neurone par des mécanismes qui impliqueraient des pertes de fonctions ou alors des gains de fonctions toxiques. Tau peut subir plusieurs modifications post-translationnelles, comme la troncation ou la phosphorylation qui, en conditions pathologiques, sont impliquées dans le changement conformationnel de tau, ce qui favoriserait l'assemblage du tau monomérique en tau oligomérique et en filaments. De plus, ces formes pathologiques peuvent être transférées de neurone à neurone, ce qui favorise la propagation de la pathologie le long de régions du cerveau qui sont interconnectées entre elles, reflétant la progression des symptômes.

Par conséquent, l'agrégation de la protéine tau et sa propagation sont deux cibles prometteuses pour de futurs traitements. Dans cette thèse, nous avons exploré les effets de composés anti-agrégants, ainsi que d'un vaccin qui cible le tau phosphorylé aux sites Ser396/404, dans un modèle de souris surexprimant le tau humain 4R0N non muté par AAV8.

Dans la première partie de la thèse, nous avons montré un aperçu d'un potentiel mécanisme d'action des composés anti-agrégants dans un modèle de souris surexprimant tau dans le cortex entorhinal. Même si nous n'avons pas observé d'effet sur l'agrégation de tau, nous avons observé une augmentation de la phosphorylation sur les sites Ser202/205 dans le cortex entorhinal et l'hippocampe ipsilatéral. De plus, une augmentation dans la densité de microglies et une diminution du niveau de tau total humain dans le liquide céphalo-rachidien de la souris suggère un effet des composés sur l'élimination de tau, qui impliquerait la neuroinflammation.

Dans la seconde partie de la thèse, nous avons montré que le vaccin contre l'épitope phospho-Ser396/404 induit une réponse du système immunitaire dans des souris sauvages. Grâce au marqueur PHF-1, nous avons montré que les anticorps reconnaissaient l'épitope prévu. Par ailleurs, nous avons montré que le vaccin diminuait le nombre de neurones positifs pour HT7 dans l'hippocampe contralatéral, suggérant un effet sur la propagation de neurone à neurone. Nous avons également observé que les souris sauvages traitées par le vaccin

présentaient une dégénérescence moindre du gyrus denté, ce qui suggère un rôle neuroprotecteur du vaccin.

Dans cette thèse, ces deux traitements ont montré des effets bénéfiques sur des marqueurs de la pathologie tau dans un modèle de surexpression de cette protéine induite par l'injection d'un vecteur viral de type AAV8. De manière encourageante, des études ont permis de corréler certains de ces marqueurs de tauopathie avec le déclin cognitif chez les patients. Des investigations plus approfondies seront toutefois nécessaires pour déterminer si ces traitements sont susceptibles d'avoir des effets thérapeutiques sur la progression de ces maladies qui affectent tant de personnes dans le monde.

Mots-clés: Tauopathie, Tau, Vaccin, Anti-agrégation, Neuroprotection, AAV8, démence, maladie d'Alzheimer

# Table of Contents

# List of Figures

# Chapter 1 : Introduction

## Social and political aspects of dementia

### The challenge posed by dementia

With a total burden of 50 million people[1] currently living with a diagnosis of dementia around the world, leading to an estimated economic annual cost of 818 billion dollars[2], the international community is facing a growing challenge. Not only does dementia cause disability and dependency among affected individuals but it also overwhelmingly impacts families and communities. Family caregivers suffer themselves from high level of stress and are more prone to psychological and physical morbidities[3]. Depression, anxiety, cardiovascular diseases, higher propensity to smoke and drink alcohol are among the problems that are more prevalent in family caregivers of dementia patients[3,4].

On an economic level, growing healthcare costs related to dementia pose a significant challenge to healthcare systems[5], social services and society in general. Inaction over national public health and finance policies would mean an increase in debt and an obstacle to economic growth. This threat towards social and economic development triggered the realization in the international community of the urgent need to have a collective and coordinated global action to tackle the issue of dementia, with the involvement of all stakeholders[6]. To better understand the importance of the challenge posed by dementia and the importance of the context of research on dementia, we will explore first, how dementia impacts society and what solutions the policymakers found to tackle this issue.

### Addressing dementia: an international struggle

Early international resolutions and commitments were not specifically addressing the issue of dementia, although it was included as part of the global phenomena associated to the ageing societies in developed countries. Along with issues such as the respect of human rights of elderly people or old age poverty, dementia was addressed through the scope of mental health[7] and non-communicable diseases affecting older persons[8].

The world assembly on ageing in Vienna in august 1982 was the first time that the international community gathered together and discussed the challenges of an ageing

society. They came up with an international plan of action on ageing with several recommendations for member states. Recommendation 4 of the Vienna plan of action specifically draws the attention of governments to provide appropriate healthcare to old people suffering from "mental disorders and failure to adapt to the environment"; and avoid social isolation by supporting families and caregivers[7]. This plan of action was endorsed by the United Nations (UN) General Assembly in December 1982[9] and lead to the United Nations Principles for Older Persons adopted in 1991 by the General Assembly[10].

At the dawn of the XXI$^{st}$ century, a second world assembly on ageing was decided by the international community[11] and gathered in Madrid in 2002. It resulted in a second plan of action, called "Political Declaration and Madrid International Plan of Action on Ageing", where the importance of an early diagnosis of "Alzheimer's Disease and related disorders" was mentioned in the list of actions recommended by the Madrid plan of action, along with recommendations to undertake research on those disorders and to provide national programs that addresses the needs of patients with dementia[8].

Dementia was gradually being acknowledged as an important challenge on itself by nations and international organizations. The World Health Organization (WHO) characterized dementia as "a priority condition" in the Mental Health Gap Action Program published in 2008[12] and similarly, the UN General Assembly adopted in 2011, the resolution A/RES/66/2 in which, member states recognized in clause 18 of the political declaration annexed to the resolution that Alzheimer's Disease and other mental and neurological disorders greatly contribute to the burden of non-communicable diseases, that were acknowledged as a threat to social and economic development in the world[13].

Furthermore, the WHO affirmed in a 2012 report that dementia should become a public health priority worldwide because of the burden it represents to the patients, the families, the society and the economy[14]. It was becoming clear that dementia would require urgent action from political leaders around the world and collaboration between governments, policy makers, international organizations, scientists, clinicians and private non-governmental organizations representing patients with dementia, such as Alzheimer's Disease International who raises awareness on this condition.

The calls for action were heard and the resolution to act picked up steam at the highest levels of power. Political leaders pledged to address this issue, beginning with the G8 Dementia Summit held in London in 2013. The Health and Science ministers of the United Kingdom,

France, Canada, Germany, Italy, Japan, Russia and the United States of America came up with a declaration for global action against dementia, where they made the commitment to find a cure or a disease-modifying treatment by 2025, to share information about the research and develop an international research plan[15]. This was shortly followed in 2015 by the first WHO Ministerial Conference on global action against dementia. Health ministers from around the world, non-governmental organizations, advocacy groups, scientists and healthcare providers gathered in Geneva and committed to decrease the burden of dementia by preventing it and improving the quality of the care provided to patients. Furthermore, they also stressed the importance of finding a cure or a disease-modifying treatment against dementia by improving the scientific collaboration among member states and increasing the investment in research[16]. Following those international commitments and high-level meetings, the WHO created in December 2017, the Global Dementia Observatory which is an online platform to share data, knowledge and track the progress on raising awareness and on research advances. In addition, the WHO published a guide in June 2018 that would help member states to implement national plans against dementia[2,17]. The framework of the WHO action plan is centered around seven action areas:

1) Dementia as a public health priority: 75% of countries should have national plans against dementia by 2025
2) Dementia awareness and friendliness: All countries should have campaigns to raise awareness among the population
3) Dementia risk reduction: Countries should achieve the prevention of dementia by reducing the risk factors
4) Dementia diagnosis, treatment and care: Increasing the ability to diagnose dementia so that in at least 50% of the countries, 50% of people living with dementia are correctly diagnosed
5) Support for dementia care givers: 75% of countries should provide support and better training for healthcare providers and families by 2025
6) Information systems for dementia: at least 50% of the countries should have a database collecting relevant information on dementia by 2025
7) Dementia research and innovation: the number of outputs from global research should double between 2017 and 2025.

2025 is only a few years away and yet this ambitious plan to tackle dementia shows the importance of acting right now in order to be prepared to face the exponential increase in epidemiologic numbers that are predicted for 2050.


*Figure 1: Timeline of international commitments and resolutions to address dementia*

## The case of Switzerland: an example of a national plan against dementia

In Switzerland, where dementia affects an estimated 148,000 people according to the non-profit organization "Alzheimer Suisse"[18], the debate on dementia was initiated in 2009 by two Swiss parliament members. Jean-François Steiert, one of the two members of parliament, raised a motion that would require the Swiss Federal Council to monitor the costs and prevalence of dementia in order to define priorities and a strategy to address the issue of dementia in a national health policy[19]. Shortly after this motion, another motion was raised by parliament member Reto Wehrli that would call upon the Swiss government to come up with a national strategy that would set the priority on research on dementia, development of new treatments, prevention and detection, support of healthcare providers and caregivers, and the planning and optimization of infrastructures for dementia[20]. Those two motions were adopted by the Swiss Parliament in 2012 and resulted in the publication of the National Dementia Strategy 2014-2019[21].

This document describes four main areas of actions and nine objectives focused on raising awareness of dementia in the general public, improving the healthcare services and infrastructures, promoting better quality and ethical management of dementia patients from early onset of the disease to the late stages, providing better training for healthcare professionals for detection, diagnosis, treatment and follow up of dementia patients, and encouraging research on dementia.

Switzerland is involved in improving the quality of life of people living with dementia and their relatives. The Swiss approach to dementia is very humane, as it is patient centered and not disease centered. This has been emphasized at the First WHO ministerial conference on global action against dementia in 2015. In their general statement[22], the swiss delegation called the international community to act for the people living with dementia and not just against dementia as a disease.

Interestingly, the Swiss delegation emphasized the importance of research and data sharing. In their fifth point of their general statement, they rightfully said that "…*there is an urgent need for understanding what went wrong so far in drug development and why we have failed in finding an adequate treatment so far. Funding in research is driven by high impact publications that celebrate positive findings and innovation. Maybe it is about time we dare to share these negative results that would enlighten. The importance of collaboration also*

*includes sharing and learning from our mistakes*". This powerful statement from the swiss delegation encourages the academic community to not only publish positive findings but also negative results.

# Clinical aspects of dementia

## Definition of dementia

Dementia is described by the International statistical classification of diseases and related health problems, 11th Revision (ICD-11) and by the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), published by the American Psychiatric Association (2013) as an acquired clinical syndrome with the evidence of a progressive loss of cognitive features in at least one or more domains such as memory, language, executive functions, attention, social cognition and judgement, psychomotor speed and visuospatial abilities. Those cognitive deficits should have a significant negative impact on the activities of daily life of the patients, jeopardizing their independence. Furthermore, another criteria mentioned in the DSM-5 is that the cognitive impairment is not explained by delirium or another mental disorder such as schizophrenia[23,24].

To understand the need of research on dementia, we will explore the impacts of dementia in the clinic and how physicians face the need for better diagnostic tools, better understanding of the pathophysiology and eventually the need for new drugs.

## Diseases causing dementia

Several diseases can have a clinical presentation of dementia as a symptom[24,25]. Alzheimer's Disease (AD) is the most common cause with 60-70% of dementia cases[26]. Other important causes of dementia are vascular dementia (estimated 15% of cases)[27], followed by Lewy Body Dementia (LBD) (estimated 10% of cases)[28], Frontotemporal Dementia (FTD) (estimated 5% of cases)[24], and Traumatic Brain Injury (TBI). Dementia can also be caused by alcohol or drug abuse, HIV infection, other neurodegenerative disorders (Parkinson's Disease, Huntington Disease, Prion disease), as well as other reversible medical conditions like vitamin B1, B6 or B12 deficiencies, disorders of thyroid, parathyroid and adrenal glands, liver failure, kidney failure, hypoxemia, neurosyphilis, brain tumor, normal pressure hydrocephalus and heavy metal intoxication[25].

## Approach to the patient with dementia[25,29]

Because of the plethora of diseases causing dementia, physicians face some real challenges, first to recognize the suspicion of dementia, then to find a cause of dementia in order to adopt the appropriate management plan and rule out reversible causes. History of the patient is important for the diagnosis and it often starts with the complaint from the patient or a relative that there is a change in some cognitive functions, such as memory (for example, the patient reports that he/she has difficulty remembering appointments) or a change in the behavior (aggressive behavior, irritability, etc). According to the Harrison's Principles of Internal Medicine, 18th edition, it is important for the physician to know the type and onset of the symptoms, their duration and their progression. Indeed, an acute cognitive impairment would most probably indicate delirium or reversible causes of dementia that might require urgent action.

To narrow down the type of dementia and precise the condition that is causing it, a detailed and comprehensive physical examination is essential. The gait, the posture and motor skills of the patient are important to evaluate in order to identify neurological signs that are associated with diseases causing dementia. Some conditions such as Corticobasal Degeneration typically affect motor abilities, whereas Alzheimer's Disease most often does not present with motor symptoms. The presence of resting tremor would suggest parkinsonism related disorders like the Lewy Body Dementia or Parkinson's Disease.

Neuropsychiatric tests like Folstein's Mini Mental State Exam (MMSE)[30], the "clock drawing" test[31] or the Montreal Cognitive Assessment test (MoCA)[32] are useful tools that physicians can use to assess the mental and cognitive state of patients. Although often useful to assess dementia, those tests have limitations, because a decreased scoring is not sufficient to confirm the diagnosis of dementia and on the other way around, a normal scoring does not necessarily exclude dementia. Hence the importance of the clinical expertise of the physician to interpret test results by taking into account the full history, physical examination and laboratory investigation results. Laboratory tests aim to rule out reversible and treatable causes of dementia. Those tests include full blood count, glycated hemoglobin, creatinine clearance, levels of hepatic enzymes, vitamin B12, folic acid and Thyroid Stimulating Hormone (TSH), serology for HIV and syphilis. Lumbar puncture might be indicated to rule out infectious causes.

In addition to laboratory tests, neuroimaging is first indicated to exclude secondary causes of dementia that might explain the symptoms, such as the presence of brain tumors, any subdural hemorrhage or normal pressure hydrocephalus. In addition, neuroimaging can be used to observe the presence of atrophy in certain areas of the brain that would help specify the type of degenerative disease that is causing dementia. More recently, the use of Positron Emission Tomography (PET) scan for diagnostic purpose is increasingly being considered as a diagnostic tool, mainly with biomarkers of Alzheimer's Disease[33], as discussed later.

With the exception of reversible and secondary causes, dementia caused by neurodegenerative diseases does not have any disease-modifying treatment yet. Pharmaceutical management of those cases is purely symptomatic. Therefore, the management of dementia consists in the appropriate psychosocial care to help patients keep a good quality of life as much as possible and provide supportive counselling to relatives who will become informal caregivers.

Currently, definitive diagnosis of the cause of a neurodegenerative disease is done at autopsy. In most cases of dementia linked to neurodegeneration, an abnormal aggregation of protein is found as a pathological hallmark. Whether it is Alzheimer's Disease, Lewy Body Dementia, Traumatic brain injury, Frontotemporal Dementia or Huntington Disease, all have their own characteristic pathological protein accumulation in the brain. However in most cases, there is one common feature found, either as a primary pathological hallmark or as a secondary feature in the presence of another pathology: the involvement of the protein tau[34–38].

# Clinical aspects of Tauopathies

## Definition of tauopathies

Neurodegenerative diseases characterized by the aggregation of tau protein in the brain are called by the umbrella term of "tauopathy"[39]. Tauopathies are a category of neurodegenerative diseases with a wide spectrum of clinical presentations[40], among which dementia. It is important to highlight that dementia is a clinical syndrome involving cognitive impairment, whereas tauopathy is a class of neurodegenerative diseases with several clinical presentations, but the common feature of which is the presence of tau aggregates in the brain. Clinical symptoms of tauopathies range from dementia to motor disorders[41], with some tauopathies presenting with more predominant cognitive decline and others presenting with a predominant motor disorder.

Tauopathies are separated in primary tauopathies and secondary tauopathies[42]. When a disease has tau aggregation as its main pathological feature without the association of any other protein, it is considered a primary tauopathy. Such diseases include Pick's Disease, Progressive Supranuclear Palsy (PSP), Corticobasal Degeneration (CBD), Argyrophilic grain disease, Age-related tau astrogliopathy, globular glial tauopathy, Primary age-related tauopathy (PART) or Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)[43,40].

Secondary tauopathies are diseases where tau pathology is associated with another protein pathology or if tau is not the main component of the neurodegeneration. According to this definition, Alzheimer's Disease is a secondary tauopathy since both amyloid beta and tau pathologies are implicated. Similarly chronic traumatic encephalopathy (CTE) is a tauopathy that is sometimes associated with TAR DNA-binding protein 43 (TDP-43)[44]. Parkinson's Disease, Lewy Body Dementia and Huntington's Disease can be considered as secondary tauopathies, as tau pathology is found in some cases of those neurodegenerative diseases along with the deposition of other pathological proteins, namely alpha-synuclein for PD and LBD, and huntingtin for Huntington's Disease[45,43,46,38].

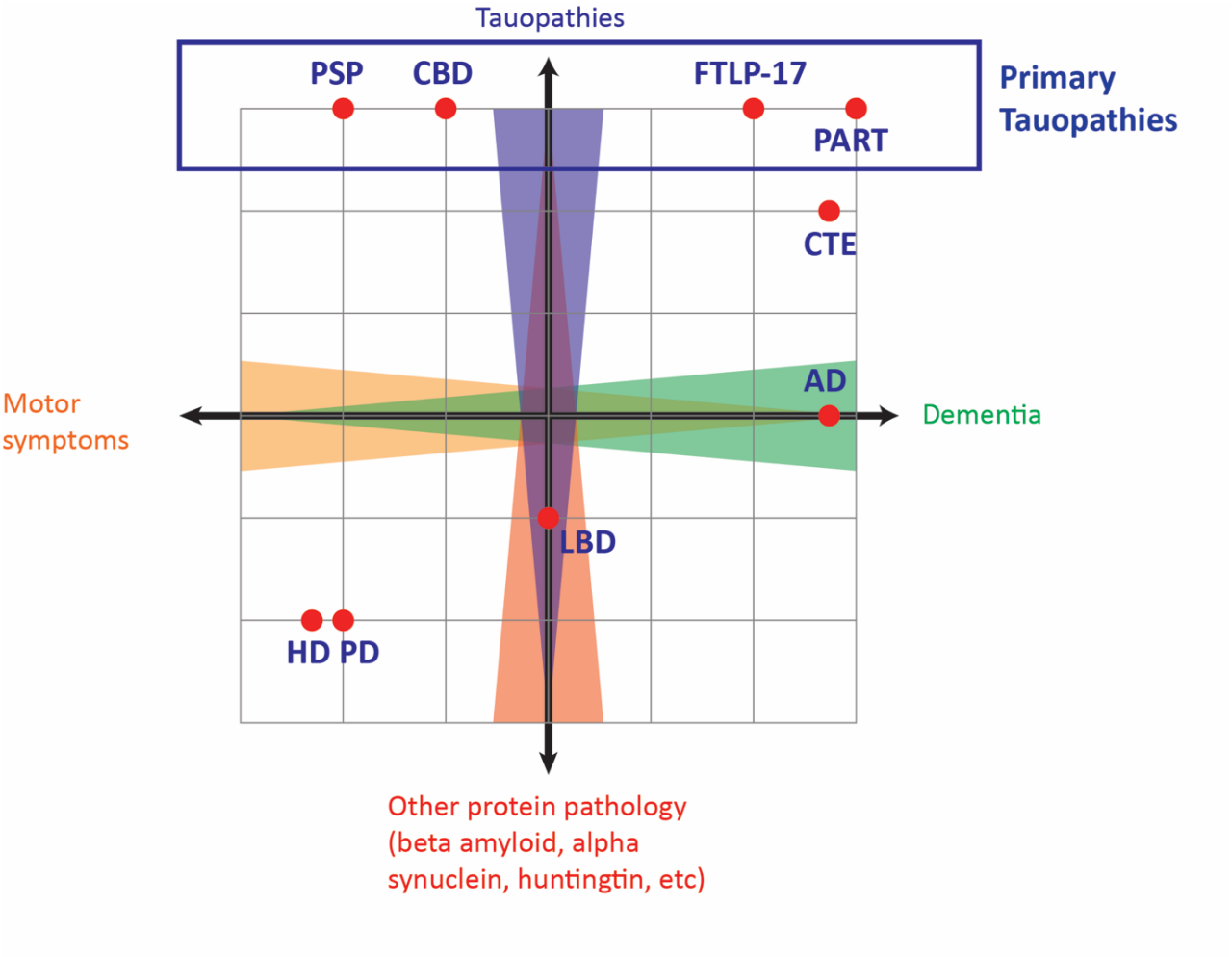



*Figure 2: Spectrum of symptoms in tauopathies.*

Chart visually explaining the notion of spectrum of symptoms on the X-axis and the continuum of presence of tau pathology in regards to some other protein pathologies on the Y-axis. Diseases at the very top of the Y-axis only have tau pathology and therefore are primary tauopathies. AD is on the middle of the Y-axis because tau and amyloid beta are equally predominant pathological hallmarks of the disease. On the X-axis, AD is on the right side, because it manifests predominantly by dementia symptoms. HD and PD are on the left side, as their predominant symptoms are motor symptoms. LBD presents with parkinsonism and dementia, therefore it sits in the middle of the spectrum.

AD: Alzheimer's Disease, PSP: Progressive Supranuclear Palsy, CBD: Corticobasal Degeneration, FTLP-17: Frontotemporal Dementia and Parkinsonism Linked to Chromosome 17, PART: Primary Age Related Tauopathy, CTE: Chronic Traumatic Encephalopathy, LBD: Lewy Body Dementia, HD: Huntington's Disease, PD: Parkinson's Disease.

Loosely adapted from Lebouvier et al.[40]

## Approach to the patient with a suspected tauopathy

Since the definitive diagnosis of a tauopathy can only be made post-mortem, physicians often rely on the history of patients and the results of their physical and neuropsychiatric tests to make a putative diagnosis of tauopathy. History is key for diagnosis and in addition to onset, duration and progression of symptoms, physicians typically enquire about personal events that might have occurred, like a head trauma, and also about any family history of the same trouble, suggesting a genetic factor of the disease. Different tauopathies manifest themselves with their own constellation of symptoms between motor disorders and cognitive deficits. Some sets of symptoms are pathognomonic of a tauopathy, such as the Richardson Syndrome, which is the association of symptoms that begins with gait and balance disorders resulting in frequent falls, followed by oculomotor disorders leading to loss of vertical eye movements as well as body axial rigidity[42]. This syndrome is typical of progressive supranuclear palsy. Other combination of symptoms are less evident and cannot be diagnosed antemortem, like primary age-related tauopathy or PART which has a similar clinical presentation than Alzheimer's Disease but lacks the involvement of the amyloid beta pathology[47].

Motor symptoms frequently associated with tauopathies are[42] vertical gaze palsy, axial rigidity, falls, speech apraxia, limb apraxia, dysarthria, stiffness of muscles, dystonia, action myoclonus and gait freezing at movement initiation. Cognitive symptoms found in tauopathies typically are memory loss, as well as changes in behavior or personality.

Those symptoms are often associated with depression, anxiety, constipation, urinary incontinence and loss of smell.

### *Importance of biomarkers for tauopathies*

Currently, there is no approved clinical or laboratory test to specifically identify a tauopathy[40,42,48]. However, research on tau biomarkers focuses on two main approaches: the development of a cerebrospinal fluid (CSF) biomarker and tau radioligand for Positron Emitting Tomodensitometry (PET) imaging. The use of reliable biomarkers is important to make a correct and timely diagnosis of a tauopathy.

## CSF Biomarkers

CSF biomarkers are mostly used for Alzheimer's Disease as studies show a significant increase in CSF tau in AD patients which correlates with the histopathological staging of tau accumulation in the brain[49,50,48]. However, CSF analysis for other tauopathies is not fully reliable yet, since the correlation between tau levels in CSF and the stage of the pathology is not well established[40]. Although a recent study by Irwin et al. showed some correlation between the level of phosphorylated tau in CSF and FTLD-Tau[51], other tauopathies do not show a similar correlation[52]. Therefore, the use of CSF biomarkers currently only discriminates Alzheimer's disease through the detection of phosphorylated tau, total tau and amyloid beta peptide 1-42. However, it does not help pinpointing the exact tauopathy that is affecting the patient.

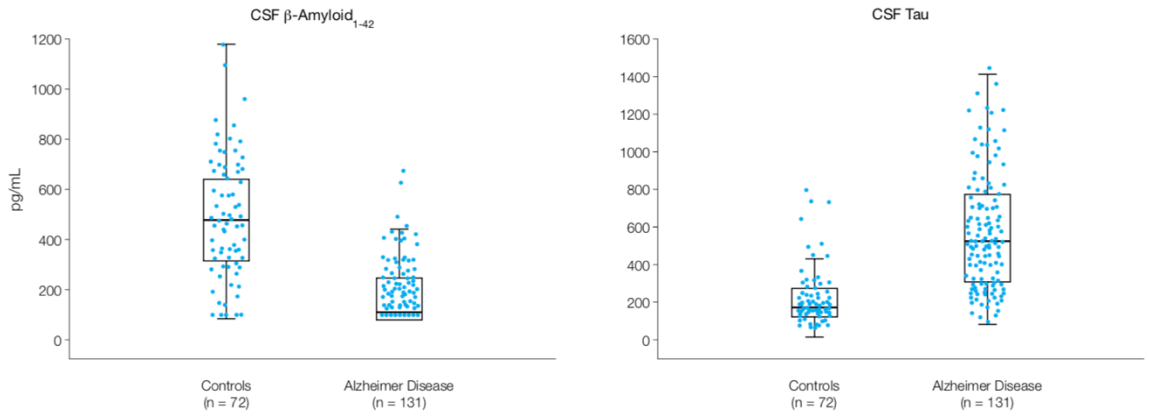



*Figure 3: CSF biomarkers in AD*

The level of beta-amyloid peptide 1-42 is decreased in the CSF in AD patients compared to controls (left graph). The level of tau protein in the CSF is increased in AD patients compared to controls. (adapted from Sunderland et al.[53])

## Neuroimaging

On the other hand, neuroimaging offers a promising avenue for the diagnosis of tauopathies. Several radioligands have been developed, among which $^{18}$F-flortaucipir, also known as [$^{18}$F]AV1415[54]. It is the radioligand that is the most studied[55] and it binds to tau aggregates[56] found in AD. Tau radioligands for PET imaging offer a real hope to diagnose tauopathies and follow the progression of the pathology, because in the case of AD, [$^{18}$F]AV1415 signal pattern is closely correlated to the pattern of spread of the pathological tau aggregates found in the

brain of AD patients[57,58]. Furthermore, the signal detected by [$^{18}$F]AV1415 seems to correlate with the cognitive decline observed in AD patients[59]. $^{18}$F-flortaucipir has been used in the context of other tauopathies as well, namely PSP and FTD, showing increased signal in regions of the brain that are most affected by the tau pathology. However, the results are not as convincing as in AD, because of off-target signal[40].

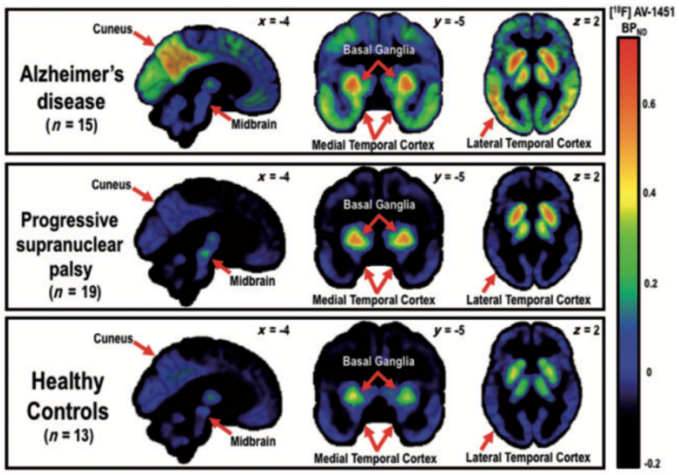
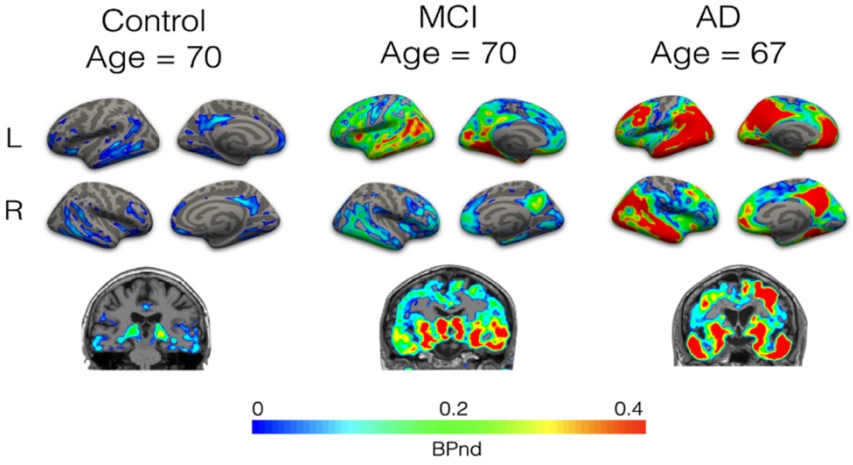



*Figure 4: Tau PET signal in AD patient, PSP patient and healthy control*

[$^{18}$F]AV1415 signal is increased in medial temporal lobes and neocortex in AD patients compared to controls.
[$^{18}$F]AV1415 signal is increased in the midbrain and the basal ganglia in PSP patients.
Controls also have increased signal in basal ganglia, although lower than PSP, suggesting an off target effect.
Adapted from Passamonti et al.[57]

*Figure 5: Tau PET signal reflects the extent of tau pathology*

[$^{18}$F]AV1415 signal in a healthy control, in a patient with Mild Cognitive Impairment and in a patient with AD. The extent of the signal correlates with the extent of tau pathology. Healthy controls show some tau deposition in the medial temporal area. In MCI, tau deposition includes the temporal and posterior cingulate region. In AD, tau pathology has spread to the temporal and parietal cortices. Adapted from Hall et al.[60]



## Frequent tauopathies

### *Alzheimer's Disease*

As mentioned previously, AD is a neurodegenerative disease, which is by far the most common cause of dementia, accounting for 60% to 70% of cases. AD patients often present with memory loss, spatial disorientation, language trouble or neuropsychiatric disorders.

The diagnostic criteria include loss of memory with impairments in learning new information or recalling previously memorized information. Memory loss has to be associated with at least one of either aphasia (trouble speaking), apraxia (trouble executing motor functions without any motor impairment), agnosia (trouble recognizing objects), or executive functions disorders (planning tasks). In addition, those disorders should have a significant impact on daily tasks and on social and professional activities.

| AD Diagnostic criteria |
| --- |
| • Anterograde amnesia<br>and at least one of<br>• Agnosia<br>• Apraxia<br>• Aphasia<br>• Disturbance in executive functions<br>Symptoms should be progressive and affect daily activities |

*Table 1: AD diagnostic criteria*

#### Risk factors of Alzheimer's Disease

Identifying modifiable risk factors of AD from those that are not modifiable is essential in order to prevent the disease. Reducing the risk factors is one of the action areas in the World Health Organization action plan against dementia, as seen previously[2]. Modifiable risk factors of Alzheimer's Disease include cardiovascular risks[61] such as hypertension, hypercholesterolemia, diabetes, obesity, physical inactivity or smoking. Depression[62], history of head trauma[63,64,65] and low education level[25,66,67] are also found to increase the risk of AD. Since a cure against dementia and AD is inexistent, strategies to reduce its prevalence are focused on prevention. A study by de Bruijn et al.[68] indicated that a theoretical reduction of all modifiable risk factors of dementia could reduce the prevalence of dementia cases by a third approximately. Another study by Norton et al.[66] predicted that a 10% decrease per decade of the prevalence of risk factors of AD could reduce the worldwide prevalence of AD by 8.3% by 2050 (representing 8.8 million less people suffering from AD by 2050). The decrease of prevalence of AD even reaches 15% (representing approximately 16 million less people with AD) with a 20% reduction per decade of the prevalence of modifiable risk factors.

The most important risk factor of AD is ageing[69]. The prevalence of the disease doubles every 5 years from 65 years old.

Gender is also identified as a risk factor, women having a two-fold increased risk compared to men[70].

Several genes have been identified as risk factors as well, the gene coding for Apolipoprotein E4 (ApoE4) is the most important genetic risk factor for sporadic and late-onset cases of AD. ApoE4 is a protein involved in the clearance of amyloid beta protein. Other genes are associated with AD, among which TREM2, a gene that is involved in the microglial response[67]. It is interesting to observe that although tau protein is an important pathological hallmark of AD, the gene MAPT that codes for tau is not strongly associated with AD[71]. This suggests that the tau pathology found in AD is not caused by a genetic mutation in MAPT but rather by another cause that might trigger the pathology.

Most cases of AD are late-onset (i.e after 65 years old) sporadic cases. However, there is a minority of AD cases (0.1%) that are inherited with an autosomal dominant genetic mutation[72]. People affected by a familial form of AD often develop the disease earlier than 65 years old. It has been shown that mutations in the genes encoding the amyloid precursor protein (APP), Presenilin 1 (PSEN 1), or Presenilin 2 (PSEN 2) cause inheritable forms of Alzheimer's Disease[73]. APP is a protein whose cleavage produces the amyloid beta (A$\beta$) protein which is found in the senile plaques that characterize AD. The two presenilin proteins are associated to the $\gamma$ secretase enzyme which is involved in the cleavage process of the APP protein[67].

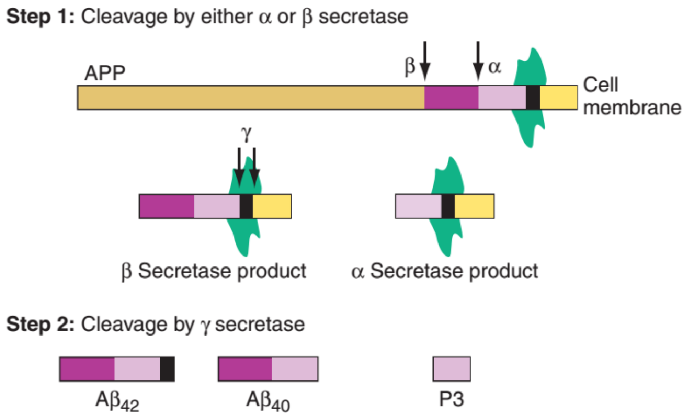



*Figure 6: Production of Amyloid beta peptides through cleavage of Amyloid Precursor Protein*

APP is a transmembrane protein that is cleaved by either α secretase or β secretase. The resulting peptides are cleaved by γ secretase. Cleavage of the α secretase product by γ secretase leads to the non-toxic peptide P3. Cleavage of the β secretase product by γ secretase leads to either the $A\beta_{40}$ peptide or to the more toxic amyloidogenic $A\beta_{42}$ peptide, found in Aβ plaques.
Adapted and modified from Harrison's Principles of Internal Medicine, 18th edition

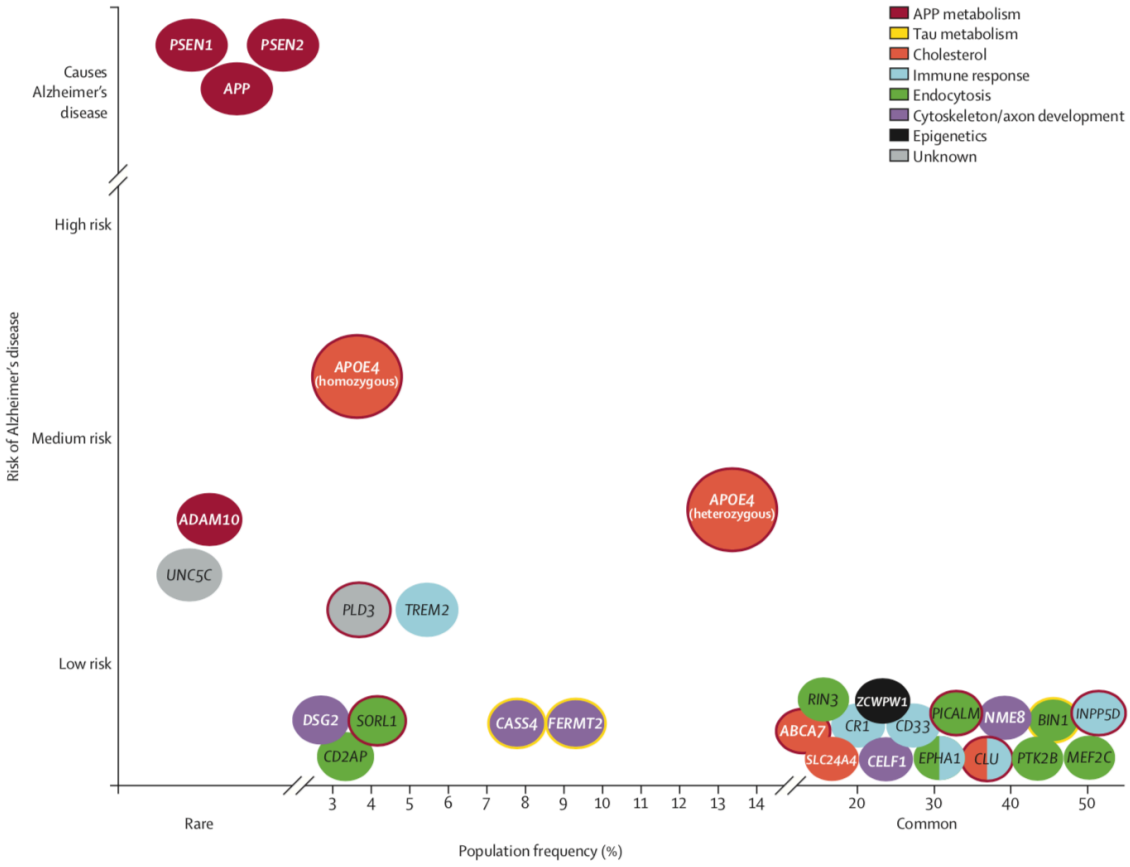



*Figure 7: Genes linked to Alzheimer's Disease*

Graph with all the genes that are linked to AD. Genes circled in red are involved with the APP metabolism and genes circled in yellow are involved with the tau pathway. The colors indicate the corresponding metabolism that is associated with the gene. Adapted from Scheltens et al.[67]

## Physiopathology of Alzheimer's Disease

When brain samples of people who suffered from AD are analyzed, two pathological features are systematically present: extracellular plaques formed by Aβ aggregation and intracellular neurofibrillary tangles (NFT) formed by aggregated tau[74].

The apparition and progression of Aβ plaques and NFTs follow each their own very specific pattern that is well described by Braak and Braak, who gave their name to the staging of the progression of the pathology[74].

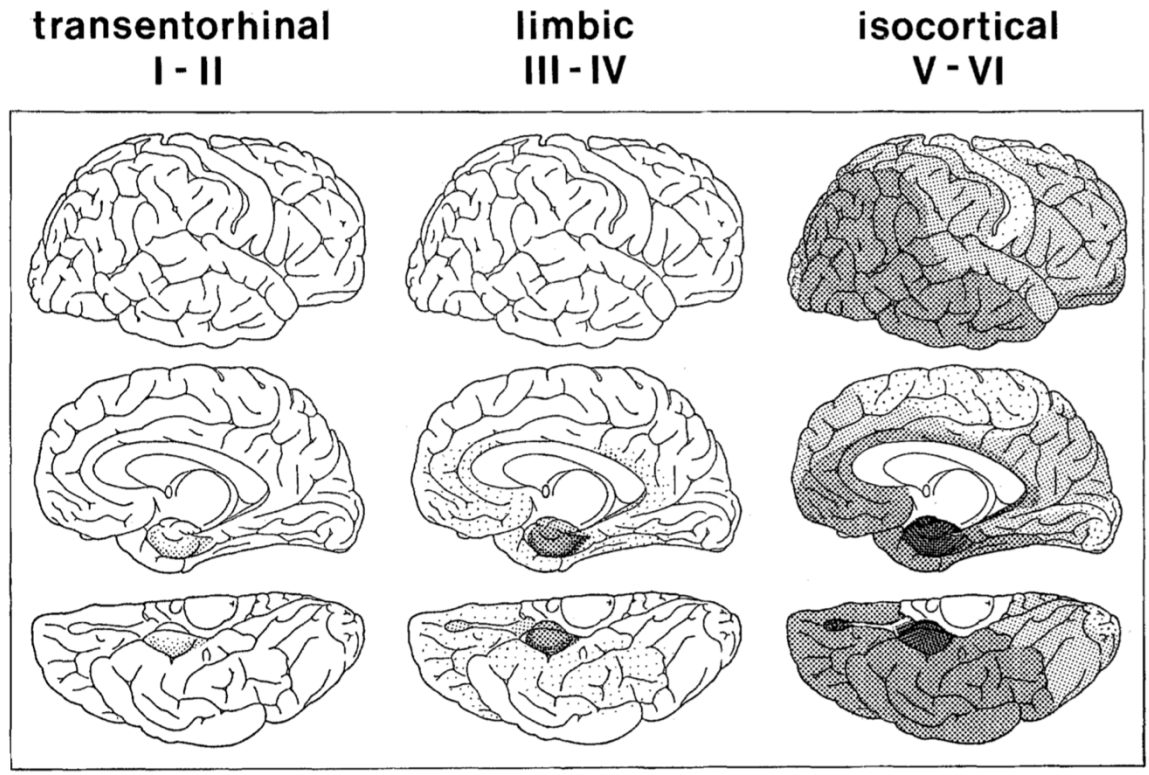



*Figure 8: Neuroanatomical progression of NFTs that correspond to the six levels of the Braak stages. (adapted from Braak and Braak[74])*

It is important to highlight that out of the two patterns of progression of both pathologies, it is the pattern of spread of tau NFTs that is strongly correlated to the neuronal loss[34] and to the cognitive decline[73,75]. NFTs are reported to appear in the Locus Ceruleus area in the brain stem as early as beginning of adulthood[76]. From there, NFTs spread to the transentorhinal cortex, then to the hippocampus and other limbic regions and finally they spread to the neocortex affecting the primary sensory, motor and visual regions[77].

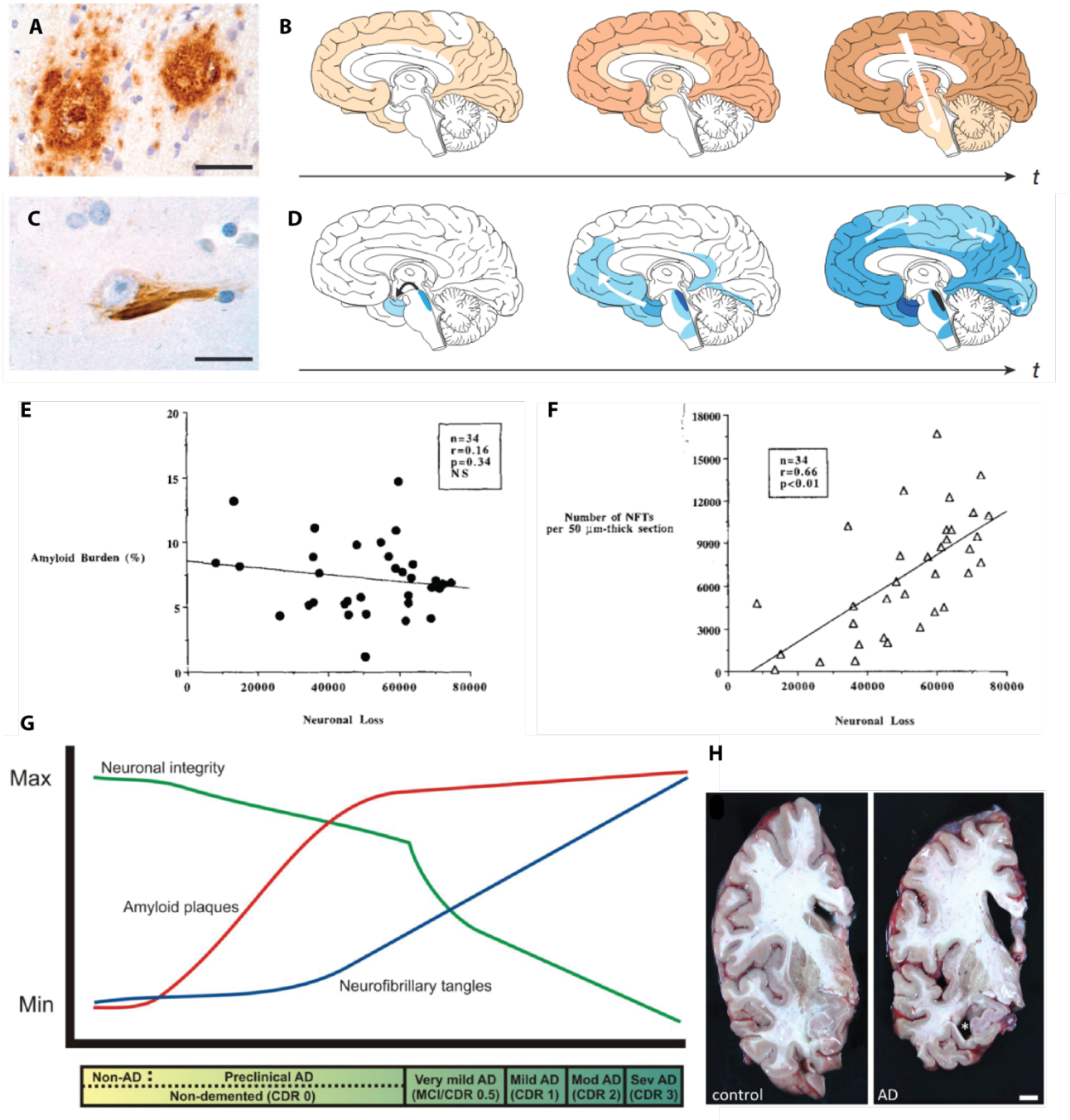



*Figure 9: Pathological features of Alzheimer's Disease*

A): Amyloid beta plaque. Scale bar: 50µm. B) Progression of amyloid beta plaques over time. C) Tau neurofibrillary tangle. Scale bar 20µm. D) Progression of NFT overtime. NFT start in locus ceruleus, followed by transentorhinal cortex, hippocampus and limbic areas, and spread to the neocortex. A, B, C and D are adapted from Jucker et al[78]. E) Amyloid beta plaque load is not correlated with neuronal loss unlike NFTs (F). E and F are adapted from Goméz-isla et al.[34] G) Graph showing the progression of amyloid plaques and neurofibrillary tangle in parallel of neuronal loss. The stage of the cognitive decline is shown below the graph. NFT progression is correlated to neuronal loss as well as the severity of the cognitive decline (adapted from Perrin et al.[79]). H) Atrophy of the brain found in AD patients. Note the atrophy of the hippocampus shown by the star (scale bar: 1cm, adapted from Spires-jones et al.[80])

The interplay between amyloid beta and tau pathologies is not fully understood. Whether amyloid beta pathology is driving the tau pathology or the reverse is subject to debate. However studies tend to agree that both pathologies synergistically enhance each other[81,82,83]. What is known is that amyloid beta pathology requires tau pathology for its own toxic effects[81,84,85] at the level of synapses[80] and that amyloid beta causes mislocalization of tau at the synapses[86,87], where tau interacts with the tyrosine kinase Fyn and the postsynaptic protein PSD-95, that regulates the NMDA receptors. This leads to overexcitation of the synapse that provokes downstream excitotoxicity in neurons. This observation tends to place amyloid beta overproduction and deposition as the initial event that leads to the vicious circle of both pathologies acting on each other. Another reason why amyloid beta and not tau is believed to be the trigger is because the mutations of APP, PSEN1 and PSEN2 genes directly cause pathological amyloid beta to accumulate and aggregate, leading to AD with both amyloid beta and tau pathologies being present. Whereas a mutation in the gene of tau MAPT only triggers tau pathology without triggering the amyloid beta pathology[67].

Another important feature of this toxic interaction is the involvement of neuroinflammation mediated by microglial cells.

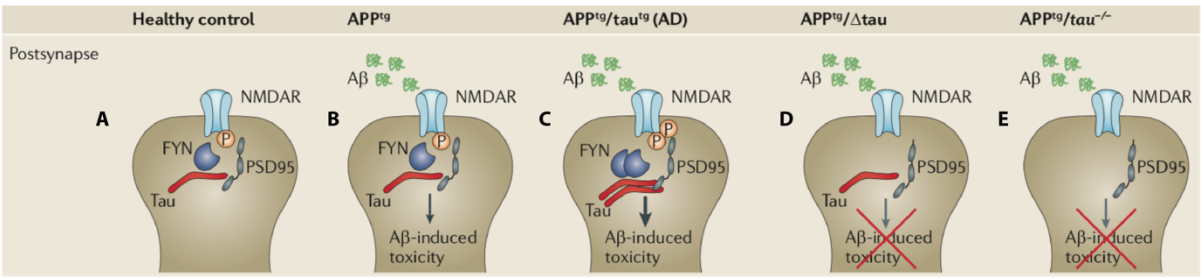



*Figure 10: Mechanism of the interplay between amyloid beta and tau at the synaptic level in mouse models*

In healthy controls **(A)**, a small amount of tau is localized in the somatodendritic compartment, where it is bound to Fyn kinase and leads to a complex with PSD95 that phosphorylates NMDA receptors, potentiating its effects. This leads to excitotoxicity in the presence of Aβ **(B)**. Pathological tau accumulation leads to an increased effect of NMDA activation that enhances excitotoxicity **(C)**. In a model with truncated tau that does not bind to Fyn kinase, the complex with tau and PSD95 is not formed, thus protecting from Aβ induced toxicity **(D)**. In a tau depleted model **(E)**, the PSD95, Fyn and tau complex is not formed, therefore Aβ does not induce toxicity. Adapted and modified from Ittner et al.[82]

## *Frontotemporal dementia*

Frontotemporal dementia (FTD) is a common cause of dementia and is a syndrome that groups several symptoms, spanning between behavioral disorders, aphasia and motor symptoms. These symptoms are caused by various diseases characterized by the atrophy of the frontal and/or temporal lobes of the brain. Because of that feature, diseases which cause this specific atrophy are grouped under the class of Frontotemporal lobar degeneration (FTLD). FTD is used to describe the FTLD diseases that are characterized by behavioural changes and cognitive executive disorders. At the level of pathology, FTLD diseases can have different pathological inclusions formed from various proteins. The most prevalent feature of FTLD is tau aggregation (FTLD-Tau), accounting for 45% of all FTLD diseases. The remaining FTLD are caused by either accumulation of TDP-43 (FTLD-TDP) or inclusions of Fused in Sarcoma (FUS) (FTLD-FUS)[40,25].

### FTD variants

FTD is a syndrome with so many heterogenous symptoms that it has been classified in different subsets of syndromes, according to their main clinical manifestations. More than half[88,37] of the FTDs are part of the behavioural variant (bvFTD) which presents with mainly personality changes, apathy or social disinhibition causing impulsivity. Memory is relatively spared in FTD.

Other variants that are relevant to FTLD-tau are the semantic dementia (SD) variant of FTD and the progressive non fluent aphasia (PNFA) that affect the understanding of the meaning of words (for SD) and the speaking ability of the patients (for PNFA). Another variant of FTD that is found is the motoneuron disease variant or FTD-MND. However it is caused by the aggregation of other proteins than tau[25,89].

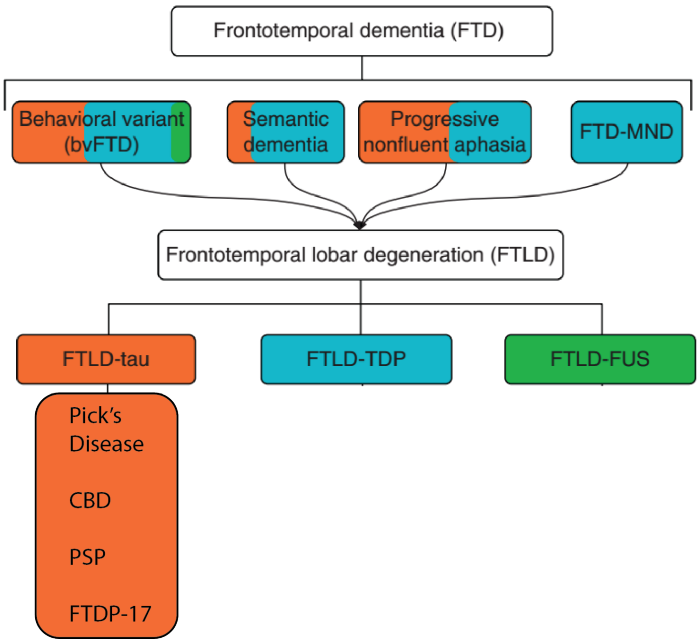



*Figure 11: FTD and FTLD*

Representation of FTLD pathologies and their different clinical presentations as variants of frontotemporal dementia. FTLD-tau (orange) is caused by several diseases listed. Those diseases have different symptomatic presentations, ranging from bvFTD, to SD and PNFA.
In turn, those syndromes are caused by either FTLD-tau, TDP-43 or FUS for bvFTD, FTLD-tau and TDP-43 for SD and PNFA. The frequency of each pathology is represented by the proportion of the appropriate color.
Adapted and modified from Harrison's Principles of Internal Medicine, 18th edition.[25]

### Frontotemporal Lobar Degeneration due to tau inclusions (FTLD-tau)

FTLD-tau are the diseases where tau inclusions are found in various patterns and localizations. They are by definition primary tauopathies. The heterogenicity of clinical symptoms of FTLD-tau diseases is explained by the different neuroanatomical localizations and patterns of spread of tau inclusions[37].

Among FTLD-tau diseases, we find conditions such as corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), Pick's disease as well as "Frontotemporal Dementia and Parkinsonism linked to chromosome 17" (FTDP-17). The latter disease is interesting because it is inherited in an autosomal dominant manner with several mutations in the MAPT gene linked to it[90].

### Risk factors

Most FTD cases are sporadic, however with 40% of autosomal dominantly inherited cases, FTD is one of the most common dementia associated with genetic mutations and one of the most common form of early onset dementia[88]. Eight genes have been linked to all FTDs, among which the MAPT gene is one of the most commonly mutated. There are around 50 pathogenic mutations identified in the gene of MAPT, and two of the most common

mutations are P301L and P301S, that are missense mutations linked to FTDP-17 and found on exon 10 of the gene.

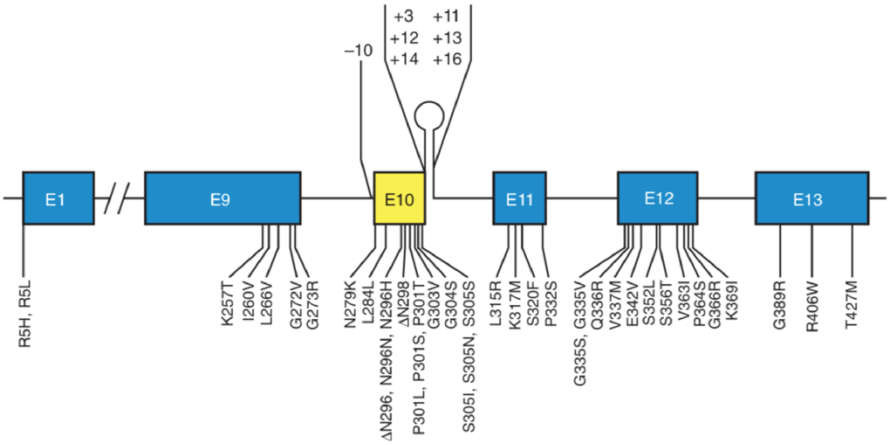



*Figure 12: Known mutations of the MAPT gene*

P301L and P301S are one of the first mutations identified and are important for animal models of tauopathy. Adapted from Goedert et al.[89]

## Pathophysiology of FTLD-tau

Genetic mutations of MAPT causes tau pathology through toxic gain of function of the tau protein, which is more prone to detach from microtubules and has a higher propensity to self-aggregate and form hyperphosphorylated filaments that accumulate in cell bodies[37].

Accumulation of pathological tau can present in several forms. There can be pathological tau inclusions in compact rounded bodies within neurons, called Pick bodies, which are a hallmark of Pick's disease. Tau can also accumulate in astrocytes in the case of progressive supranuclear palsy and form neurofibrillary tangles in other FTD linked to MAPT mutations.

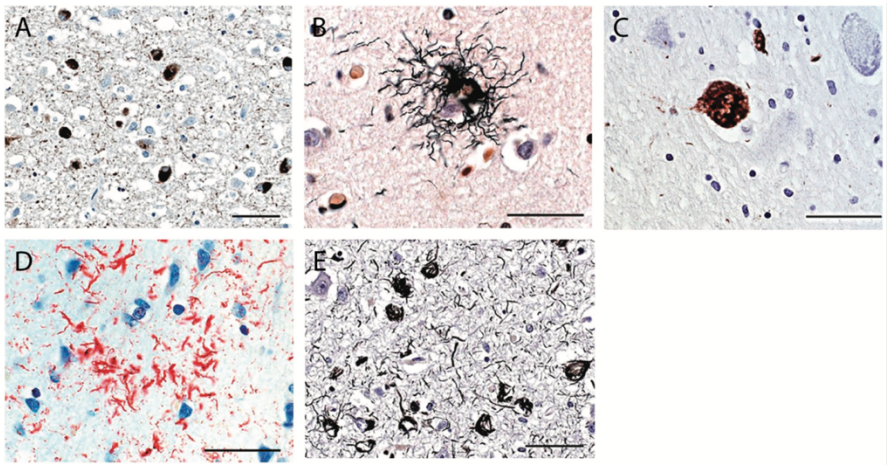



*Figure 13: Pathological hallmarks of FTLD-tau*

A) Pick bodies in a patient with Pick's disease. B) Tufted astrocytes in a patient with PSP. C) Globose tangle in PSP. D) Astrocytic plaque in CBD. E) Tau pathology in neurons and glia in a patient with MAPT gene mutation. A, C and D are tau immunohistochemistry. B and E are Gallyas-Braak silver stainings. Scale bar: 50μm. Adapted from Finger.[37]

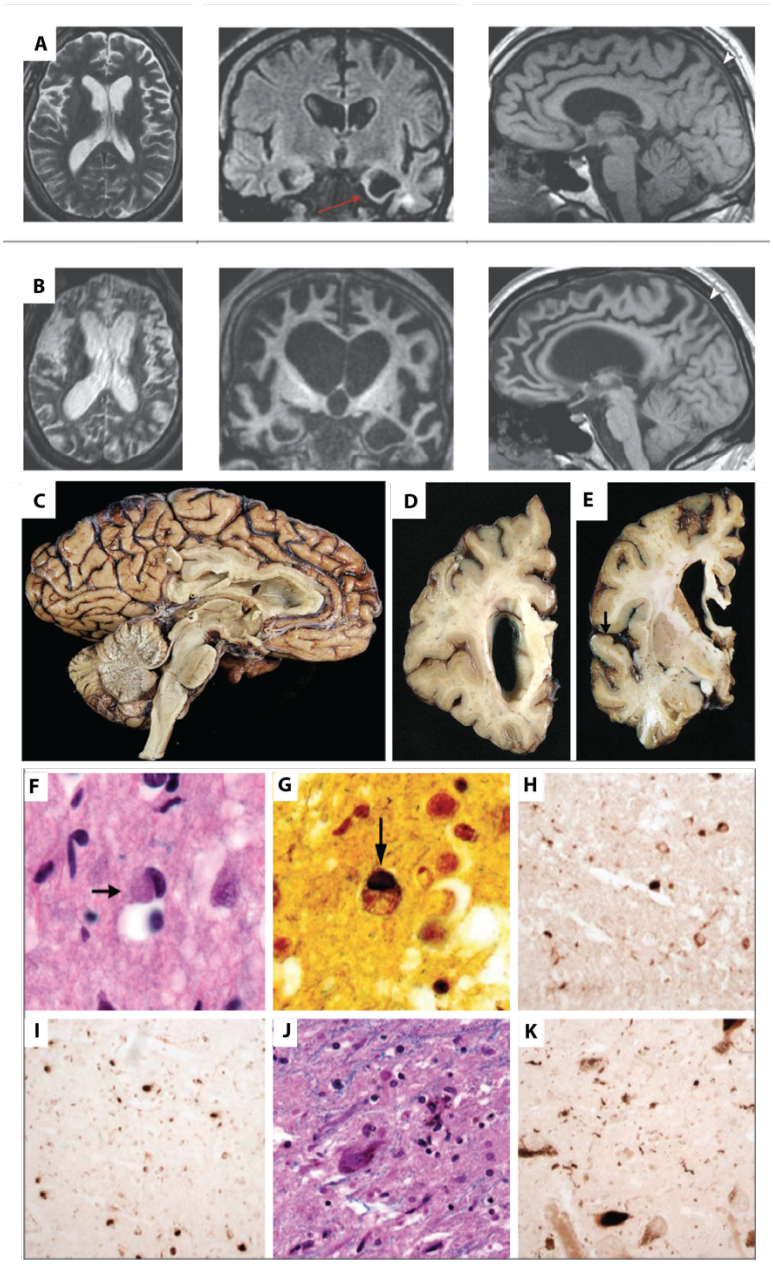



*Figure 14: From imaging to pathology in a case of Pick's disease in a 31-years old man*

A) Axial, coronal and sagittal views of MRI scans in T2-weighted, T2-weighted fluid attenuated recovery and T1-weighted showing the brain of a patient presenting with bvFTD. The ventricles (red arrow) are enlarged and cortical atrophy can be observed in the left temporal lobe and frontal lobe.
B) Axial, coronal and sagittal views of MRI scans in T2-weighted, T1-weighted and T1-weighted of the same patient three years later. The cortical atrophy progressed severely.
C) Aspect of the brain of the same patient that passed away due to the progression of the disease. Brain after fixation.
D) The brain presents with enlargement of ventricles due to cortical atrophy
E) The brain presents with frontal and temporal atrophy (arrow)
F), G), H), I), J) and K) Histopathological pictures of the same brain. Various stainings and immunohistochemistry for tau pathology reveal tau inclusions in neurons and inclusions that correspond to Pick bodies, confirming the diagnosis of bvFTD due to Pick's Disease.
The patient had a mutation in the MAPT gene.
Adapted and modified from "Case records of the Massachusetts General Hospital, Case 09-2015: a 31-year old man with personality changes and progressive neurologic decline"[91]

## *Chronic traumatic encephalopathy*

Chronic traumatic encephalopathy (CTE) is defined as a neurodegenerative disease caused by repetitive head trauma that often happens in contact sports, like boxing or American football[92]. However, Bennet Omalu, a leading pathologist that put the spotlight on CTE by reporting its presence in American professional football players, defines CTE as a "progressive neurodegenerative syndrome caused by single, episodic, or repetitive blunt force impacts to the head and transfer of acceleration-deceleration forces to the brain"[93]. This latter definition suggests that CTE can be caused by a single traumatic brain injury.

Besides contact sports, CTE is also found in patients exposed to physical abuse and in military personnel exposed to blast injuries due to explosive devices[92,94,95].

Clinically, patients suffering from CTE present with a wide spectrum of symptoms that involve an impairment in at least one domain of either cognition, mood and behavior, or motor functioning[92,93]. Neurocognitive impairment features symptoms like decreased concentration, executive dysfunction or memory and language trouble. The mood and behavior impairment involve symptoms of depression, apathy, aggressive behavior, suicidality and impulsivity. As for the motor symptoms, they present with spasticity, ataxia, tremors and gait disturbances[92].

This wide spectrum of clinical presentations shows the difficulty in diagnosing CTE in a patient, because those symptoms overlap with those of other neurodegenerative diseases like Alzheimer's Disease, Frontotemporal Dementia or Parkinson's Disease.

Although, it is widely accepted that CTE occurs following repetitive traumatic brain injuries (TBI), there is evidence that a single TBI can cause CTE, as demonstrated by a case report of a patient who was injured by a gunshot wound to the head, 42 years prior to his death, without any other history of head trauma. Pathological features of CTE were present at autopsy[96].

## Traumatic Brain Injury

Traumatic brain injury is an important public health problem because it is a major cause of death and disability, affecting approximately 57 million people around the world[97] who have been hospitalized for TBI. The incidence of TBI varies from country to country and is estimated to range between 200 and 600 cases per 100000 people per year[98], although it is believed that those numbers are underestimated because not all TBIs are reported, especially those occurring in conflict areas[99]. The major causes of TBI are road traffic accidents, falls and violence. It affects all groups of ages with a peak in incidence at early childhood, early adulthood and old people[99].

Traumatic brain injury is the consequence of an impact to the head by a blunt or penetrant object, a blast wave or an impact from acceleration-deceleration forces leading to either a change in the state of consciousness or amnesia, neurologic and neuropsychological change, a fracture to the skull or intracranial lesion[100].

TBI is characterized by a primary injury mechanism which happens immediately and results in hemorrhage, contusion, shearing of axons and blood vessels, and diffuse axonal injury[101]. It is followed by a secondary injury mechanism, after a certain time of latency that can last a few hours or even weeks after the primary injury mechanism. The secondary injury mechanism is the direct consequence of the latter and is characterized by depolarization of cell membranes, with increased concentration of intracellular calcium concentration, leading to a pathological chain of events, that results in oxidative damage, endoplasmic reticulum stress, mitochondrial dysfunction and different types of tau modifications, like hyperphosphorylation[101].

In the long term, TBI is a risk factor for dementia and neurodegenerative diseases such as AD or CTE[102].

## Pathophysiology of CTE

Like other neurodegenerative diseases, the definitive diagnosis of CTE is done at autopsy[94]. As a tauopathy, CTE brains also have deposition of pathological tau as a hallmark. However, the pattern of distribution is different from other tauopathies, suggesting that CTE is a distinct entity[92]. Even though the pathological manifestation of CTE has a high variability, with

sometimes the coexistence of other protein aggregates such as TDP-43 and even amyloid beta, there is a pathognomonic feature of CTE that is the deposition of tau aggregates in neurons or astrocytes around small blood vessels located in the depths of the brain sulci[94]. This observation along other recurring supportive observations lead a panel of neuropathologists to come up with pathological criteria for the diagnosis of CTE[94]. The required feature for the diagnosis of CTE according to the National Institute of Neurological Disorders and Stroke (NINDS)[94] is the presence of tau aggregates as described before.

In addition, other features that are supportive of the diagnosis of CTE are the presence of neurofibrillary tangles in the superficial layers II and III, the presence of tau aggregates in the CA2 area of the hippocampus and subcortical nuclei like the mammillary bodies, amygdala, substantia nigra among others. The presence of tau immunoreactive thorny astrocytes in the subpial and periventricular regions and tau immunoreactive large grain-like structures.

The presence of TDP-43 immunoreactive inclusions in neurons is also supportive of the pathological diagnosis of CTE.

Macroscopically, the brains of CTE patients tend to present dilatation of the third ventricle, septal abnormalities, atrophy of the mammillary bodies and signs of traumatic injury.

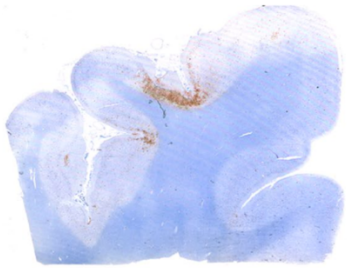


*Figure 15: CTE features in human brain cortical tissue*

AT8 staining that recognizes hyperphosphorylated tau localized at the depth of a brain sulcus. Adapted from McKee et al.[94]

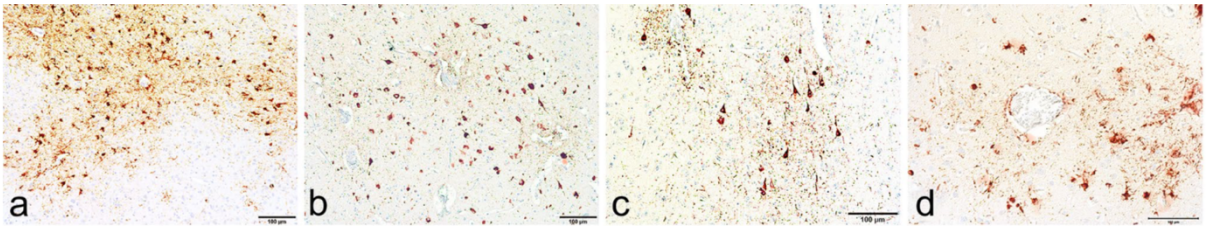



*Figure 16: Tau neurofibrillary tangles and tau inclusions around small blood vessels (a-d)*

Scale bar: 100 μm. Adapted from McKee et al. [94]

## Neuroimaging and biomarkers

Research is currently ongoing to identify diagnostic tools for chronic traumatic encephalopathy. Encouragingly, it has been observed that the presence of tau in the blood plasma is increased after a traumatic brain injury and the level of tau in the CSF is also increased in boxers[101].

PET neuroimaging is also being sought to characterize CTE. A study by Robinson et al.[103], showed in a small cohort of Iraq and Afghanistan war veterans, that there was an increase of uptake of the tau tracer Flortaucipir associated with blast exposure but not with blunt concussion, nor with symptom duration. The neuroanatomical localization of the uptake was also not corresponding to what is usually observed in CTE, showing that further research is needed.

Research on CTE is ongoing with rodent models of TBI that reproduce tau pathology to understand the underlying pathological mechanisms and identify therapeutic targets[95,104–106].

## Relevance of clinical aspects of tauopathies for research

It is frustrating to note that there is still no disease-modifying treatment available yet for tauopathies, especially when we realize the importance of the involvement of the tau protein in the clinic. The current treatments against AD only act on the cognitive symptoms without targeting the disease pathology. Only two classes of treatments are available on the market: cholinesterase inhibitors and glutamate antagonists. They offer some relief but do not prevent the progression of the disease. This highlights the urgency to understand the pathogenic mechanisms of tauopathies in order to identify therapeutic approaches.

The three conditions presented above show the importance of tauopathies in the clinic. With Alzheimer's Disease, we saw how the presence of the amyloid beta pathology can induce tau pathology that is correlated with the cognitive decline. With FTDP-17 causing frontotemporal dementia, we learned that a genetic mutation in the gene MAPT induces tau pathology, demonstrating that tau alone can cause neurodegeneration and cognitive decline, along other symptoms. In the case of Chronic Traumatic Encephalopathy, we see that tau pathology can be induced by a traumatic stress to the brain and that it can occur sporadically as a long-term effects in patients who were subjected to repetitive head trauma or even a single traumatic brain injury.

# Biology of Tau

## Genetics of tau

Tau is a protein encoded by the gene MAPT which is located on the chromosome 17q21. MAPT contains 16 exons, among which exons 2, 3 and 10 undergo alternative splicing. The protein tau is one of the microtubule-associated proteins and is expressed in neurons and in glial cells. Its structure is made of four major domains, starting with the amino (N) terminal acidic projection domain, then a proline-rich domain (PRD), followed by a microtubule-binding domain (MTBD) and finishing with the carboxy (C) terminal domain. Tau in its native and microtubule-unbound form is said to be an unfolded protein which preferentially adapts a conformation that looks like the shape of a paperclip, however, when bound to microtubules through its microtubule-binding domain, tau is straightened out and its amino terminal domain projects away from tau, possibly to modulate spacing from other neighboring microtubules.

The microtubule-binding domain is formed by a number of imperfect repeat domains that are present four times (4R) if exon 10 is present, otherwise three times (3R) if exon 10 is absent after splicing. The repeat domain in second position which is encoded by exon 10 has a propensity to form a beta-sheet structure together with the repeat domain in third position. 4R and 3R isoforms of the tau protein are equally expressed in the adult human brain, whereas only 4R tau isoforms are found in the adult mouse brain.

Exon 2 and 3 encode each for a 29 amino acids insert, both inserts being situated in the amino terminal domain. Just like exon 10, exons 2 and 3 can be expressed or not depending on their alternative splicing. Exon 3 is never expressed without exon 2, whereas exon 2 can be expressed alone without exon 3[107]. Therefore, when only exon 2 is expressed, the protein tau contains only one insert (1N) in the N terminal domain. When exons 2 and 3 are expressed, tau contains two inserts (2N). When neither exon 2, nor 3 is expressed, tau contains no insert at all (0N).

In summary, there are six different combinations possible with the alternative splicing of exons 2, 3 and 10 that form the six isoforms of the tau protein expressed in the adult human brain: 3R0N, 3R1N, 3R2N, 4R0N, 4R1N and 4R2N.

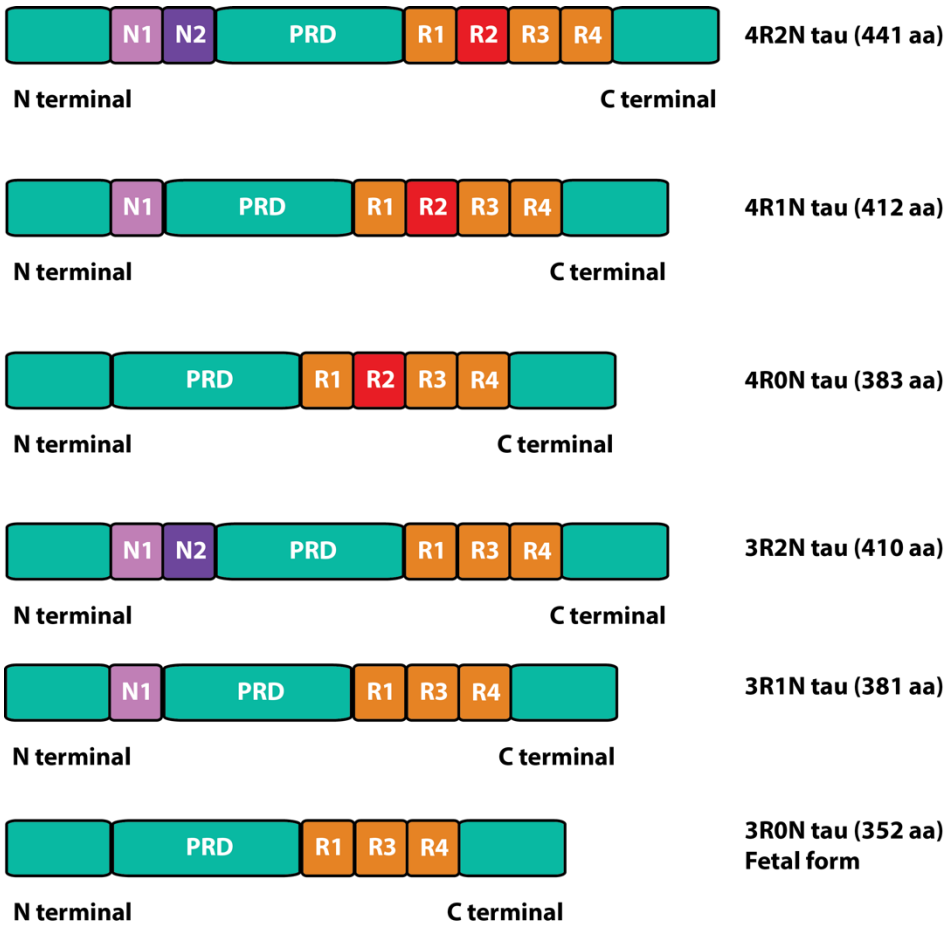



*Figure 17: The six isoforms expressed in the adult human brain*

N1 and N2 are the two possible N-terminal inserts. PRD is the Proline Rich Domain and R1-4 are the repeat domains found in the microtubule-binding domain.

## Tau isoforms

The different isoforms of tau are differentially expressed throughout the development. Indeed, the shortest isoform 3R0N is exclusively present in the fetal brain[77,108]. Whereas in adult humans, the other isoforms are expressed with an equal ratio of 3R over 4R tau[40,77,107,109]. In adult mice, only 4R tau isoforms are present.

Regarding the isoforms that contain either 0, 1 or 2 N terminal inserts, it is found that globally 1N is predominant (54%) over 0N (37%) and 2N (9%) in humans. However, it is important to note that the expression of isoforms is variable between different brain regions[109] and even within subcellular localization[110]. Overall, this heterogeneity in expression of isoforms within

the cell and between brain regions suggests that the different isoforms might have their own specific role. It has been shown that in the microtubule-binding domain, the number of repeat domains has an impact on the level of binding of tau to the microtubule. Indeed, 4R tau binds more strongly than 3R. In addition, the presence of N-terminal inserts has been associated with microtubule spacing and membrane-binding abilities[77,107,111]. Therefore, 2N isoforms would have different characteristics than the 0N isoform.

Accordingly, the pathologic role is also different between isoforms. For example, it has been shown that 4R isoforms have a higher propensity to aggregate than the 3R isoforms. This is well illustrated with the P301L mutation causing FTDP-17. Indeed, this mutation is situated in the exon 10 which encodes the R2 binding domain that is present in 4R tau isoforms only. This mutation induces aggregation and causes tau accumulation, with a shift in the 4R/3R ratio towards higher level of 4R tau. In addition, the 0N isoforms are more prone to aggregate than 1N and 2N isoforms[112].

Tauopathies seen earlier have their own specific profiles of tau isoforms found in pathologic inclusions. This allows a classification of tauopathies based on the predominant isoform found. Primary Age Related Tauopathy, Alzheimer's Disease and Chronic traumatic encephalopathy still have both 4R and 3R tau isoforms aggregates in the brain. Pick's Disease is a 3R tauopathy, since a majority of 3R tau isoform is found in the Pick bodies. Progressive Supranuclear Palsy and corticobasal degeneration are 4R tauopathies. FTDP-17 on the other hand can be 3R, 4R or 3R and 4R, depending on the location of the mutations[40,42]. Typical P301L or P301S mutations are located on exon 10, encoding for the 4R tau isoform. Therefore, FTLP-17 due to those mutations are 4R tauopathies.

## Post-translational modifications of tau

The protein tau undergoes several changes that are linked to its functions. The most widely studied post-translational modification is tau phosphorylation. It is the most known modification because of its presence in pathological tau deposition found in tauopathies[77,113–115]. Although hyperphosphorylated tau is a hallmark of pathology, phosphorylation itself occurs physiologically and is linked to the functions of tau protein[86,116,117]. Furthermore, phosphorylation is developmentally regulated, as developing neurons have an increased level of phosphorylation of tau protein, mainly on the 3R0N isoform[77,107,118]. Studies have identified

at least 85 sites on the tau protein that can be phosphorylated on either serine, threonine or tyrosine residues[77,116,119]. Phosphorylation of tau is regulated by the dynamic activity of several kinases which add a phospho group on the protein and phosphatases which dephosphorylate tau. Among the kinases involved in tau phosphorylation are the glycogen synthase kinase 3 (GSK3), the cyclin-dependent kinase 5 (Cdk5), Src kinase or Fyn kinase[77,120]. Many more kinases are involved and they can be classified in three main groups: proline-directed serine/threonine-protein kinases, non-proline-directed serine/threonine-protein kinases and tyrosine residues specific protein kinases. However, the most important kinase identified is GSK3 because it phosphorylates at least 40 out of 85 identified sites of phosphorylation and 29 of those 40 sites are found to be hyperphosphorylated in Alzheimer's Disease[77,120–122].

Protein phosphatase 1 (PP1), 2a (PP2A), 2b (PP2B), 2c (PP2C) and 5 (PP5) are involved in dephosphorylating tau, however the most important phosphatase is PP2A which accounts for 70% of all phosphatase activity[77,123–125]. Together, kinases and phosphatases work on the dynamic phosphorylation and dephosphorylation that physiologically occur in order for tau to accomplish its functions. However, an imbalance that activates kinases or impairs the phosphatases is expected to cause hyperphosphorylation, which can be linked to the tau pathology when chronically induced[119].

Other post-translational modifications of tau include acetylation which is also important regarding tau pathology, because acetylation has been linked to the mislocalization of tau in the somatodendritic compartment[126–128].

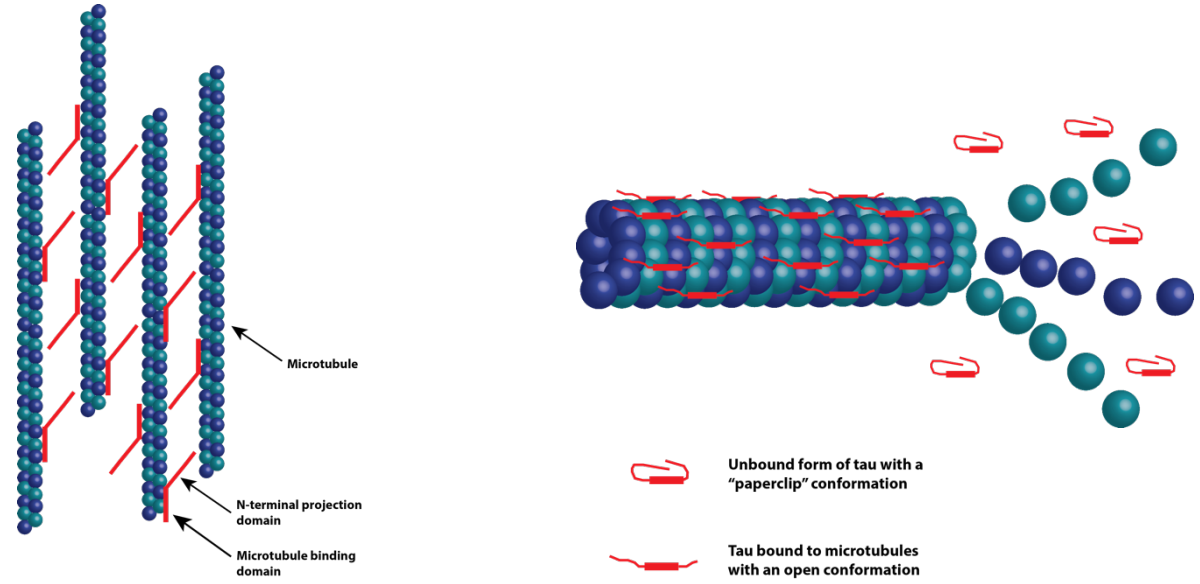
In addition, tau can undergo truncation[129], glycosylation[130], ubiquitinylation[131], glycation[132,133], sumoylation[134], oxidation[135] or nitration[136] among others. All those modifications have been described and all have a physiological role, as well as a role in tau pathology[77,116,137,138].

## Localization and functions of tau in neurons

One way to explore the roles played by the protein tau is to determine the subcellular localization of tau, as well as its molecular interactions. Localization of tau is known to be developmentally regulated, as tau is mainly present in axonal and somatodendritic compartments in immature neurons. In mature neurons, however, tau is mainly found in the

axonal compartment, where it is known to accomplish its most described and well-known function, which is microtubule stabilization and assembly[139,116]. Through its microtubule-binding domain, tau attaches to tubulin polymers and dynamically regulates microtubule assembly, thus playing a crucial role in stabilizing and reorganizing the cytoskeleton. While bound to microtubules, the paperclip conformation of unbound tau changes and opens up[116]. 4R tau binds with a higher affinity to microtubules than 3R tau, most certainly because of the presence of the peptide sequence "VQIINK" present only between the first repeat R1 and the second repeat R2, which makes it specific to 4R tau[107,140]. Of note, this sequence is involved in the formation of tau aggregates found in tauopathies. Binding and detachment from microtubule is regulated through phosphorylation of tau[139,113]. When tau is dephosphorylated, it has a higher affinity to microtubule, whereas phosphorylation tends to maintain tau detached from the microtubules. The microtubule stabilization and assembly role of tau highlights its importance in axonal growth and cytoskeletal structure stability in the mature neuron. Interestingly, tau is not the only microtubule-associated protein. *In vivo* studies in mice show that knocking out tau does not significantly alter the stabilization of the microtubule. This suggests early developmental compensation mechanism by other microtubule-associated proteins, mainly MAP1A which is found to be increased[141]. This compensation does not seem to occur in adult brains[141,142], which might explain why tau pathology causes neuronal microtubule instability.

Even though tau knockout mice do not show any major neurological defect, it has been observed that the diameter of axons in tau deficient mice is smaller than usual[107]. This brings us to the role of the N-terminal projection domain, which acts as a buffer to provide space between microtubules, increasing the axon diameter. Therefore, the N-terminal projection domain of tau has a role in the organization and spacing of microtubules[143].




A B

*Figure 18: Tau interactions with microtubules in the axon*

A. The N-Terminal projection domain has a role in organizing and spacing the microtubules. B. Tau protein promotes polymerization of tubulin and helps stabilizing the microtubule via interactions with the tau microtubule-binding domain. Binding to microtubules changes the conformation of the protein.

In the axon, tau has an additional role in the regulation of axonal anterograde and retrograde transport by interacting with transport proteins like dynein and kinesin[116,144]. A recent study in a *Drosophila* model showed that tau depletion affects the vesicular transport by increasing the time spent by a vesicle in a near immobile position and by decreasing the mean run length of vesicles[145], further illustrating the involvement of tau in axonal transport.

In addition, it has been shown that the inserts present in the N-terminal projection domain of the 1N and 2N tau isoforms have the ability to bind to the annexin A2 protein at the level of plasma membranes in the axons, highlighting the ability of tau to interact with cell membranes[146,147]. Through the membrane interaction, tau is believed to play a role in various cell signaling pathways by interacting with transmembrane receptors[77].

Although tau is mainly found in axons, it is also present in other compartments of neurons[116]. Tau has been found in the somatodendritic compartment, in the postsynaptic densities and

even in the nuclear compartment of cells. This suggests that tau is likely to play additional roles which could be important in neuronal physiology[146].

In dendrites and dendritic spines, tau has an important role in synaptic plasticity. Tau acts as a scaffold at the level of synapses, both in pre- and post-synaptic compartments. Tau is known to play a role in synaptic activity, as it interacts with Fyn tyrosine kinase and binds to PSD-95 which regulates the function of NMDA receptors[84,86,146].

In pathological conditions, it is precisely at the level of the postsynaptic density that tau has been proposed to exert its deleterious effects. Indeed, in Alzheimer's disease, amyloid beta causes the mislocalization of tau at the postsynaptic level, abnormally increasing tau abundance in this compartment. This may increase the interaction of tau with Fyn kinase and PSD-95, increasing the synaptic activity through NMDA receptors, which might lead to excitotoxicity. Furthermore, a study showed that tau is required to cause long-term depression (LTD) in the hippocampus[148]. This means that tau plays a role in downregulating the AMPA receptors, possibly leading to synapse pruning.

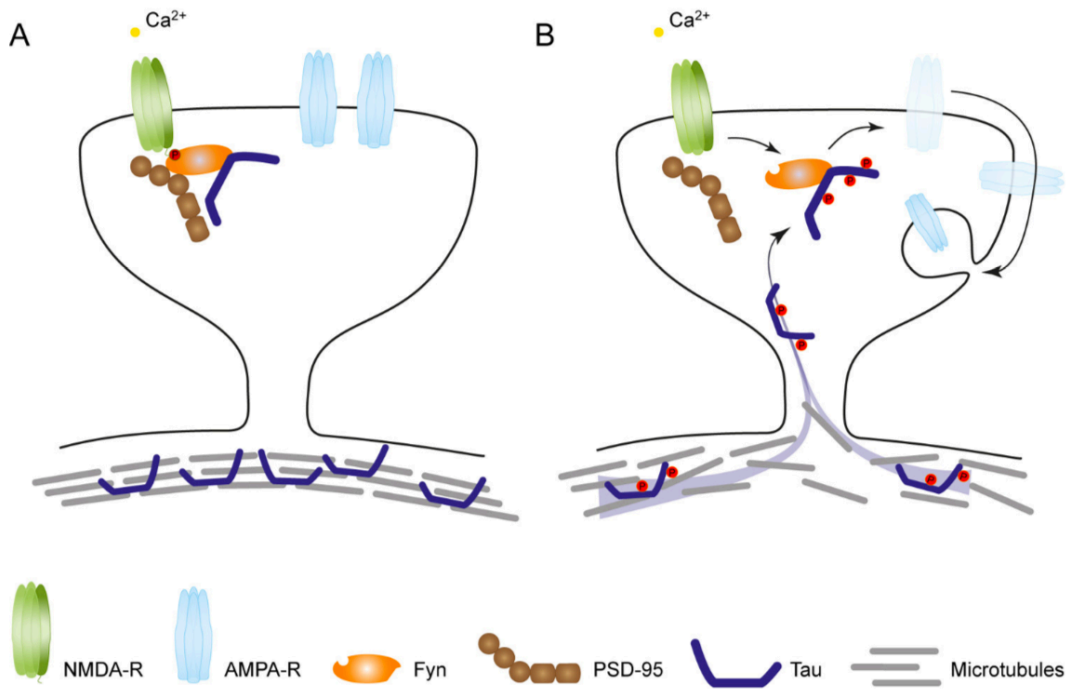



*Figure 19: Tau interaction at the synapse*

A. Tau is physiologically present at the synapse where it has a role in synaptic plasticity and in the neuronal activity signaling by interacting with postsynaptic proteins like Fyn kinase and PSD-95 to promote the activation of NMDA receptors
B. In pathological conditions, there is an increased presence of pathological tau in the synapse, over activating NMDA receptor which leads to excitotoxicity and also internalizing AMPA receptors which causes long term depression and disappearance of the synapse.
Adapted from Arendt et al.[86]

Interestingly, tau has also been found in the nuclear compartment. This is mainly the case for the 4R1N isoform, which is the most predominant isoform in the mouse brain. This observation further highlights the fact that isoforms have different roles to play and show different subcellular distribution[110].

In the nucleus, tau is believed to bind to DNA and plays a role of protection against different stressful stimuli, like hyperthermia or hypothermia which can cause DNA breakage[77]. In addition, tau has been described to play a role in the regulation of genetic and epigenetic expression by binding and changing the conformation of DNA and its binding to histones.

Furthermore, other roles have been found for tau, including a role in broader metabolism[146]. Indeed, it has been discovered that tau null mice display increased insulin signal resistance in the brain and that tau depletion resulted in gain of weight and inactivity, suggesting an anorexigenic effect of tau in the brain in physiological conditions[149], possibly via a signaling role of tau in the insulin signaling pathway.

Surprisingly, the insulin signaling pathway also includes the GSK3 kinase, which is normally inactivated by insulin, through insulin receptors[150,151]. Thus, insulin resistance similar to type 2 diabetes would prevent inactivation of GSK3, which would then be able to phosphorylate its downstream substrates, among which the tau protein in the brain[150]. Accordingly, it is known that patients with type 2 diabetes have an increased risk of developing dementia[152]. Overall, this suggests a close relationship between tau, which is broadly involved in neurodegenerative diseases and insulin resistance, and associated metabolic perturbations.

Further roles of tau have been described including a function in myelination[146]. This shows that tau is much more than a microtubule-associated protein. In pathological conditions, the perturbations of tau function may therefore affect many mechanisms that are important for neuronal physiology.

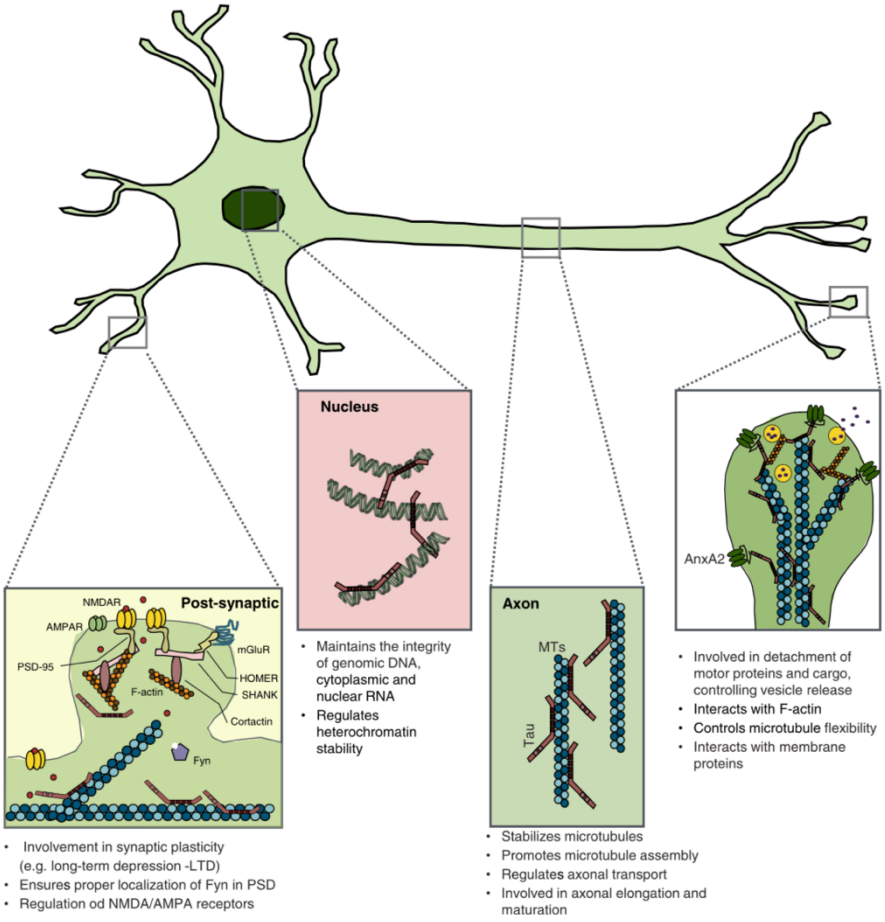



*Figure 20: Identified functions and localizations of tau in neurons*

Tau protein is located in several compartments in the neuron. In axons, tau stabilizes microtubules and regulates axonal transport. At the synapses, tau interacts with membrane proteins and plays a role in neuronal activity and synapse plasticity. In the nucleus, tau protects the DNA and nuclear RNA, while regulating the genetic and epigenetic expression. Adapted from Sotiropoulos et al.[146]

# Tau pathology

## Tau aggregation

The pathological hallmark of tauopathies is the presence in brain tissues of aggregated forms of tau forming neurofibrillary tangles. Tau is found to be hyperphosphorylated in those aggregates. It has been suggested that the process that converts monomeric forms of tau found in physiological conditions into aggregated neurofibrillary tangles is progressive and involves several modifications of tau[77,116,119,153–155].

Hyperphosphorylation, meaning an excessive number of phosphoryl groups on tau, is known to decrease the affinity of tau to microtubules and to favor the mislocalization of tau in the somatodendritic compartment. Hyperphosphorylation also changes the conformation of tau, which might expose the peptide sequences that are located near the second repeat R2 and third repeat R3 in the microtubule-binding domain[116,140,156].

These two peptide sequences have been described to have a higher propensity to form beta-sheets, which may promote high affinity aggregation with other beta-sheet structures from other misfolded tau proteins, marking the first step of a pathogenic cascade. It is important to note that hyperphosphorylation is not always described as promoting tau aggregation[157] and that it is not the only event that can cause such conformational change in tau. Indeed, tau truncation by several enzymes and tau acetylation have also been described to cause the exposition of the two peptide sequences that leads to aggregation[122].

The tau protein starts to aggregate by first forming tau dimers and oligomers. Further multimerization leads to more elaborate structures such as paired helical filaments (PHF) and straight filaments (SF). Accumulation of paired helical filaments and straight filaments leads eventually to the formation of neurofibrillary tangles.

However, the mechanism by which this aggregation process leads to neurodegeneration is not yet fully understood. The first question that arises is whether neurodegeneration is due to a toxic loss of function of tau or a toxic gain of function. For example, a toxic loss of function can lead to less binding of tau to its interacting partners like microtubules and would prevent tau to accomplish its normal functions. On the other hand, mislocalization of tau in dendritic spines, causes excitotoxicity as seen in the pathophysiology of Alzheimer's Disease, suggesting a toxic gain of function. Probably both toxic mechanisms might be involved[158].

The other question that is debated is which form of aggregated tau causes neurodegeneration? Several studies showed that neurofibrillary tangles are not the main toxic tau species, but rather represent a byproduct of a neuronal rescue mechanism. Several studies suggested that soluble tau oligomers might be a major toxic species, by disrupting the neuronal activity and seeding tau aggregation and further toxicity in neurons exposed to such oligomers[159,160,161]. Indeed, Santacruz et al., showed in P301L transgenic mice that by suppressing the expression of human tau, they induced a decrease in neuronal loss and cognitive impairment, even though neurofibrillary tangles did not decrease and even continued to progress[162], possibly by the aggregation of the remaining soluble tau oligomers.

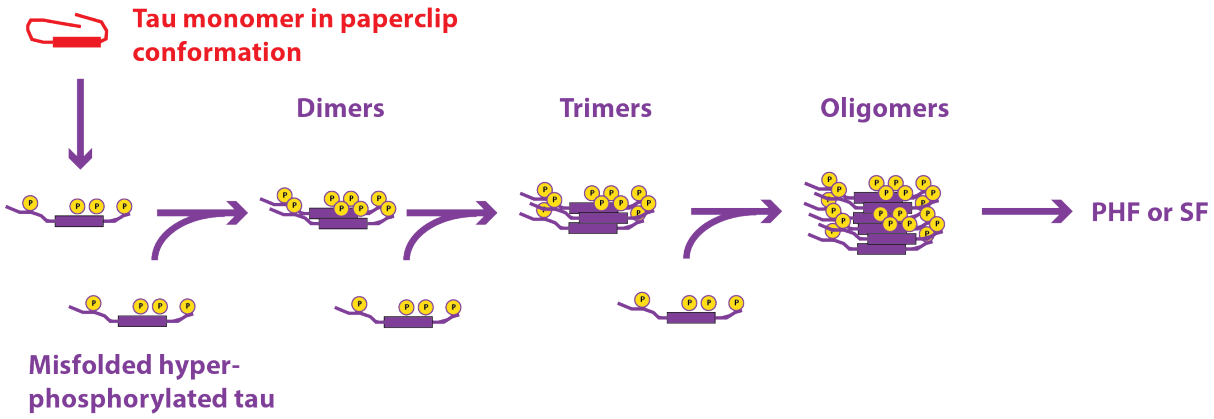



*Figure 21: Pathogenic cascade of tau aggregation*

When a tau monomer in a paperclip conformation undergoes a post translational modification such as truncation or acetylation, the conformation changes and exposes a peptide sequence that is prone to form beta sheet structures with other tau proteins. The cascade begins with two tau proteins forming dimers then recruiting more tau forming trimers, then forming oligomers. Oligomers are believed to be the toxic species of tau that seeds pathology. Eventually, oligomers form paired helical filaments (PHF) or straight filaments (SF) that are found in NFTs. Tau aggregates are often hyperphosphorylated, however the exact link between phosphorylation and aggregation is not yet fully understood.

## Tau propagation

From the observation made on autopsy of brains of patients with tauopathies, that neurofibrillary tangles and other tau aggregated bodies progress in a stereotypical neuroanatomical pattern of spread, came the hypothesis that tau might spread from neuron to neuron. Supporting this hypothesis, several experiments showed that tau is present in the extracellular compartment, that tau can be released from cells and that tau can be uptaken by recipient cells[77,163,118].

The secretion of tau has been described to happen in multiples ways. *In vitro* experiments in several cell lines, among which SH-SY5Y[164] or neurons differentiated from IPS cells[165], showed that tau is physiologically secreted in the cell culture medium, mainly through an unconventional pathway. Indeed, the vast majority (90%) of secreted tau is released as a free protein, whereas only a small fraction is bound to microvesicles such as exosomes or ectosomes[164].

The tau that is released as a free protein is secreted through mechanisms that are not fully understood, however it has been reported that tau may exit the cell through diffusion or through the formation of nanotubes[166] between cells.

The presence of tau in the extracellular compartment suggests another role of tau in intercellular signal transmission, as tau has been described to bind to M1/M3 muscarinic receptor in downstream cells, provoking an increase of calcium concentration in the cell[167].

Furthermore, it has been reported that increased neuronal activity stimulates the release of tau *in vitro*[168].

Tau phosphorylation has been reported to increase tau secretion through exosomes[164]. This finding is in line with the fact that phosphorylated tau is more available to be secreted compared to dephosphorylated forms of tau, since the latter tend to bind microtubules.

After the demonstration that tau can be released from cells, other studies showed that tau can be taken up by downstream cells. *In vitro* studies in microfluidic chambers showed that pathological tau can be internalized by recipient neurons[169,170]. The mechanism of internalization is not fully described yet, whether it is by endocytosis or simple diffusion. Nevertheless, tau is able to bind to heparan sulfate proteoglycans that internalize tau through macropinocytosis[77,118,171].

Interestingly, tau fibrils bind to the transmembrane protein APP which is involved in Alzheimer's Disease. APP increases the internalization and intracellular aggregation of tau[172]. It has been described that small oligomers are the species of tau that are spontaneously taken up by recipient cells *in vitro*, but tau monomers are excluded[173,169]. However a recent study tends to contradict this by showing in human induced pluripotent stem cell neurons that tau monomers can also be taken up by downstream cells[174].

Most convincingly, a study by Clavaguera et al.[175] showed that intracerebral injection of brain extracts from transgenic mice with P301S mutation into the brain of transgenic mice expressing human wild-type tau, not only induced tau pathology but also the spread of this pathology along a path that is neuroanatomically connected. Further studies using the injection of tau fibrils from brain extracts, or injection of preformed recombinant tau fibrils or the overexpression of tau via viral vectors showed that tau pathology developed over time in distal neuroanatomically connected regions[176,177,178,179,118]. This observation was also confirmed with the injection of tau oligomers from rat brains with traumatic brain injury (TBI) into the hippocampus of Htau transgenic mice that express human tau. The tau oligomers induced by TBI were able to propagate and induce pathology in mice[104]. Other studies with *in vivo* models of TBI confirmed that a single traumatic brain injury induces a propagative tau pathology[105,106].

In addition, the propagation of tau is closely linked to neuronal activity[170]. Indeed, an increased neuronal activity leads to an increase in the release and spread of tau. The fact that tau spreads along neuroanatomically connected tracts and that the spread of tau is neuronal activity dependent suggests that the propagation of tau is done in a transsynaptic manner[77].

Different conformers of tau with a functional diversity have been identified and described as "strains" of tau. Those strains of aggregated tau seem to be stably transferred from neuron to neuron[180]. This means that an aggregated strain of tau is able to leave a neuron, enter the recipient neuron and seed aggregation inside the new neuron in a manner that templates its conformation[181].

An additional *in vivo* study showed that the injection of brain extracts from human patients who suffered different tauopathies, recreated the same pattern of tau aggregation in the mouse brain. For example, brain extracts from a patient who suffered with progressive supranuclear palsy, induced tufted astrocytic inclusions in the mice after injection[182]. This

suggests that the variability of the clinical and pathological presentations of the different tauopathies might in fact be due to different strains of tau aggregates.

Overall, 1) the fact that tau aggregates in cells adopt a misfolded conformation and recruit other tau monomers to form bigger aggregates, 2) the fact that an aggregated oligomeric form of tau is able to travel from neuron to neuron and induce pathology in the recipient cell by templating and 3) the fact that different tau strains are stably propagated, all of this is reminiscent of the biology of the prion that seeds pathology. This similar mechanism of aggregation (or nucleation), elongation and seeding lead Stanley Prusiner to propose that tau is a prion protein, along other proteins that aggregate and propagate in a similar manner in neurodegenerative diseases, such as alpha-synuclein involved in Parkinson's Disease[183].
However, this classification is discussed in the scientific community, as tau is generally considered not as a prion but rather as a "prionoid" protein, that behaves like a prion but does not meet all the prion criteria. Indeed, tauopathies are not considered as infectious diseases for example. Another difference between tau and the prion is demonstrated by Wegmann et al. who showed that in mice lacking endogenous tau protein, the expression of human P301L tau did not halt tau propagation, although it reduced its neurotoxicity[184]. Whereas in prion disease, normal endogenous prion protein $PrP^{c}$ is required for the propagation of pathological prions[185].
An additional pathway for tau propagation involves neuroinflammation[77]. Supporting this observation, it has been reported that extracellular pathological tau triggers the activation of microglial cells that, in turn, trigger further phosphorylation of tau[186]. Furthermore, it has been shown that microglia is able to internalize tau containing exosomes that are released from cells[187]. However, the strongest evidence of the involvement of the microglia in tau propagation comes from a study by Asai et al.[188] that showed that the removal of microglia dramatically decreases tau propagation.

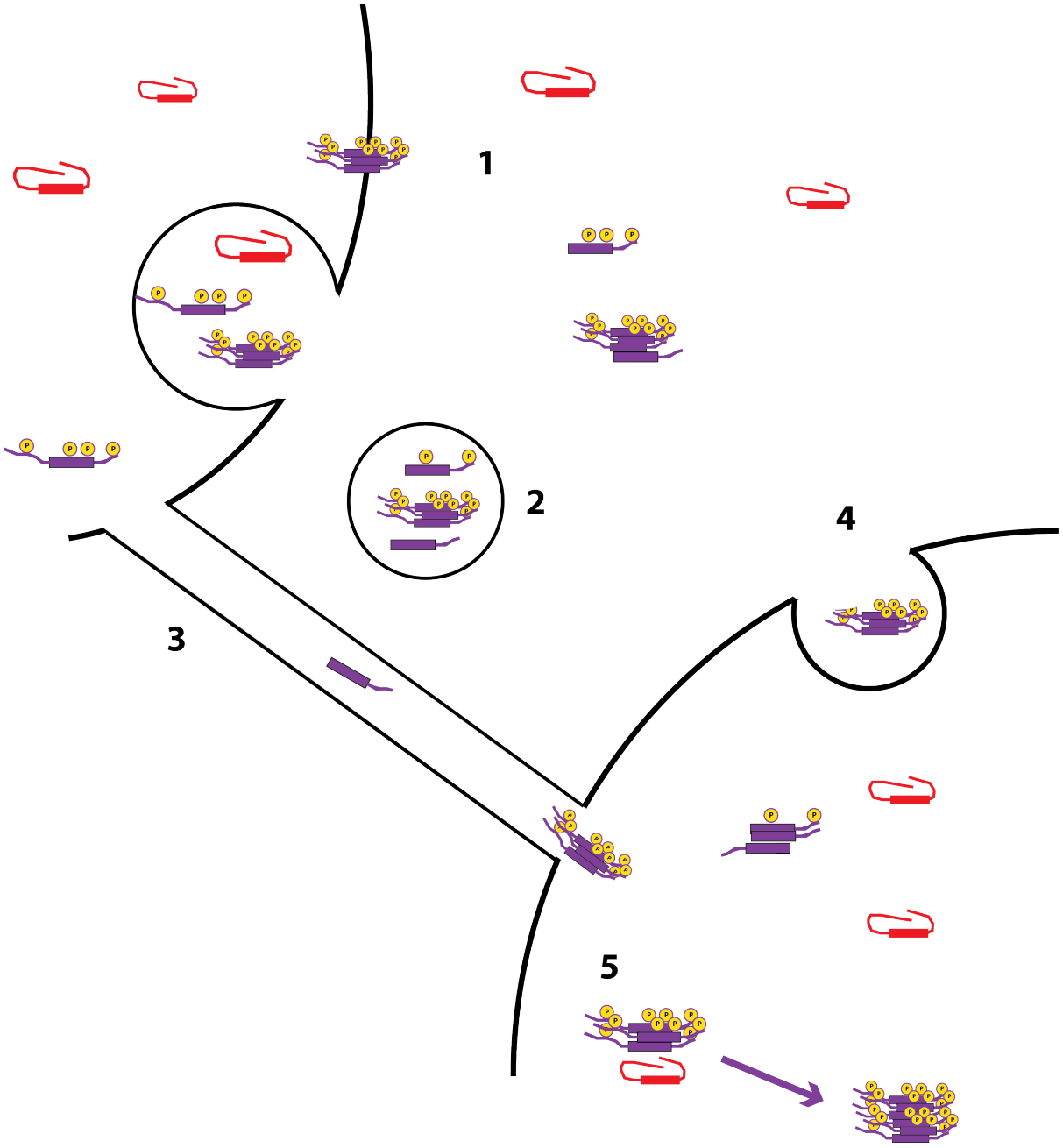



*Figure 22: Different pathways identified for tau propagation*

1) Tau is physiologically present in the extracellular compartment (in red). Approximately 90% of secreted tau is released as a free protein, not bound to any vesicle. One way tau might exit the donor cell is by diffusion through cell membranes. Toxic oligomeric tau that is truncated, acetylated and/or hyperphosphorylated might also exit the cell this way.
2) Tau can propagate inside vesicles such as exosomes or ectosomes.
3) Nanotunnels have been described and tau as a free protein might travel through those tubes to enter the recipient cell
4) Tau can bind to receptors on the recipient cell membrane and be internalized through endocytosis or micropinocytosis.
5) When internalized, toxic oligomeric forms of tau can recruit endogenous monomeric tau and template their pathological configuration and seed pathology.

## Therapeutic targets against tau pathology

Understanding the mechanism by which pathological tau causes neurotoxicity is essential in order to develop therapeutic strategies. From what we learned on the pathogenesis of tau, we can identify several steps where therapeutic agents can act, from the expression of tau to its aggregation and propagation.

Since studies in tau knock out mice did not show deleterious effects[142] and since tau presence at synapse is compulsory for amyloid beta to cause toxicity[189], the idea of reducing the expression of tau by targeting the mRNA via transcription inhibitors might be one therapeutic approach[116,190].

Another strategy to tackle pathological tau is to address the hyperphosphorylation of tau that is a post-translational modification that detaches tau from microtubules, eventually leading to tau aggregation. Hyperphosphorylation can be regulated by therapeutic agents targeting kinases and phosphatases. Inhibition of GSK3$\beta$ has been studied and several drug candidates were tried clinically[190], however the clinical trials were not successful because of lack of clinical benefit[77,116]. On the other hand, increasing the activity of phosphatases to decrease hyperphosphorylation might be another approach, however targeting specifically the phosphatase has been difficult[191].

Since pathological tau is detached from microtubules, leading to the destabilization of the cytoskeleton and impairment of axonal transport, microtubule stabilizing agents have been tested. Even though, stabilization of microtubules reduced tau pathology, neurotoxicity and axonal deficits in transgenic mice[192], no clinical benefit was noted in clinical trials[191].

Pathological tau is found in a misfolded conformation; under physiological conditions, misfolded proteins are degraded through the ubiquitin-proteasome system[190,193]. Therefore, one idea is to enhance the activity of this system by inhibiting the activity of the heat shock protein 90 (HSP90), that results in the increase in the activity of the HSP70 and CHIP (the carboxyterminal of HSP70-interacting protein). HSP70/CHIP increase the degradation of tau protein. However, tau aggregates may acquire a size that do not allow them to be cleared by this system. Aggregates get degraded through autophagy, therefore, molecules that enhance this pathway of elimination have been tested. However, the long-term use of these products does not seem to be safe[190], because of the risk of-off target effects, with the possible collateral degradation of other proteins.

The fact that pathological tau starts to self-assemble and form oligomers and aggregates in tauopathies is a strong argument to find compounds that interfere with the mechanism of aggregation. Inhibiting the aggregation process would therefore prevent the formation of toxic oligomeric tau. It is important that putative compounds inhibit aggregation at early stage and not just the formation of advanced fibrils, since oligomeric tau has been identified as one of the species potentially causing neurotoxicity[161]. The most advanced therapeutic agent so far is the derivative of methylene blue, LMTM (leuco-methylthioninium bis-hydromethanesulfonate) which is undergoing phase III clinical trial.

Last, since pathological tau propagates from neuron to neuron in a prionoid manner, one promising strategy is to intercept with antibodies the tau seeds when they are in the extracellular compartment and block propagation. Therefore, anti-tau immunotherapy is one important therapeutic approach to consider, either through active or passive immunotherapy. Several vaccines and antibodies targeting various epitopes have been developed and are currently in various stages of clinical trials[190,191]. Most of these immunotherapies target epitopes of tau that are specific to pathological conditions. So far, recent studies in mice showed that anti-tau immunotherapies effectively reduced tau pathology[194], suggesting a possible beneficial effect on the progression of tauopathies.

# Chapter 2: Aims of the thesis

Tauopathies and in particular Alzheimer's disease pose a serious challenge to society by the magnitude of their prevalence in the world and the threat of an exponential increase predicted because of population ageing. The extent of tauopathies stands in stark contrast with the absence of disease-modifying treatment available to patients. This fact highlights the urgency of conducting research to better understand the pathophysiology of tau protein involved in tauopathies and define new strategies for therapeutic approaches.

In this work, we used an approach based on the intracerebral injection of AAV vectors to induce local tau pathology by overexpressing the human 4R0N tau protein in brain regions which are critically involved in Alzheimer's disease, such as the hippocampus and the entorhinal cortex. A number of assays were established to quantify the development of the tau pathology, both at the immunohistological and biochemical levels. We also used this vector system to generate a quantitative model of the rate of transfer of the human tau protein from the site of tau overexpression in the injected hippocampal CA3 region to the contralateral hippocampal formation.

This animal model approach was used with the objectives to assess the efficacy of two types of therapies that target different facets of the tau pathology, according to the following two main aims:

***Aim 1***: The first aim of this thesis is to specifically address, in the context of tauopathies, the question of the efficacy of aggregation inhibitor compounds that target the formation of beta-sheets in various proteins. Indeed, these compounds have been designed to target proteins with a propensity to misfold, including the human tau protein, in order to prevent the propensity of tau to form oligomers and/or fibrils which lead to neurotoxicity. Here, we characterize their effects on pathological markers of tau in a mouse model based on the overexpression of human tau in the entorhinal cortex using an AAV8 vector. This aim is addressed in Chapter 3.

***Aim 2***: The second aim of this thesis is to assess the efficacy of an active immunotherapy approach that targets tau phosphorylated at Ser396/404 residues. In particular, our objective

is to evaluate if this approach can prevent the neuron to neuron transfer of human tau and the neurodegenerative effects of human tau accumulation. Indeed, vaccines engineered to elicit an anti-tau antibody response have been proposed to target the neuron to neuron transfer of pathological tau, to prevent further seeding of the pathology in distally connected regions of the brain, which may contribute to disease progression. Towards this aim, we use a quantitative AAV8 based mouse model of tau propagation, both in presence and absence of the amyloid pathology, exploiting the interhemispheric hippocampal connectivity. This aim is addressed in Chapter 4.

# Chapter 3: Pan-protein aggregation inhibitor compounds increase tau phosphorylation and microglial response possibly leading to tau clearance

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2) AC Immune SA

Abstract:

Intraneuronal accumulation of tau aggregates is the hallmark feature of several neurodegenerative diseases that are called tauopathies. Tau is a microtubule-associated protein that is in a soluble monomeric form in physiological conditions. Through a mechanism that might involve several post-translational modifications in tau, such as truncation or hyperphosphorylation, tau monomers start to assemble with each other, forming oligomers and more elaborate fibrillar structures that become insoluble in pathological conditions. The process of pathological tau assembly is thought to contribute to neurotoxicity through a gain of toxic function and/or loss of function. Therefore, interfering with the assembly of tau into oligomers and aggregates is an important target for therapeutic strategies. Here, we developed a model of sporadic tauopathy in mice using AAV-mediated overexpression of human 4R0N wild-type tau in the entorhinal cortex to assess the efficacy of two pan-protein aggregation inhibitors.

No effect of the compounds were seen on markers of conformational changes (MC1), formation of tau multimers (HT7/HT7 AlphaLISA) and late stage pathological phosphorylation (Ser396/404). Nevertheless, we found by immunohistochemistry and biochemistry that the aggregation inhibitor compounds increased the level of phosphorylation of tau at the Ser202/Thr205 sites in the entorhinal cortex and in the distal synaptically connected hippocampus. In addition, we found a trend towards an increase in microglial area coverage in the entorhinal cortex of compound-treated mice. The increase in microglial density was found to be significant in the hippocampus, suggesting that microglia was activated following phosphorylation of tau at the residues Ser202/Thr205. Remarkably, a significant decrease in human tau in the CSF of mice treated with the compounds suggests an effect on the clearance of tau.

# Introduction

The intraneuronal deposition of tau aggregates is the hallmark feature of many neurodegenerative diseases, collectively named as "tauopathies"[39,77], among which Alzheimer's Disease is considered as the most prevalent tauopathy. Tau aggregates typically accumulate as fibrillar deposits known as neurofibrillary tangles, in a stereotypical spatial temporal pattern. In Alzheimer's Disease, those neurofibrillary tangles typically start to appear in the entorhinal cortex, followed by the hippocampus and the neocortical areas of the brain, as described by the Braak staging[74]. Although the exact role of neurofibrillary tangles in tauopathies remains unclear[160], it has been suggested that the chain of events leading to the oligomerization of tau and the formation of tau aggregates is key in the pathogenic cascade causing neurodegeneration and cognitive decline[195].

The mechanisms that trigger tau monomers to misfold and self-assemble into dimers, trimers, and oligomers, to finally form more elaborate structures like paired helical filaments are not fully understood yet[140,155]. It has been shown that the microtubule-binding domain of tau is at the core of tau aggregation[77], with a propensity to form beta-sheet structures that interact with other tau proteins in oligomers. It has been shown that the core of the formation of beta-sheet structures are two hexapeptide sequences, which are "VQIINK" located at the beginning of the second repeat domain and "VQIVYK" located at the beginning of the third repeat domain of tau protein[140]. These hexapeptide sequences are essential in the formation of tau fibrils and the exposition of these sequences by a conformational change in tau may initiate the process of aggregation and fibril formation[116]. Tau post-translational modifications such as nitration, truncation, acetylation and hyperphosphorylation are likely to be involved in the initial steps of this pathogenic cascade[77,116]. Tau misfolding and aggregation may lead to neuronal toxicity either through the gain of toxic activities, and/or via the loss of physiological tau functions, among those the stabilization of microtubules.

Therefore, interfering with tau misfolding and aggregation has been identified as an important target for potential disease-modifying therapies. *In vitro* studies identified small molecules that can interfere with the misfolding of tau into beta-sheets, and therefore possibly prevent the assembly of tau monomers into oligomeric and aggregated forms of tau. *In vitro* assays demonstrated that small molecules can successfully inhibit tau aggregation, and even showed that tau aggregates can be disaggregated [191,196,197]. However, it remains to

be proven if these small anti-tau aggregation compounds can provide clinically beneficial effects.

The LMTM (leuco-methylthioninium bis-hydromethanesulfonate) is so far the only one of such compounds to have reached phase III clinical trial. It was shown to improve cognition in patients with mild to moderate AD treated with LMTM as a monotherapy. In contrast, patients treated with LMTM as an add-on to their regular symptomatic treatment for AD showed no significant improvement[198].

Since the mechanism of action of aggregation inhibitors is not fully understood *in vivo* yet, it is therefore important to further characterize their mode of action in animal models of tauopathy.

Here, we tested *in vivo* two aggregation inhibitor compounds by oral administration. Those compounds have been originally designed as potent amyloid-beta (Abeta) aggregation inhibitor compounds (Patent WO2011/128455 "Novel compounds for the treatment of diseases associated with amyloid or amyloid-like proteins"[199]), and previously been identified to show *in vitro* activity on the aggregation of multiple misfolded protein targets, such as amyloid beta or Tau[200–202]. Thus, these compounds are considered as pan-protein aggregation inhibitors. The *in vivo* potency activity of these compounds was herein evaluated in a mouse model of tauopathy based on the injection of an adeno-associated viral (AAV) vector to overexpress human wild-type tau in the entorhinal cortex. Our aim was to promote conditions leading to tau aggregation by overexpressing human tau in this brain region, which is involved in the early stages of tau pathology in Alzheimer's disease. We assessed the effects of these compounds on the level of tau as well as pathological modifications of the protein in both the entorhinal cortex and hippocampus. We also explored the effects of the compounds on local microglial activation and on the accumulation of tau in the cerebrospinal fluid (CSF). In the CSF, tau has been described to mostly accumulate as truncated forms, rather than as a full-length protein[203–205].

# Results

## Overexpression of human wild-type 4R0N tau and 4R2N tau in entorhinal cortex leads to more tau aggregation than in hippocampus

Entorhinal cortex and hippocampus are two brain regions that are affected in tauopathies. However, tau pathology, and in particular its propensity to aggregate, depends in which brain region tau is expressed[206]. To assess the development of tau aggregation as a function of the brain region, we injected 1.5E10 vg of AAV8-PGK-tau vector in either the lateral entorhinal cortex (EC) or the CA3 area of the hippocampus of 11 weeks-old female C57BL/6 WT mice. We also used two vectors to compare different tau isoforms (AAV8-PGK-4R0N tau and AAV8-PGK-4R2N tau). Overall, mice were included in four different groups: overexpression of tau 4R0N in CA3, 4R0N in EC, 4R2N in CA3 and 4R2N in EC. We then euthanized the animals three months after the vector injection for immunohistochemical and biochemical analyses. In this study, the wild-type form of tau was chosen over other mutated forms of tau in order to reproduce conditions closer to sporadic tauopathies.

We observed a consistent expression of human tau in both regions, detected with the human tau specific HT7 antibody (Fig. 23 A-D). We then assessed the presence of tau aggregation. For that purpose, we measured the abundance of human tau multimers in total protein extracts of the same brain regions, using an AlphaLISA assay based on acceptor and donor beads both coupled to the same human tau-specific HT7 monoclonal antibody, as described previously[207]. Convincingly, when the level of HT7/HT7 tau was normalized to the amount of total human tau determined by AlphaLISA using the HT7/Tau13 pair of antibodies, tau multimerization was found to be significantly higher in the entorhinal cortex than in the CA3 hippocampus ($P = 0.04$). This was observed for both tau isoforms, further confirming that wild-type tau has more propensity to accumulate as misfolded and/or multimeric forms when overexpressed in the EC (Fig.23 E).

## Overexpression of human wild-type 4R0N tau in entorhinal cortex and in the CA3 area of the hippocampus leads to more aggregation than the overexpression of human wild-type 4R2N tau in those regions

Tau isoforms have been reported to have their own characteristics in terms of propensity to aggregate in vitro[112]. Indeed, the 0N isoform aggregates at lower concentrations than the 2N isoform. In addition, the same study showed that tau proteins that contain the sequence encoded by exon 3, which is specific to 2N isoforms, are less prone to aggregation.

To confirm this observation in our model of tau overexpression, we performed an AlphaLISA assay to compare the level of tau multimers that reflect aggregated forms of tau in the mice injected with AAV8-PGK-4R0N WT Tau and AAV8-PGK-4R2N WT Tau in either CA3 area of the hippocampus or the entorhinal cortex, as described before.

Biochemical analysis by AlphaLISA confirmed that there is a significant increase ($P = 0.04$) of aggregated tau with 4R0N isoform compared to 4R2N isoform, confirming that overall, 4R0N tau has higher propensity to aggregate than 4R2N tau (Fig.23 E).

Based on these data, we decided to inject the AAV8-PGK-4R0N tau vector in the EC of WT mice as a model system to explore the effects of compounds that inhibit tau aggregation.

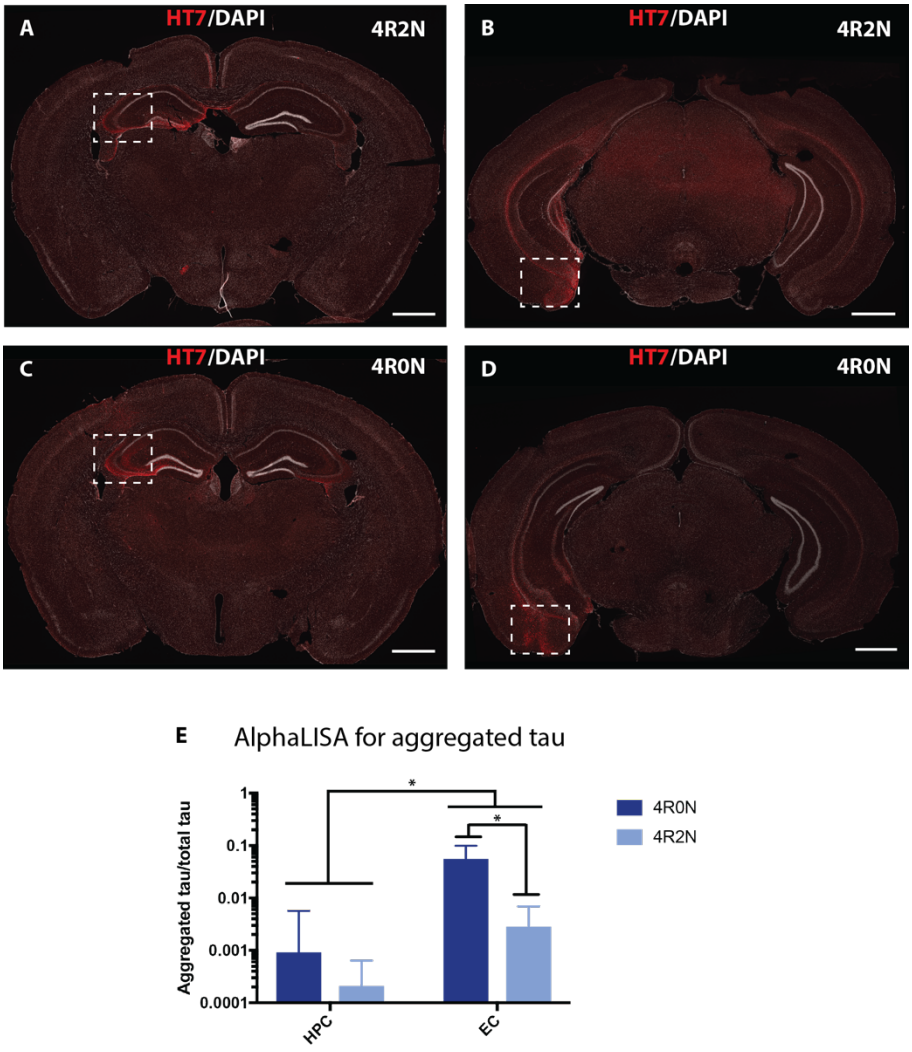



*Figure 23: Brain region and tau isoform are two parameters influencing tau aggregation*

1.5E10 vg of either AAV8-PGK-4R0N hTau WT or AAV8-PGK-4R2N hTau WT are injected in two different brain regions: the CA3 area of the hippocampus or the EC of WT mice. Expression and aggregation of WT tau is analyzed 3 months after vector injection. **(A,B)** HT7 immunohistochemistry shows expression of human tau 4R2N following injection either in hippocampal CA3 (A) or in the EC (B). **(C,D)** HT7 detection of human tau 4R0N either in hippocampal CA3 (C) or in the EC (D). The dashed box indicates the site of AAV8 injection. Scale bars: 1 mm.

**(E)** AlphaLISA analysis of aggregated human tau: multimeric tau (HT7-HT7 signal) is normalized to total human tau (Tau13-HT7 signal). Note the higher level of aggregated tau in the injected EC as compared to HPC. The aggregation is further increased for the 4R0N isoform as compared to the 4R2N isoform in the EC. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison test. N=2-4 per condition.

*P<0.05, **P<0.01, ****P<0.0001. Data represent mean ± SEM. AlphaLISA analysis was performed by collaborators.

## Mice overexpressing human 4R0N tau in the entorhinal cortex are orally treated with pan-protein aggregation inhibitor compounds

We injected with 2E10 vg of the AAV8-PGK-4R0N Tau WT vector a cohort of 75 female wild-type C57BL/6 mice. Five additional female wild-type C57BL/6 mice were injected with the same dose of AAV8-PGK-MCS empty vector as a control group. All mice were injected in the EC brain region to promote tau misfolding and aggregation.

Starting one week after injection, the mice received a treatment with a pan-protein aggregation inhibitor compound by daily oral gavage. A group of 25 mice received a daily dose of pan-protein aggregation inhibitor compound (Compound 1) for five days a week, with a total weekly dose of 60 mg/kg. A second group of 25 mice received a second pan-protein aggregation inhibitor compound (Compound 2) with a similar dosing and administration. A third control group of 25 mice, as well as the mice injected with the empty vector both received only the vehicle solution (Fig. 24 A).

Blood and brain samples were analyzed to confirm the presence of the treatment compounds by looking at the levels of blood and brain exposures by liquid chromatography – mass spectrometry. Overall, both treatment compounds were detected in the blood and the brain, with compound 2 showing higher levels of exposure than compound 1 (Fig. 24 H and I).

Three months after injection, we analyzed the expression of 4R0N human tau WT by immunohistochemistry with the HT7 staining that specifically recognizes total human tau. All animals injected with AAV8-PGK-4R0N tau WT showed a consistent expression of total human tau in the EC, with human tau-positive neuronal projections visible in the ipsilateral hippocampus anterior to the site of injection, which are likely part of the perforant pathway connecting the entorhinal cortex and the hippocampus[208] (Fig. 24 B-G). Notably, HT7-immunoreactive neuronal cell bodies were also observed in the anterior part of the hippocampus, most notably in the granular layer of the dentate gyrus, suggesting that human 4R0N tau was spreading from AAV8-injected EC to neurons located in the anterior hippocampus.

Furthermore, we quantified the level of total human tau by HT7/Tau13 AlphaLISA assay and observed no effect of the compounds on AAV-mediated tau expression levels.

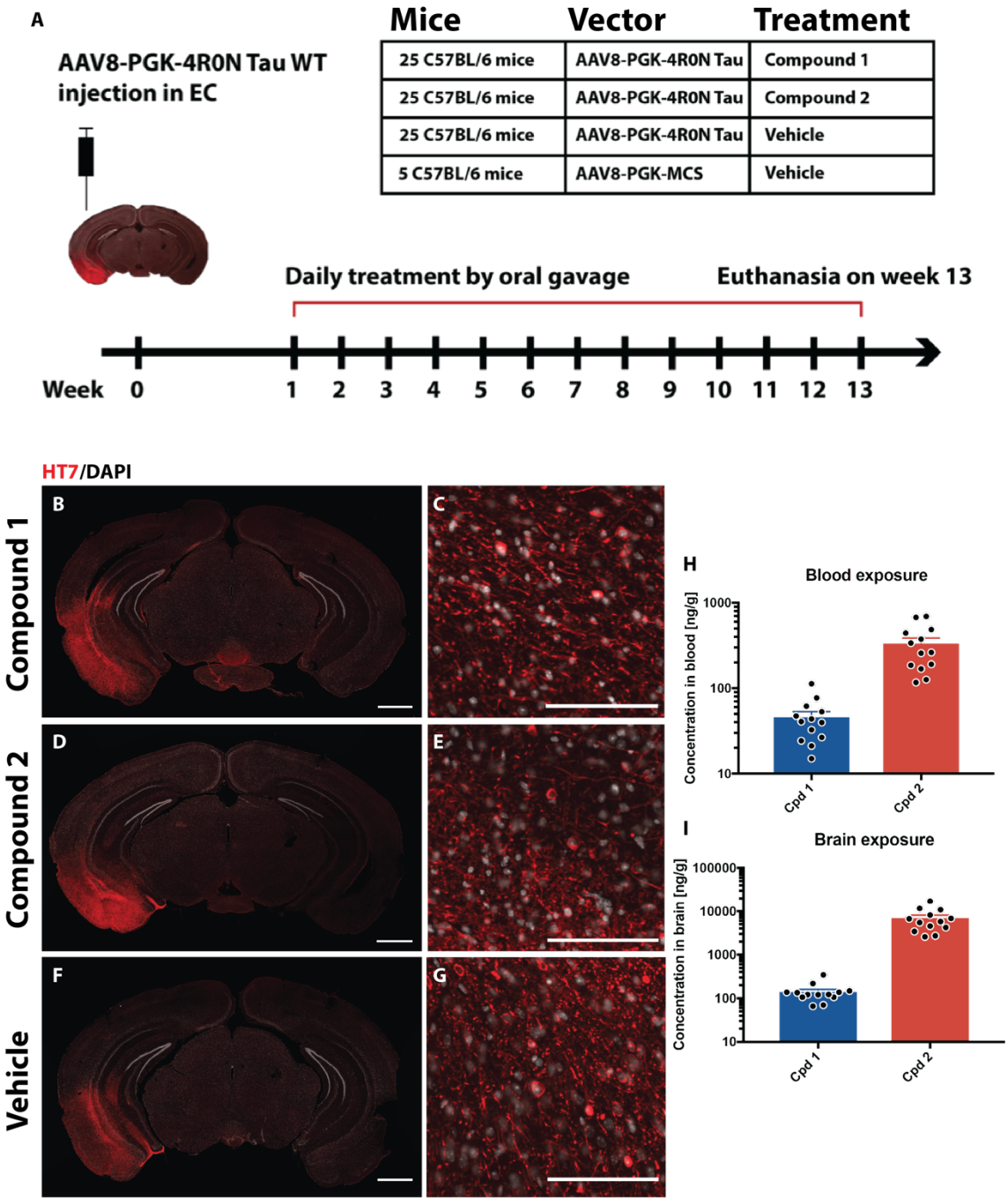



*Figure 24: Experimental design and overexpression of 4R0N Tau WT in the entorhinal cortex of C57BL/6 mice*

**(A)** Experimental design: 3 groups of 25 C57BL/6 wild-type mice were injected with AAV8-PGK-4R0N Tau WT and a group of 5 C57BL/6 wild-type mice were injected with the control vector AAV8-PGK-MCS. The mice are treated for 13 weeks. 15 mice per group and 3 mice injected with the empty vector are analyzed by immunohistochemistry and the remaining mice are used for biochemical analysis.
**(B-G)** HT7 immunohistochemistry shows that stereotaxic injection of AAV8-PGK-4R0N Tau WT in the entorhinal cortex of C57BL/6 mice induces a local overexpression of human 4R0N tau WT in all three injected groups. **(C, E, G)** Higher magnification showing neuronal cell bodies and axons expressing human 4R0N tau WT at the site of vector injection. Scale bars in A, C, E: 1 mm; and in B, D, F: 100 μm.
**(H, I)** Exposure levels of compounds 1 and 2 detected in the blood (G) and in the brain (H) by liquid chromatography – mass spectrometry (LC-MS). Data represent mean ± SEM. LC-MS for exposure levels was performed by collaborators.

## Compound effects on tau aggregation and tau misfolding

To determine the effects of the pan-protein aggregation inhibitor compounds on tau misfolding, we performed MC1 immunohistochemistry (IHC) on a few animals per group, on brain sections near the injection site (Fig. 25 A-H). We found no significant effect of the treatment with compounds on MC1 immunoreactivity. However, MC1 immunoreactivity also appeared to be similar between the mice injected with the AAV8-PGK-4R0N Tau WT vector and the empty vector. This suggests that the wild-type form of tau overexpressed in our model has a low propensity to accumulate as misfolded species (Fig. 25 I).

Next, the HT7/HT7 AlphaLISA assay was performed to assess tau aggregation by measuring the amount of human tau multimers. Values were normalized to the level of total human tau, assessed by Tau13/HT7 AlphaLISA assay. Again, no significant difference was observed between the treatment groups and the group administered with the vehicle (Fig.25 J). Therefore, the administration of the anti-aggregation compounds does not appear to induce detectable effects on the accumulation of tau multimers in this model based on wild-type tau, which is characterized by low aggregation levels.

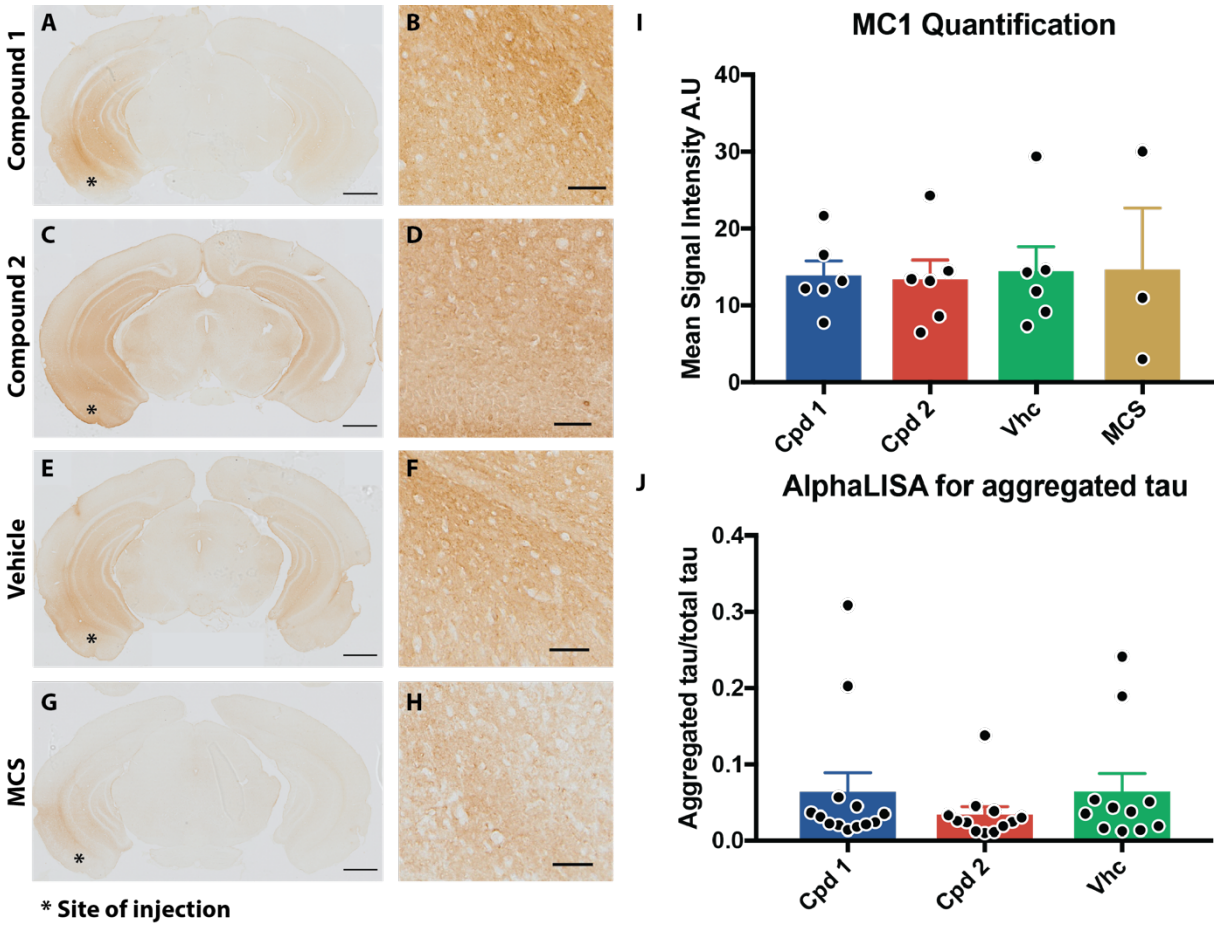


*Figure 25: Quantification of the conformational change of tau protein*

**(A-H)** Representative sections show immunohistochemistry with the conformational MC1 antibody to detect misfolded tau. Note the MC1 immunoreactivity in the injected entorhinal cortex (left hemisphere). Scale bar: 1mm). **(B, D, F, H)**: Higher magnification of the MC1 immunoreactivity near the site of vector injection. Scale bar: 50 μm.
**(I)** Quantification of MC1 immunoreactivity (with background correction) in the injected entorhinal cortex. Statistical analysis: one-way ANOVA. N=3-6 mice per group.
**(J)**: AlphaLISA analysis HT7/HT7 tau multimers in protein extracts from the injected entorhinal cortex. Values are normalized to total human tau levels measured by Tau13/HT7 AlphaLISA. There is no significant difference between the groups. Statistical analysis: one-way ANOVA. N=11-13 mice per group.
Data represent mean ± SEM. AlphaLISA analysis was performed by collaborators.

## Pan-protein aggregation inhibitor compound 1 induces an increase in hyperphosphorylated tau at Serine 202 and Threonine 205

In order to further explore the effects of the compounds on tau post-translational modifications, we proceeded to explore the effects of the pan-protein aggregation inhibitor compounds on the level of hyperphosphorylation of the protein tau. Indeed, tau hyperphosphorylation is considered as an early event which may affect the pathogenic cascade leading to the formation of tau aggregates. We performed IHC on the brain sections with the AT8 and PHF-1 antibodies. AT8 specifically recognizes early-stage phosphorylation of tau at the residues Serine 202 and Threonine 205 (Fig. 27 A-H). The PHF-1 antibody specifically recognizes tau hyperphosphorylated at the residues Serine 396 and Serine 404, which is often associated with tau aggregates (Fig. 26 A-H). To semi-quantitatively assess tau phosphorylation, we measured the mean signal intensity of the AT8 and PHF-1 signals on 3 brain sections around the site of vector injection.

With the PHF-1 antibody, we did not find any significant change between groups with the PHF-1 signal, although there was a trend towards higher PHF-1 signal in the group treated with compound 1, when compared to the vehicle-treated group (P = 0.3) and the MCS control group injected with the empty vector (P = 0.1) (Fig. 26 I).

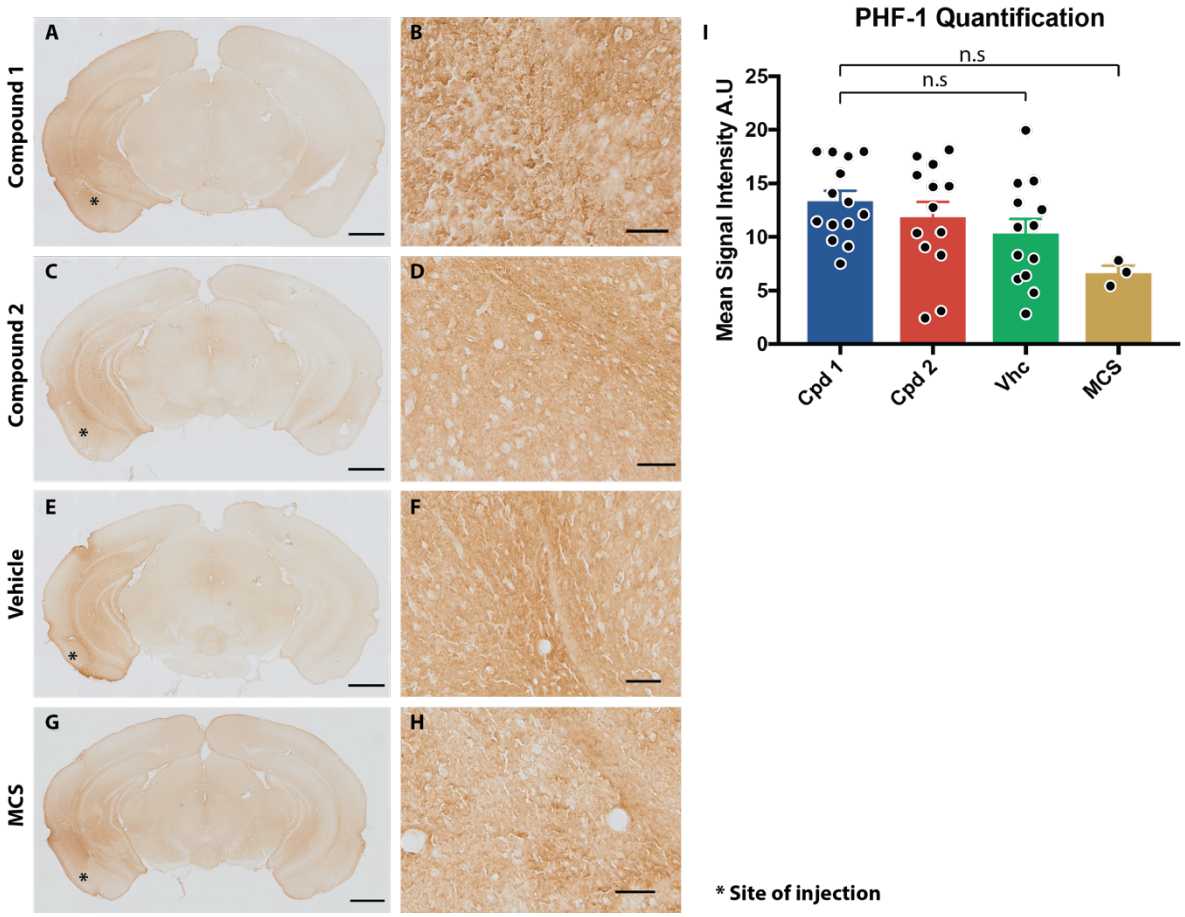



*Figure 26: Quantification of the hyperphosphorylation of the tau protein at the site Ser396/404*

**(A-H)** PHF-1 immunohistochemistry to assess the effects of the pan-protein aggregation inhibitor compounds on tau hyperphosphorylation at the sites Ser396/Ser404. Note the local immunoreactivity in the injected left hemisphere. **B, D, F, H**: Higher magnification of PHF-1 immunoreactivity near the site of vector injection in the entorhinal cortex. Scale bars: A, C, E, G: 1mm; and B, D, F, H: 50µm.
**(I)** Semi-quantitative analysis of PHF-1 immunoreactivity (with background correction) in the injected entorhinal cortex. Statistical analysis: one-way ANOVA. N=13-14 mice in the groups injected with the AAV8-PGK-4R0N Tau WT vector; N=3 in the empty vector injected group.
injected groups who received compound 1, compound 2 and the vehicle. (P = 0.3 between compound 1 and vehicle groups and P = 0.1 between compound 1 and MCS groups, by one-way ANOVA and Tukey's multiple comparison test).

Strikingly, AT8 immunohistochemistry in the EC revealed a significant increase in the immunoreactivity measured in the group treated by the compound 1, as compared to the group administered with the vehicle (P=0.006) (Fig. 27 I). In contrast, the compound 2 treated group did not show any significant effect on AT8 immunoreactivity when compared to vehicle-treated mice (P = 0.27). This result shows that compound 1 increases the phosphorylation of tau at Serine 202 and Threonine 205 at the site of vector injection.

To further explore this effect, we performed an AlphaLISA assay to analyze the phosphorylation of Serine 202 and Threonine 205 residues on overexpressed human tau (Fig. 27 J). Again, the level of AT8-positive human measured in the EC was significantly increased in the compound 1 treated group as compared to the vehicle-treated group (P = 0.028). Treatment with compound 2 induced a non-significant increase in AT8 phosphorylation.

Next, we analyzed the AT8 phosphorylation signal as a function of total human tau level in the EC (Fig. 27 K). We found a significant correlation between the levels of total human tau and human tau Ser202/Thr205 phosphorylation in both the compound 1- (P = 0.004) and the vehicle-treated (P = 0.01) groups. In the vehicle-treated mice, AT8 signal only mildly increased with higher levels of human tau. In the compound 1 treated group however, the slope of the correlation was significantly increased (P = 0.013). This clearly demonstrates that compound 1 increases AT8 phosphorylation of the human tau protein in the treated brains.

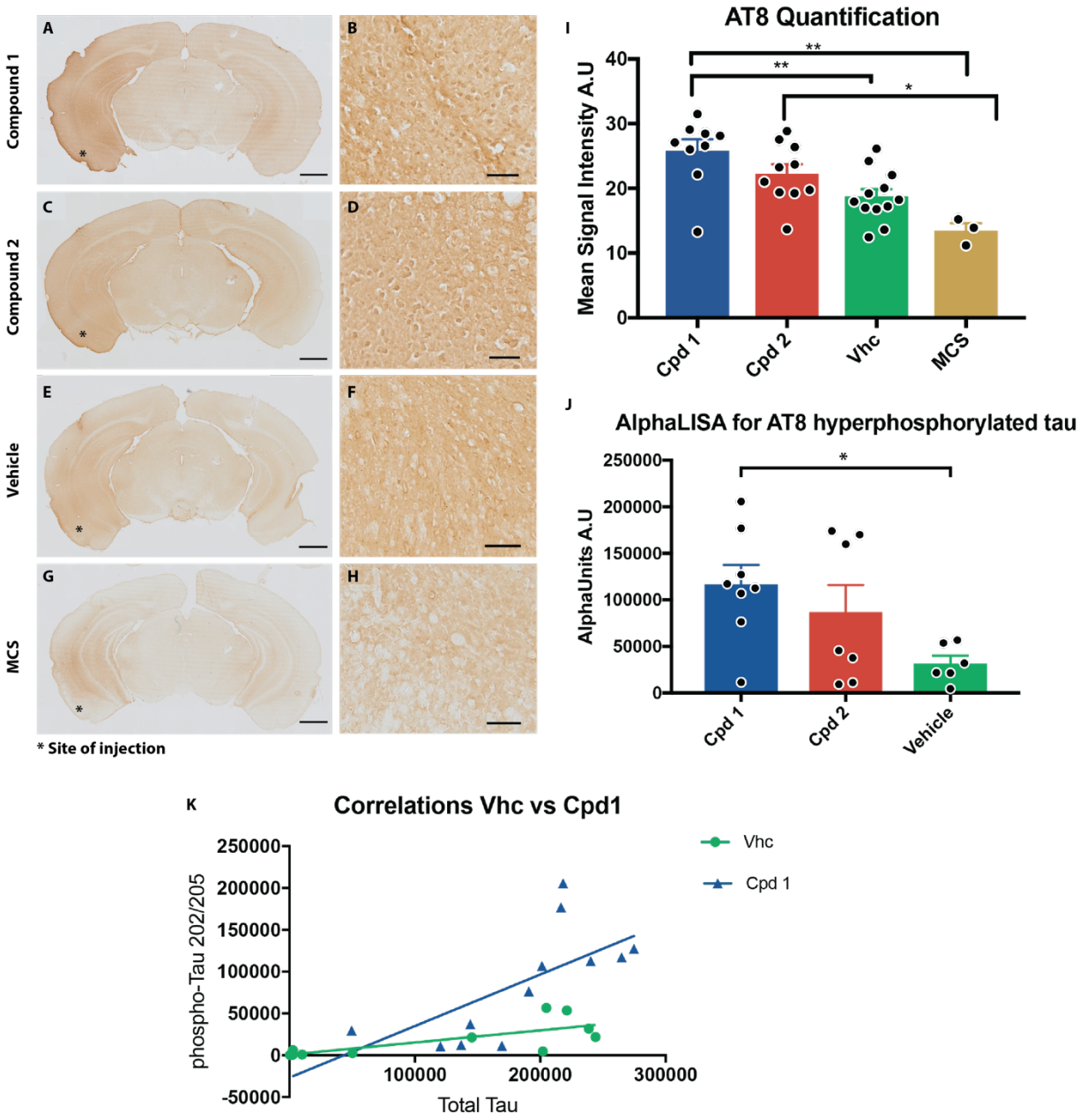



*Figure 27: Quantification of the hyperphosphorylation of the tau protein at the site of Ser202/Thr205*

**(A-H)** Representative brain sections for AT8 immunohistochemistry (Ser202/Thr205 phosphorylation). Note the increase in the AT8 signal in the vector-injected left hemisphere of the brain treated with the pan-protein aggregation inhibitor compound 1. **B, D, F, H**: Higher magnification of AT8 immunoreactivity near the site of vector injection in the entorhinal cortex. Note the presence of AT8-immunoreactive neuronal cell bodies in the compound-treated mice. Scale bars: A, C, E, G: 1 mm; and B, D, F, H: 50 µm.
**(I)** Semi-quantitative analysis of AT8 immunoreactivity (with background correction) in the injected entorhinal cortex. Statistical analysis: P = 0.006 between Cpd1 and Vhc, P = 0.001 between Cpd1 and MCS, P = 0.026 between Cpd2 and Vhc (one-way ANOVA with Tukey's post hoc test). N=9-12 mice in the groups injected with the AAV8-PGK-4R0N Tau WT vector; N=3 in the empty vector injected group. Some mice were removed from the analysis because of the poor quality of the tissue sections.
**(J)** AlphaLISA analysis of total protein extracts from the entorhinal cortex. The graph shows the level of AT8 phosphorylation on overexpressed human tau captured with the HT7 antibody. Note the higher AT8 signal in mice treated with compound 1. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test with respect to vehicle-injected mice. N=6-8 per group. Samples with total human tau levels under 100,000 AlphaUnits were excluded from the analysis. AlphaLISA analysis was performed by collaborators.

**(K)** Linear regression analysis of the correlations between total human tau and AT8 phosphorylated human tau. Both correlations are significant (P=0.01 for vehicle and P=0.004 for compound 1). Note that in the group treated with compound 1, the slope of the correlation AT8/total human tau is significantly increased in the compound 1 treated group with respect to the vehicle-treated group (P = 0.0129). Correlative analysis was performed by collaborators.
*P<0.05, **P<0.01. Data represent mean ± SEM.

## Pan-protein aggregation inhibitor compound 1 increases Ser202/Thr205 tau phosphorylation in the anterior hippocampus distally to the site of vector injection

Next, we sought to determine if the increase in tau phosphorylation at Serine 202/Threonine 205 residues induced by compound 1 was also present in the hippocampus, the main projection areas of the neurons located in the EC. We measured by immunohistochemistry the mean signal intensity of the AT8 signal in the hippocampus ipsilateral to the site of AAV8-PGK-4R0N Tau WT vector injection, approximately 1 mm anterior to the site of injection (Fig. 28 A-H). We found a significant increase in the AT8 signal with the compound 1 treated group compared to the group administered with vehicle (P=0.029) (Fig. 28 I), in line with the results obtained in the entorhinal cortex. AT8 signal in the compound 1 group was also significantly increased with respect to compound 2 treated group (P=0.025). This result showed that pan-protein aggregation inhibitor compound 1 has similar effects on tau phosphorylation in the hippocampal projection areas of the entorhinal cortex.

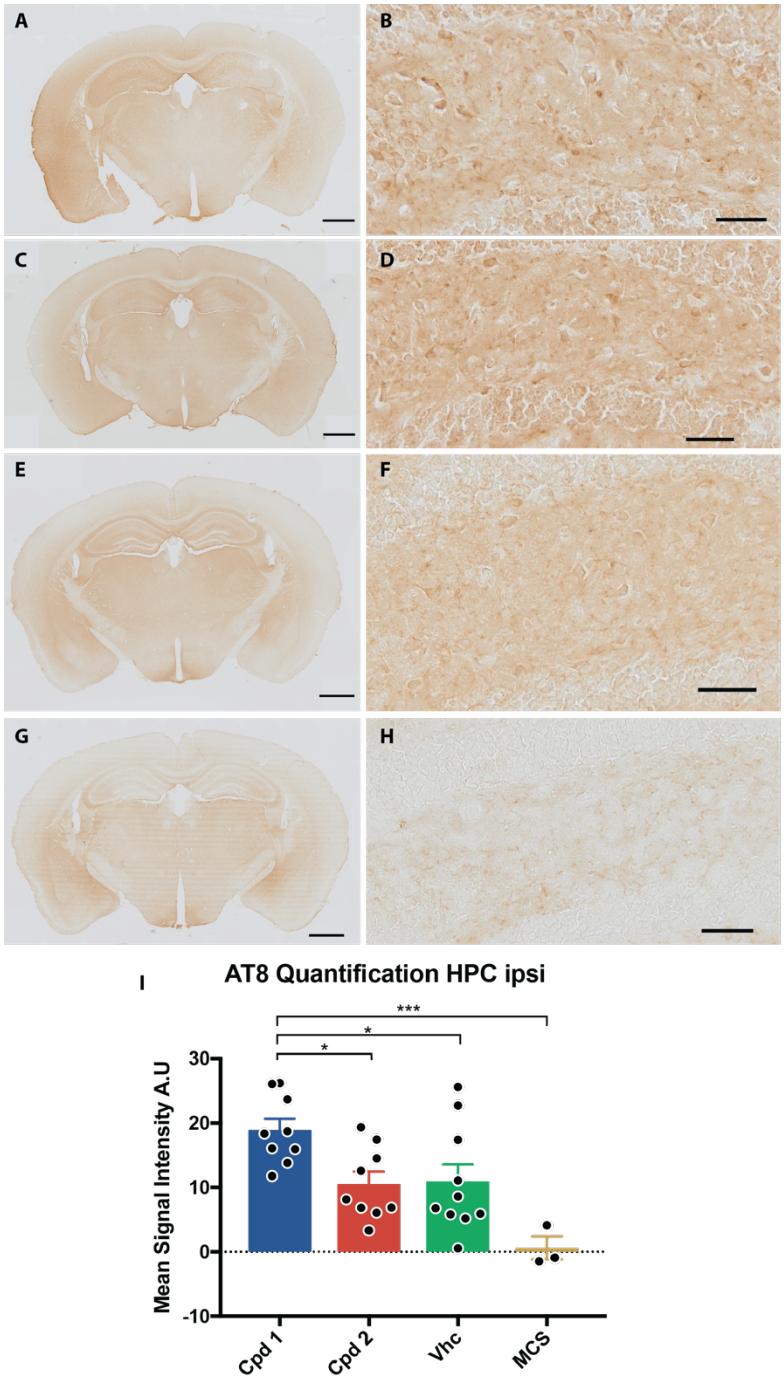



*Figure 28: Pan-protein aggregation inhibitor compound 1 increases tau Ser202/Thr205 phosphorylation in the ipsilateral hippocampus*

**(A-H)** Representative sections for AT8 immunohistochemistry (Ser202/Thr205 phosphorylation), anterior to the site of vector injection. Note the increase in the AT8 signal in the hippocampus ipsilateral to vector injection, in the brain treated with the pan-protein aggregation inhibitor compound 1. **B, D, F, H**: Higher magnification of AT8 immunoreactivity in the dentate gyrus area of the hippocampus. Scale bars: A, C, E, G: 1 mm; and B, D, F, H: 50 μm.
**(I)** Semi-quantitative analysis of AT8 immunoreactivity (with background correction) in the hippocampus ipsilateral to vector injection in the entorhinal cortex. Statistical analysis: P = 0.0006 between Cpd1 and MCS, P = 0.029 between Cpd1 and Vhc and P = 0.025 between Cpd1 and Cpd2 (one-way ANOVA with Dunnett's multiple comparison test with respect to compound 1 treated mice). N=9-10 mice in the groups injected with the AAV8-PGK-4R0N Tau WT vector; N=3 in the empty vector injected group. Some mice were removed from the analysis because of the poor quality of the tissue sections. *P<0.05, ***P<0.01. Data represent mean ± SEM.

## Pan-protein aggregation inhibitor compounds tend to increase the microglial response near the site of vector injection

In Alzheimer's disease, neuroinflammation is involved in the pathogenesis of the disease and is associated with hyperphosphorylated tau and tau aggregates, suggesting a role of the microglia in tau pathology[187]. Since we observed a clear effect of the pan-protein aggregation inhibitor compounds on AT8 hyperphosphorylation, we sought to determine if the treatment compounds have an effect on the neuroinflammatory response. We performed immunohistochemistry for Iba1, a common marker for microglial cells, CD68 which is a marker used to assess the phagocytic activity of microglia and GFAP to assess the level of astrogliosis (Fig.29 A). To assess the inflammatory reaction, we first quantified the fluorescent signal area coverage at the site of vector injection in the entorhinal cortex. We observed a trend towards an increase in the Iba1 signal area coverage for the groups treated with the pan-protein aggregation inhibitor compound 1 ($P = 0.077$) and compound 2 ($P = 0.089$) when compared to the vehicle group, suggesting a potential effect of the treatment compounds on neuroinflammation (Fig.29 B).

Next, we measured the signal overlap between the Iba1 and CD68 signals to assess the phagocytic activity of the microglia. Here, there was a non-significant trend towards an increase in phagocytic activity in the brains of the mice treated by the compound 2 ($P = 0.076$), compared to both the vehicle-treated and empty vector injected control groups (Fig.29 C). Quantification of the area covered by the GFAP signal did not reveal any significant difference between groups, although a fraction of the mice injected with the tau-expressing vector showed a clear increase in GFAP immunoreactivity indicative of astrogliosis (Fig.29 D).

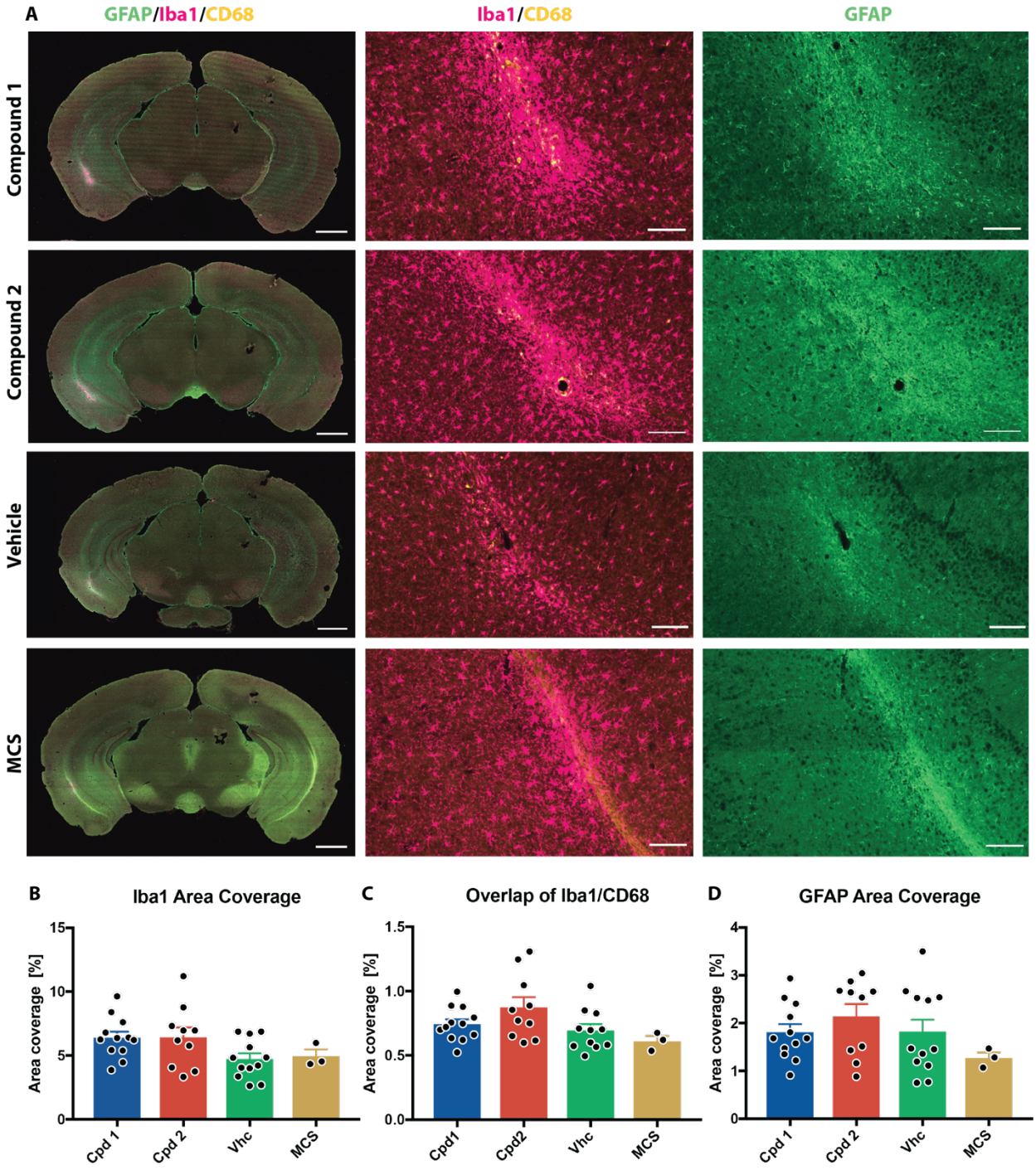



*Figure 29: Effect of the pan-protein aggregation inhibitor compounds on markers of neuroinflammation*

**(A)**: We performed immunohistochemistry for astrogliosis (GFAP) and microgliosis (Iba1 and CD68) to assess the effect of the treatment compounds on neuroinflammation. We observed an increased signal for all three stainings at the site of injection. Scale bar for brain sections: 1mm, scale bar for close up of the site of injection: 100 μm.
**(B)**: To assess the level of microglial activity, we measured the area covered by the Iba1 signal that is expressed in microglial cells. We found a non significant trend towards an increase in Iba1 with compound 1($P = 0.077$) and compound 2 treated groups ($P = 0.089$) compared to the vehicle group (one-way ANOVA and Dunnett's multiple comparison test).
**(C)**: To assess the phagocytic activity of the microglia, we stained for the CD68 marker and we measured the area covered by the overlap of the Iba1 signal and CD68 signal. We observed a non-significant trend towards

an increase in phagocytic activity in the compound 2 treated group compared to the vehicle group (P = 0.076 one-way ANOVA, Dunnett's multiple comparison test).
**(D)**: We then assessed the effect of the treatment on astrogliosis by measuring the area covered by the GFAP staining signal. We did not observe any significant trend in the difference in astrogliosis between the three groups injected with tau compared to MCS. Although a fraction of the mice overexpressing tau showed an increase in the GFAP signal area coverage, suggesting astrogliosis.
Data represent mean ± SEM.

## Compound 1 pan-protein aggregation inhibitor increases the microglial density in the ipsilateral hippocampus distal to the site of vector injection

As changes in AT8 tau hyperphosphorylation were most evident in the hippocampus, we proceeded to analyze the microglial response in this brain region. As compared to the mice injected with the empty vector, some animals injected with the tau-expressing vector indeed displayed microglial activation in the ipsilateral hippocampus, in which the abundant presence of human tau-positive axonal fibers was also observed (verified by HT7 immunostaining) (Fig. 30 A). When we measured the area coverage of the Iba1 signal and of the Iba1/CD68 co-staining, we again found a trend towards increased microglial activity in the groups treated with the pan-protein anti-aggregation compounds 1 and 2 (Iba1: P=0.16 for compound 1 and P=0.0738 for compound 2 compared to the vehicle group; Iba1/CD68: P=0.13 for compound 1 and P=0.077 for compound 2 compared to the vehicle group) (Fig.30 B-C).

However, the density of Iba1-positive microglial cells was significantly increased in the compound 1 treated mice, when compared to both the vehicle-treated mice injected the tau-expressing vector (P=0.004), as well as the empty vector injected mice (P=0.026) (Fig. 30 D). This result indicates that the overexpressed 4R0N tau exposed to the pan-protein anti-aggregation compound 1 is more prone to induce microglial activation in the hippocampal region.

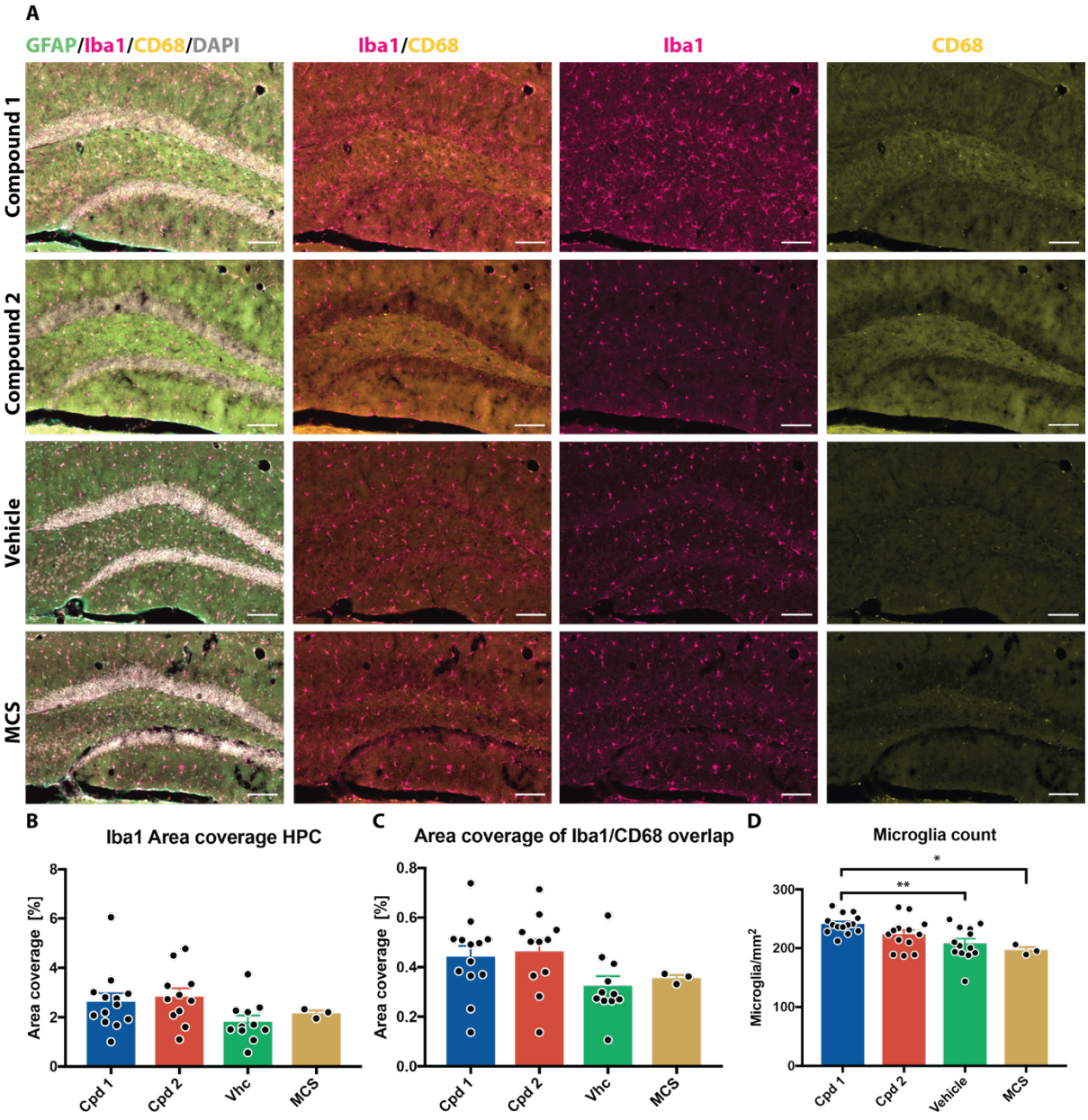



*Figure 30: Pan-protein aggregation inhibitor compound 1 increases the microglial density in the hippocampus*

**(A)**: To determine the effect of the hyperphosphorylation of tau on the microglial response, we looked in the hippocampus distal to the site of vector injection for markers of microglia (Iba1) and microglial phagocytic activity (Iba1/CD68) and we visually observed an increase in the Iba1 signal in the ipsilateral hippocampus. Scale bar: 100 μm

**(B)**: We quantified the microglial cells by measuring the area coverage of the Iba1 signal and found a non significant trend towards an increase in the area coverage with both the compound 1 (P=0.16) and compound 2 (P=0.074) treatment group, compared to the vehicle group (one-way ANOVA and Dunnett's multiple comparison test).

**(C)**: We quantified the phagocytic activity of microglial cells by measuring the area covered by the overlapping signals of Iba1 and CD68 in the hippocampus and we found the same non significant trend of increase in the signal with both compound 1 (P=0.13) and compound 2 (P=0.077) compared to the vehicle group (one-way ANOVA with Dunnett's multiple comparison test).

**(D)**: We proceeded to manually count the microglial cell bodies in the hippocampus to measure the microglial density and we found a significant increase in the density of microglia in the compound 1 treated group compared to the vehicle group (P=0.004 by one-way ANOVA and Tukey's multiple comparison test) and compared to the MCS group (P=0.026 by one-way ANOVA and Tukey's multiple comparison test).

## Pan-protein aggregation inhibitor compound 1 and 2 decrease the level of total human tau in the CSF of treated mice

In Alzheimer's Disease[53] and in traumatic brain injury[101], there is an increase in the levels of total tau in the cerebrospinal fluid that can be used as a biomarker of the ongoing tau pathology. Therefore, we sought to determine whether there were any changes in the level of tau in the CSF of the mice injected with the human tau-expressing vector. Indeed, using the Gyros technology, which is based on an immunoassay that allows quantification of proteins in small volumes of fluids, we measured total tau levels and we found that there was a near significant (P=0.058) increase in the CSF of the mice injected with AAV8-PGK-4R0N tau WT vector as compared to mice injected with the empty vector (Fig. 31 A).

Remarkably, we found that the levels of total human tau present in the CSF of the mice treated with compound 1 and 2 was significantly reduced as compared to the vehicle-treated group (Fig. 31 A). This result demonstrates that the anti-aggregation compounds have a clear effect on the levels of tau in the CSF, possibly by increasing tau clearance.

Furthermore, as the level of human tau in the CSF is likely to be related to the brain level of tau overexpression, we performed a correlative analysis to compare these two variables. The level of total human tau at the site of vector injection (entorhinal cortex) was assessed by Tau13-HT7 AlphaLISA, which recognizes monomeric human tau. We observed a clear trend (P=0.08) towards a positive correlation between the level of human tau measured in the CSF, and the level of human tau measured in the brain samples obtained from the vehicle-treated group of mice (Fig. 31 B). However, in the tau-overexpressing mice treated with either the compound 1 or 2, this correlation was changed. In particular, in the compound 1 treated group, there was no positive correlation between these two values, showing that the compound 1 clearly affects the release of human tau in the CSF of these mice.

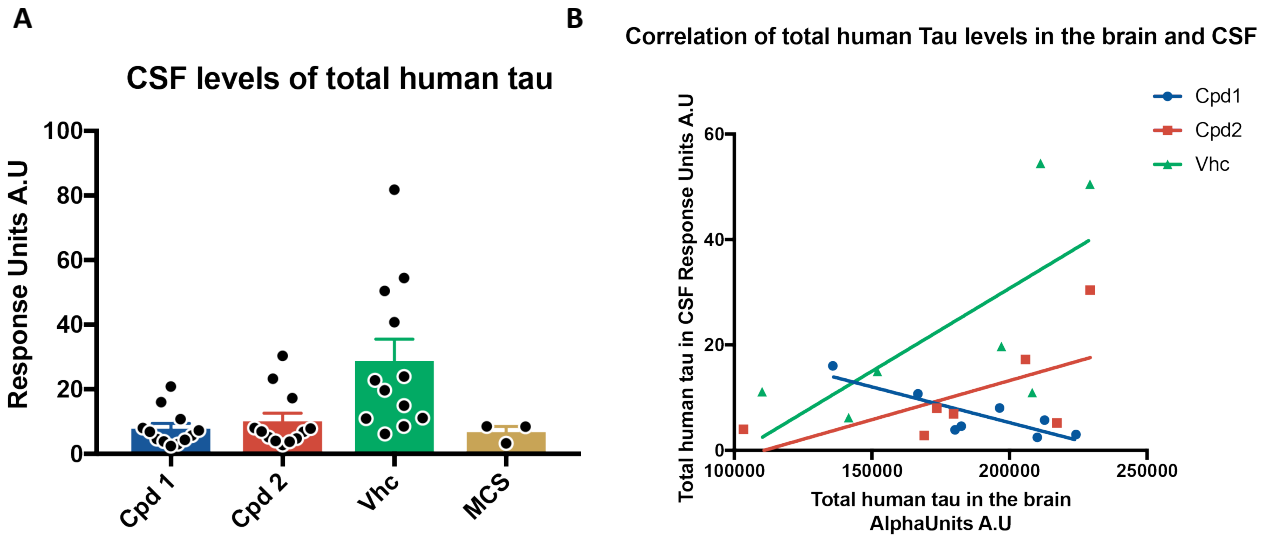



*Figure 31: CSF levels of total human tau are decreased by the anti-aggregation compounds*

**(A)** The levels of total human tau in the CSF of the mice treated with compound 1 and compound 2 are significantly decreased compared to the vehicle group (P=0.0053 for compound 1 and P=0.015 for compound 2 by one-way ANOVA and Tukey's post hoc analysis). This result suggests a role of the pan-protein aggregation inhibitors in the clearance of tau.
**(B)** Correlative analysis between the levels of total human tau in the entorhinal cortex assessed by AlphaLISA and the levels of total human tau in the CSF. There is a trend for a positive correlation in the vehicle-treated group (P=0.08). Note the negative correlation between CSF and brain tau levels (P=0.0092) in the compound 1 treated group of mice.
CSF analysis and correlative analysis were performed by collaborators.

## Compounds 1 and 2 do not affect total tau propagation assessed in the ipsilateral hippocampus

To determine whether the treatment compounds would have an effect on the propagation of the total tau protein towards connected areas by either disaggregating tau aggregates into soluble monomers that would transfer from neuron to neuron or by interfering with the tau bound to microtubules, we manually quantified the number of HT7 immunoreactive cell bodies in the ipsilateral hippocampus of all coronal brain sections that were anterior to the ventralization of the curvature of the hippocampus (Fig. 32). We saw no significant difference in the number of HT7 positive cell bodies between the compound 1, compound 2 and vehicle treated groups. This suggests that the compounds do not affect propagation of total tau.

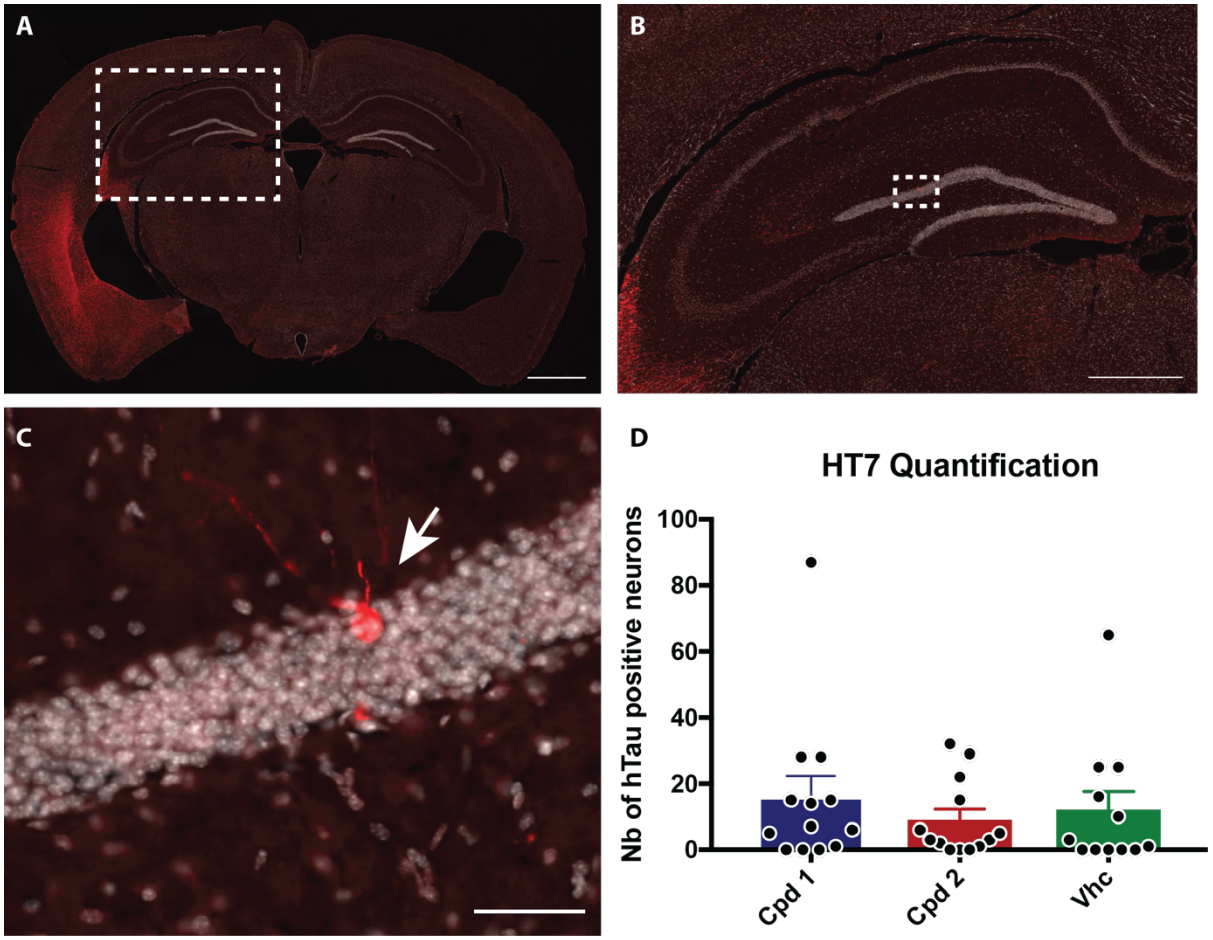



*Figure 32: Quantification of neuronal cell bodies positive for human tau in the anterior ipsilateral hippocampus*

**(A)** To assess the effect of the compounds on the propagation of tau, neuronal cell bodies positive for HT7 were counted manually in the ipsilateral hippocampus of all the brain sections situated anteriorly to the ventralization of the hippocampus, approximately 1 mm distal and anterior to the site of injection. (Scale bar: 1 mm)
**(B)** and **(C)**: Example of a neuron positive for human tau found in the granule cell layer of the dentate gyrus that is synaptically connected to the entorhinal cortex via the perforant pathway. (Scale bar 500 μm, 50 μm)
**(D)**: Graph showing the result of the quantification of HT7 positive cell bodies in the ipsilateral hippocampus, distal and anterior to the site of injection. There is no significant difference between compound 1 treated mice, compound 2 treated mice and mice who received the vehicle. This suggests that the compounds do not affect total human tau propagation.

# Discussion

## Importance of tau aggregation inhibitors as a therapeutic strategy

The oligomerization of tau protein forming toxic aggregates that further recruit and corrupt other tau monomers into a pathogenic cascade is a key pathological event leading to tauopathies. Therefore, targeting tau aggregation is an important strategy to develop disease-modifying treatments against tauopathies.

## Relevance of our model of wild-type 4R0N tau overexpression in the entorhinal cortex to explore the effects of tau aggregation inhibitor compounds

Here, we use an *in vivo* model of early tau pathology by overexpressing human wild-type 4R0N tau in the entorhinal cortex. Our results show that conformational changes and aggregation are more pronounced when overexpressing 4R0N compared to 4R2N tau, which made 4R0N tau the most suitable isoform to assess the effect of pan-protein aggregation inhibitors. Furthermore, our results indicate that the entorhinal cortex is a region of the brain that is more prone to tau aggregation as previously reported[206]. In addition, the entorhinal cortex is the earliest region involved in the Braak stages of Alzheimer's Disease[74], hence its importance as a region to model tau pathology.

Using this model of tau pathology, we treated C57BL/6 wild-type mice with two different compounds to determine the efficacy of pan-protein aggregation inhibitors to prevent the formation of tau aggregates. In our model of tauopathy, we detected low levels of tau multimers and tau misfolding. The mild pathology observed might be due to the low propensity of wild-type tau to misfold. To increase the propensity of tau to aggregate, a mutated form of tau such as P301S or P301L could have been used instead[207,209]. However, a mutated form of tau might not faithfully replicate the pathogenic cascade leading to sporadic tauopathies, which are also characterized by the formation of toxic tau oligomers and tau aggregates. Instead, these FTD-linked tau mutants are spontaneously prone to aggregation and the mutation may also affect tau interaction with microtubules[210]. Therefore, we would not be able to assess whether the aggregation inhibitor compounds also interfere with the

interaction between tau and microtubules in physiological conditions. Overall, the mild level of pathological aggregation that we observe in our model might represent the situation of an early stage of tau pathology. Unexpectedly, we found out that the aggregation inhibitor compounds did not induce detectable changes in the levels of tau misfolding and tau aggregation at this early stage of pathology. Nevertheless, our early model of pathology allowed us to highlight the effect of the aggregation inhibitor compounds on markers of hyperphosphorylation at the site of Ser202/Thr205 and microglial activity that are possibly linked to mechanisms of tau clearance.

## What is the role of compound-induced AT8 hyperphosphorylation of tau?

In this study, we found that the aggregation inhibitor compounds modify the pattern of tau phosphorylation, which is an early event occurring before the formation of tau aggregates. The aggregation inhibitor compound 1 induces a significant increase in tau hyperphosphorylation revealed by the AT8 marker, which corresponds to the phosphorylation of the Ser202 and Thr205 residues. This is observed both at the site of vector injection, where overexpressed tau is expected to be at a maximal level and accumulates in the neuronal cell bodies, but also in the hippocampus, a distal region where human tau is mainly present in axonal projections.

At first sight, this effect of the aggregation inhibitor appears counterintuitive, as AT8 hyperphosphorylation is most often referred as an early pathological marker in Alzheimer's Disease[211,212], and is considered to be an histological indicator of the presence of neurofibrillary tangles[213]. Furthermore, hyperphosphorylation of tau is known to have an inhibitory effect on the binding of tau to microtubules, which may facilitate tau aggregation[77,119,155]. However, other studies have challenged the notion that phosphorylation of tau always favors aggregation by demonstrating that certain forms of tau phosphorylation can actually inhibit aggregation and provide neuroprotective effects[155,157,210]. For example, phosphorylation of critical residues may prevent tau-tau interaction, in the same way phosphorylation can limit tau-microtubule interactions, and therefore inhibit the formation of tau aggregates[157]. The putative mechanism(s) by which phosphorylation of certain sites prevents the formation of aggregates are unclear, although it is possible that phosphorylation at certain residues facilitates conformational changes that block the assembly of the core

peptide of PHF aggregates present in the microtubule-binding domain[157]. In that context, phosphorylation of tau at the Ser202/Thr205 sites might play a role towards inhibition of aggregation of the tau protein. Accordingly, a study by Cao et al. showed *in vitro* that pseudo-phosphorylation of tau at Ser202/Thr205 sites decreased the truncation of tau at the site of Aspartate 421 (D421)[214]. Cleavage of tau at D421 is known to be associated with tau aggregation leading to tangle formation[215]. Therefore, specific phosphorylation at Ser202/Thr205 might indirectly inhibit tau aggregation by inhibiting its cleavage at D421, thus potentially having neuroprotective effects.

Another study by Ittner et al. highlighted the neuroprotective effect of site specific phosphorylation in the context of amyloid beta pathology, by showing that phosphorylation of Threonine 205 in the tau protein decreases the neurotoxic effects of amyloid beta oligomers, by disrupting at the post-synaptic level the complex formed by the PSD95/Fyn/tau proteins[216]. Threonine 205 is one of two sites of hyperphosphorylation recognized by the AT8 marker. Similarly, a study by Frandemiche et al. showed that synaptic activation induces phosphorylation of the threonine 205 residue on tau proteins located in the synaptic compartment[217], suggesting a physiological role of the phosphorylation at this particular site. However, the study showed that exposing amyloid beta oligomers to neurons *in vitro*, changes the profile of phosphorylation of tau, by decreasing the Threonine 205 phosphorylated tau and increasing the level of phosphorylation at the Serine 404 residue, which is a site recognized by the PHF-1 marker. Phosphorylation of the Ser396/404 residues is one of the early events occurring in Alzheimer's Disease, leading to the formation of tau aggregates[218]. This suggests that the different patterns of phosphorylation of tau might have different roles in either promoting tau pathogenic cascade or having an inhibitory effect on the pathological chain of events[219]. This is well illustrated by a recent study by Despres et al. which showed that the phosphorylation of the Ser202/Thr205 residues only has an inhibitory effect on the formation of tau aggregates[220]. However, when the Ser208 residue is phosphorylated in combination to the phosphorylation of Ser202/205 residues, tau is induced into forming fibrils. Similarly, and as previously stated, the study by Cao et al. showed that pseudo-phosphorylation of Ser202/Thr205 sites had inhibitory effects on tau truncation at D421; however, when the Ser396/404 sites are pseudo-phosphorylated in addition to Ser202/Thr205, then the opposite effect happens, where truncation of tau at D421 is increased, possibly favoring tau aggregation[214]. Overall, this suggests that phosphorylation of

tau is not always associated with the formation of aggregates and that specific patterns of phosphorylation of the different sites on tau might have an importance in determining whether it has a deleterious effect by facilitating tau aggregation or, on the contrary, inhibiting it.

In our study, the mechanism by which tau is phosphorylated at Ser202/Thr205 residues in presence of the aggregation inhibitor remains unknown. One hypothesis is that the compounds induce tau to adopt a conformation potentially modifying kinase/phosphatase activities to favor Ser202/Thr205 phosphorylation. In light of the studies described earlier, increased AT8 phosphorylation of wild-type tau may therefore reflect possible interactions of the compounds with tau before the protein undergoes terminal aggregation, which is expected to be a slow process in this animal model.

## A possible link between AT8 hyperphosphorylation of tau and microglial activation

In the group treated by the pan-protein aggregation inhibitor compound 1, our results showed a significantly increased number of microglial cells in the hippocampus, which is concomitant with the enhanced AT8 signal observed in the same brain region. In the ipsilateral hippocampus where this observation was made, human tau is mainly expressed by axonal projections stemming from the vector-injected entorhinal cortex. A recent study indicates that increased tau hyperphosphorylation may cause changes in microglial activity, possibly explaining the observed effects on microglial density in the projection area. Indeed, both *in vitro* and *ex vivo* experiments reported by Luo et al. show that the exposure of microglia to AT8 hyperphosphorylated tau induces its internalization and subsequent degradation by microglia[221]. Therefore, the aggregation inhibitor compound 1, by inducing tau phosphorylation at serine 202 and threonine 205 residues, may lead to microglial activation and tau clearance. Furthermore, a study by Yamada et al. showed that the turnover rate of intracellular soluble tau phosphorylated at Ser202/Thr205 was faster than total soluble tau, suggesting that this particular phosphorylation pattern increases the rate of clearance[222].

## Pan-protein aggregation inhibitor compounds possibly induce the degradation of tau leading to its decrease in the cerebrospinal fluid

Our results show that the aggregation inhibitor compounds induce a decrease in the levels of total human tau in the CSF. In Alzheimer's Disease and traumatic brain injury, total tau is found to be significantly increased in the CSF and is correlated with the pathology[53,101,223]. Therefore, a reduction of tau in the CSF might reflect a decrease in overall brain pathology. In our model of tauopathy, the control group of mice injected with the vector locally overexpressing 4R0N wild-type tau and administered with the vehicle shows a clear trend towards increased levels of human tau in the CSF when compared to the group of mice which do not overexpress human tau (injected with the empty vector). Statistical significance was not achieved most likely because of the low number of mice included in the latter group. Therefore, local human tau overexpression in the entorhinal cortex leads to a detectable increase in the level of human tau measured in the CSF, similar to AD patients. Remarkably, treatment with the aggregation inhibitor compounds partially prevents the accumulation of tau in the CSF levels measured in the treated mice. These results suggest that compounds that interfere with tau conformation may affect either the release of tau, or its clearance by mechanisms which remain to be identified. Of note, we did not observe any obvious neurodegeneration in the tau-overexpressing EC/hippocampus region, which may have contributed to the release of tau in the extracellular compartment. Nevertheless, it will be important to further explore whether changes in microglial activity may contribute to tau clearance in this animal model.

Overall, although our model did not demonstrate an effect of the treatment compounds on tau aggregates, because the model is based on wild-type tau that only show mild pathology, we nevertheless showed an insight on a possible mechanism of action of those compounds at an early stage of the pathology, where neurofibrillary tangles and other tau deposits are not well developed yet.

Further research is needed to better characterize the effects of the compounds. Future work could possibly look into the effect of the compounds on soluble tau oligomers with specific markers, or highlighting the phagocytosis of tau by microglial cells. To specifically address the

question of the aggregation inhibitors being able to disaggregate tau deposits in vivo, a more aggressive model of tau pathology might be considered by overexpressing a mutated form of tau for example.

# Chapter 4: Active immunotherapy against phospho-Ser396/404 tau decreases tau hyperphosphorylation and propagation in a viral vector-based model of human tau overexpression

Authors:

Sameer Nazeeruddin, Pamela Valdés, Patrick Aebischer, Bernard Schneider

Abstract:

In Alzheimer's Disease (AD), the spread of neurofibrillary tangles formed by tau aggregation follows a stereotypical pattern along neuroanatomically connected brain regions. This phenomenon, best described by the Braak staging of neuropathology, correlates with the progression of cognitive impairments in AD patients. *In vivo* and *in vitro* studies have shown that neuron-to-neuron transfer of the tau protein may contribute to the spread of tau fibrillar deposits. Tau transfer occurs in both physiological and pathological conditions through various pathways. In tauopathies, pathological tau is secreted in the extracellular space and taken up by recipient neurons, in which pathological forms of tau can further seed pathology by recruiting tau monomers. Therefore, interfering with the interneuronal transfer of tau by active immunization against pathological tau is a promising strategy which aims at slowing down the progression of the disease.

Here, we assessed the efficacy of an anti-phospho Ser396/Ser404 vaccine in reducing the propagation of tau in an AAV8-based model of interhippocampal neuron to neuron transfer of tau, in both wild-type (WT) and 5xFAD AD mice. The WT mice developed a strong immune response with high antibody titers against the vaccine epitope, whereas this response was found to be milder in 5xFAD mice. In the vaccinated WT mice, we showed by immunohistochemistry a decrease of the signal of the PHF-1 marker that recognizes the phospho-Ser396/404 residues, suggesting that the antibodies raised by the vaccine effectively bind to the targeted epitope in the hippocampus. Furthermore, by determining the number of human tau-positive neurons present in the contralateral hippocampus, distally to the site of vector injection inducing human tau overexpression, we showed that the vaccine significantly decreased the rate of interneuronal transfer of human tau, as a function of the titers of anti-phospho-Ser396/404 antibodies measured in the plasma. This result highlights a possible effect of the vaccine on the propagation of human tau. Remarkably, WT mice with high antibody titers also showed a partial rescue of tau-induced neurodegeneration in the dentate gyrus, which may indicate neuroprotective effects of the anti-phospho-Ser396/404 vaccine.

# Introduction

Tauopathies are neurodegenerative diseases causing a variety of symptoms ranging from dementia to motor symptoms[40,42]. The pathology of these various diseases reveals one common feature that is the abnormal aggregation of the protein tau, a microtubule-associated protein[146], expressed mainly in neurons and astroglial cells[39,77,86]. The most common tauopathy is Alzheimer's Disease, which is characterized by the extracellular accumulation of amyloid beta deposits and the intraneuronal accumulation of neurofibrillary tangles made of aggregated tau.

The spread of the neurofibrillary tangles follows a stereotypical anatomical progression over time, starting in the entorhinal cortex, then spreading to the hippocampus, followed by a progression towards the neocortical areas[211]. This progression of the pathological features of the disease allowed Braak et al. to delineate different stages of the progression of the disease based on the distribution of amyloid plaques and NFTs[74]. Remarkably, and in contrast to amyloid plaques, the progression of the neurofibrillary tangles is correlated with cognitive decline, suggesting a key role of tau in the symptomatology of the disease[75].

In line with these observations, it has been demonstrated that pathological tau aggregates are transferred from neuron to neuron and seed pathology in the recipient cell in a manner that is reminiscent of prions, by recruiting tau monomers into fibrillar aggregates[155,163,180,224,225]. In a pioneering study, Clavaguera et al. showed that when brain homogenates from P301S transgenic mice or from tauopathy patients' brains containing pathological tau aggregates were injected in the mouse brain, they triggered a tau pathology spreading to distally connected regions[175,182]. Several studies confirmed the propagative feature of tau pathology using various model systems, including transgenic mice or local injection of viral vector to induce human tau overexpression[118,176,178,180,226]. Furthermore, it has been determined that the spread of tau to distal regions is driven by neuronal connectivity[177], following various mechanisms of interneuronal transfer such as free diffusion, exosomes or tunneling nanotubes[118].

Since cognitive decline is correlated to the propagation of pathological tau in humans, assessed by neuroimaging[59,227], halting this propagation represents an important target for the development of potential disease-modifying treatments. Active immunotherapy is a promising approach to block the propagation of pathological tau from neuron to neuron,

especially when released, considering that pathological tau would be targeted by antibodies in the extracellular space[194]. However, tau is also present in physiological conditions, and neuron to neuron transfer of tau, may also happen in the normal brain[118,167,228]. Therefore targeting all tau species propagating might be deleterious as it might interfere with a potential role of tau[149,229]. Therefore, it is important to target an epitope that is present on the tau protein mainly in pathological conditions.

Various posttranslational modifications of tau such as truncation, acetylation or the more broadly studied hyperphosphorylation[77,154,228,230] are associated with the formation of pathological tau oligomers. Antibodies directed against sites carrying these modifications may be able to selectively target forms of tau capable to seed pathology, while not interfering with the propagation of physiological tau from neuron to neuron.

Several epitopes have been selected for active immunization studies, among which the hyperphosphorylated Ser396/404 site that is involved in NFTs of AD patients[218,231]. This epitope is exposed by the tested vaccine in this work (called Vaccine 1) which harbors a peptide sequence that encompasses the hyperphosphorylated Ser396/404 sites. No study yet quantified the efficacy of the Vaccine 1 against propagation of tau.

Here, in order to determine the efficacy of this treatment on preventing the propagation of tau, we developed a quantitative model, using local injection of an AAV8 vector to overexpress human wild-type 4R0N tau unilaterally in the CA3 area of the hippocampus. We used the interconnectivity towards the contralateral hippocampus[232,233] to quantify the number of recipient neurons positive for human tau, which reflects the rate of transfer from neuron to neuron.

# Results

## Stereotactic injection of AAV8-PGK-GFP in the CA3 area of mouse hippocampus leads to only local expression of GFP positive cell bodies in the ipsilateral CA3 and dentate gyrus

The hippocampus is a region involved in the progression of tau neurofibrillary tangles in Alzheimer's Disease. Both hippocampi of the brain have a well-described interconnectivity between hemispheres[232–234], mainly from the pyramidal cells of the CA3 area that project axons to the contralateral hippocampus. Hence, this brain region is well suited to study the neuron-to-neuron transfer of the protein tau through inter-hemispheric connections. Our aim was to achieve local human tau overexpression by injecting an AAV vector in the CA3 hippocampus and explore propagation of the protein to the projection areas.

To induce local gene delivery for tau overexpression and minimize AAV-mediated transduction of distal neurons, we used the serotype 8 AAV vector, which has been previously described to have lower capability for retrograde transduction as compared to other serotypes[235]. To assess AAV8-mediated targeting of the mouse hippocampal CA3, we unilaterally injected 1.4E10 vg of the vector AAV8-PGK-GFP and analyzed transgene expression 1.5 months post-vector injection. In coronal sections, we clearly observed GFP-positive neurons at the site of vector injection, in the ipsilateral CA3 and dentate gyrus, with dense GFP-positive neurites that cross the interhemispheric midline and project to the contralateral hippocampus (Fig. 33 A-B). Although there was dense network of neurites in the contralateral hippocampus, we did not observe any GFP-positive cell bodies in these areas. Therefore, injection of the AAV8-PGK-GFP vector did not lead to any detectable transgene expression in distal neurons, suggesting that the viral particles did not reach the contralateral hippocampus.

Next, we conducted a similar experiment, using the same with the AAV8-PGK-4R0N human tau WT injected at a dose of 2.8E10 vg in the CA3 hippocampus. To identify the neurons transduced by AAV8, we performed an *in situ* hybridization assay with a probe against the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) to detect the AAV-expressed mRNA (Fig. 33 C). While an intense WPRE signal was observed in the injected

CA3 and dentate gyrus, there was no detectable signal in the contralateral hippocampus. This observation confirmed that the vector did not spread to the contralateral hippocampus, three months after injection.

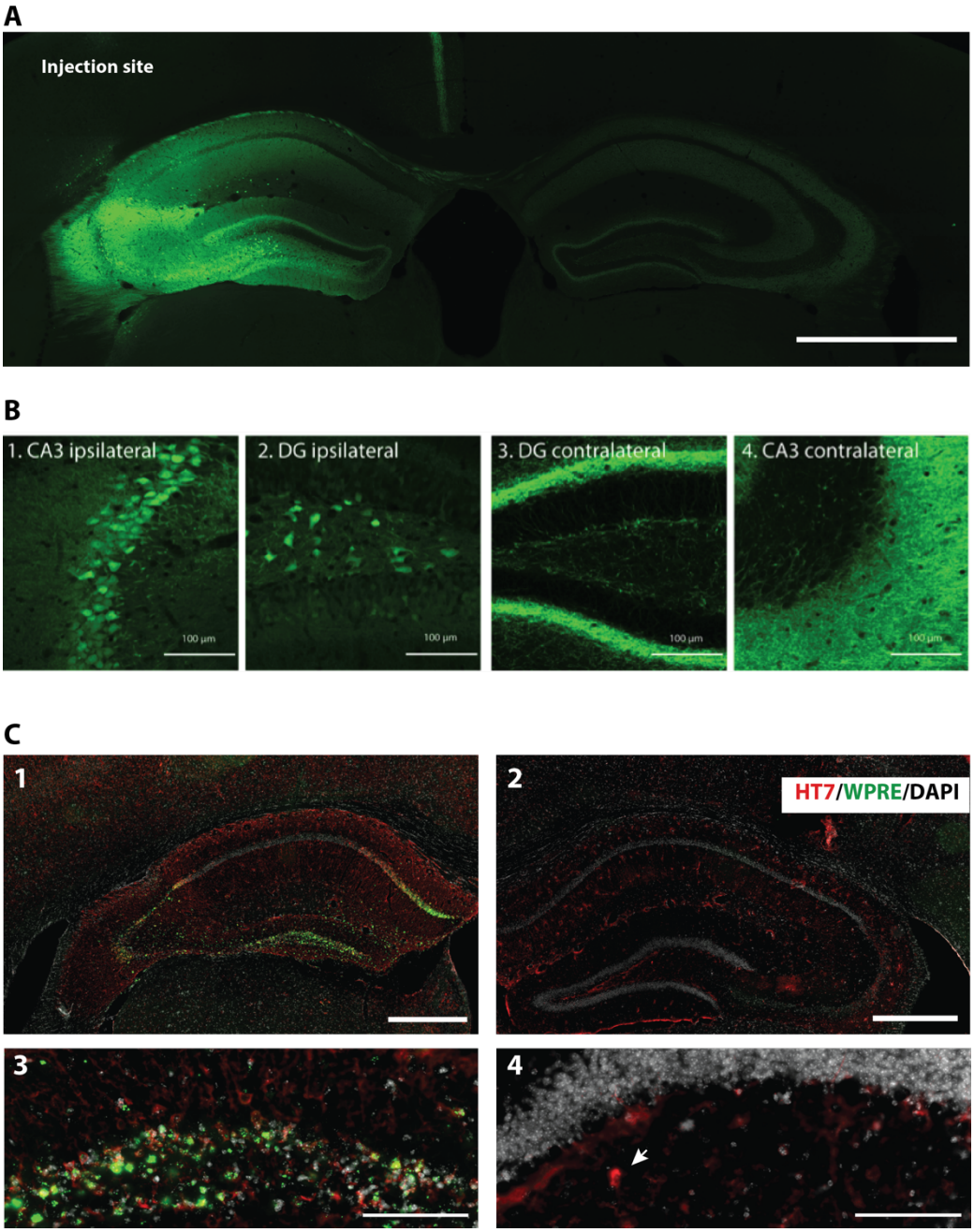



*Figure 33: Absence of GFP positive neurons and of transgene mRNA in the contralateral hippocampus confirms that AAV8 only locally transduces neurons following injection in the CA3 hippocampus.*

**(A)** Coronal section of a mouse brain injected with AAV8-PGK-GFP in the CA3 area of the hippocampus. GFP-positive fibers cross the midline and project to the contralateral hippocampus. Scale bar: 1mm
**(B)** Although GFP positive neurons are visible in the ipsilateral CA3 **(1)** and dentate gyrus **(2)**, no GFP-positive neurons are seen neither in the contralateral CA3 **(3)** nor in the contralateral dentate gyrus **(4)**. Scale bars: 100 µm for (1-4).
**(C)** *In situ* hybridization against the WPRE sequence shows transgene expression in the ipsilateral hippocampus only **(1).** There is no evident signal in the contralateral hippocampus, while there is a strong HT7 positive signal demonstrating human tau expression **(2)**. **(3)** The ipsilateral dentate gyrus is strongly positive for the WPRE

probe. **(4)** No WPRE in situ signal is detectable in the contralateral dentate gyrus, whereas a HT7 positive cell body is visible with DAPI counterstaining (arrow). RNAscope assay was performed by the Histology Core Facility at EPFL. Scale bars: 500 μm for 1-2 and 100 μm for 3-4.

To further explore the interhemispheric connectivity between hippocampi and to analyze the distribution of the overexpressed tau protein, we injected 2.8E10 vg of the AAV8-PGK-4R0N WT Tau-EGFP vector and used the CLARITY method to assess the fluorescent signal of human 4R0N tau fused to the GFP protein, which allowed to easily trace tau distribution throughout axonal projections in three dimensions. We confirmed the high connectivity of the injected site in the hippocampus to the contralateral hemisphere mainly through two interhemispheric commissures: one anterior and one posterior. Dense connectivity was also seen toward the entorhinal cortex regions, both ipsilateral and contralateral to the site of vector injection (Fig 34).

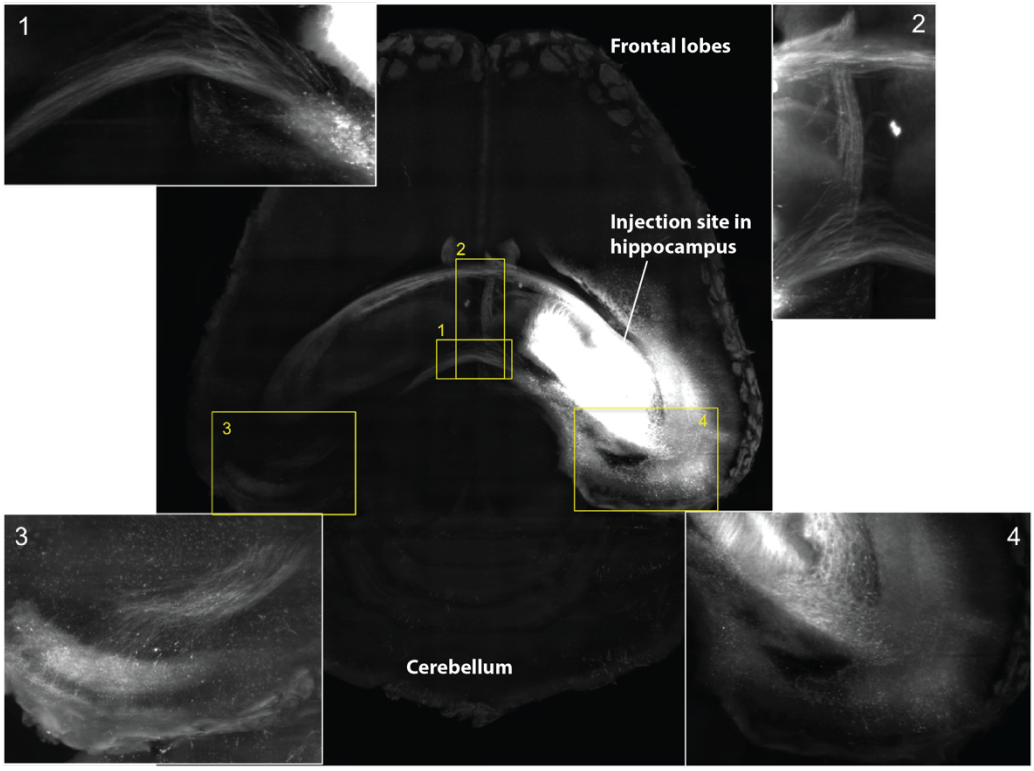



*Figure 34: 3D imaging of the hippocampus connectivity using CLARITY*

Mouse brain injected with AAV8-PGK-4R0N Tau-EGFP in the CA3 hippocampus. GFP fluorescence is imaged using the CLARITY method, showing consistent expression of tau-EGFP at the site of injection. Note the axons positive for tau-EGFP crossing the midline through two hippocampal commissures **(1-2)** and reaching the contralateral hippocampus. Tau-GFP signal is also seen in hippocampal axons projecting to the ipsilateral **(4)** and contralateral **(3)** entorhinal cortices. Image: courtesy of the Microscopy Facility at the Wyss Center for bio- and neuroengineering, Geneva.

## AAV8 overexpression of human 4R0N tau in the CA3 area of mouse hippocampus leads to the transfer of tau to neurons located in the contralateral hippocampus

When we overexpressed human tau by stereotactic injection of 1.4E10 vg of the AAV8-PGK-4R0N human tau WT vector in the CA3 area of the hippocampus, we consistently observed a high expression of human tau with a high density of neurites and neuronal cell bodies that were specifically recognized by the HT7 IHC antibody, 1.5 months after injection (Fig. 35 A). These cells were mainly visible in the CA3 area and the dentate gyrus of the hippocampus ipsilateral to the site of injection, similarly to the previously observed GFP expression. In addition, in coronal brain sections, axonal projections positive for human tau (HT7) were found to cross the midline and reach the contralateral hippocampus, which displayed a high density of human tau-positive neurites . Remarkably, HT7 immunostaining consistently revealed neuronal cell bodies positive for human tau in specific neuronal populations located in the hilar region and the granule cell layer of the dentate gyrus and in the CA3 area of the contralateral hippocampus, distal to the site of vector injection (Fig. 35 B). This showed that human tau overexpressed in the neurons located in the CA3 hippocampus accumulated in the axonal projections and was also transferred to recipient neurons in synaptically connected regions of the contralateral hippocampus.

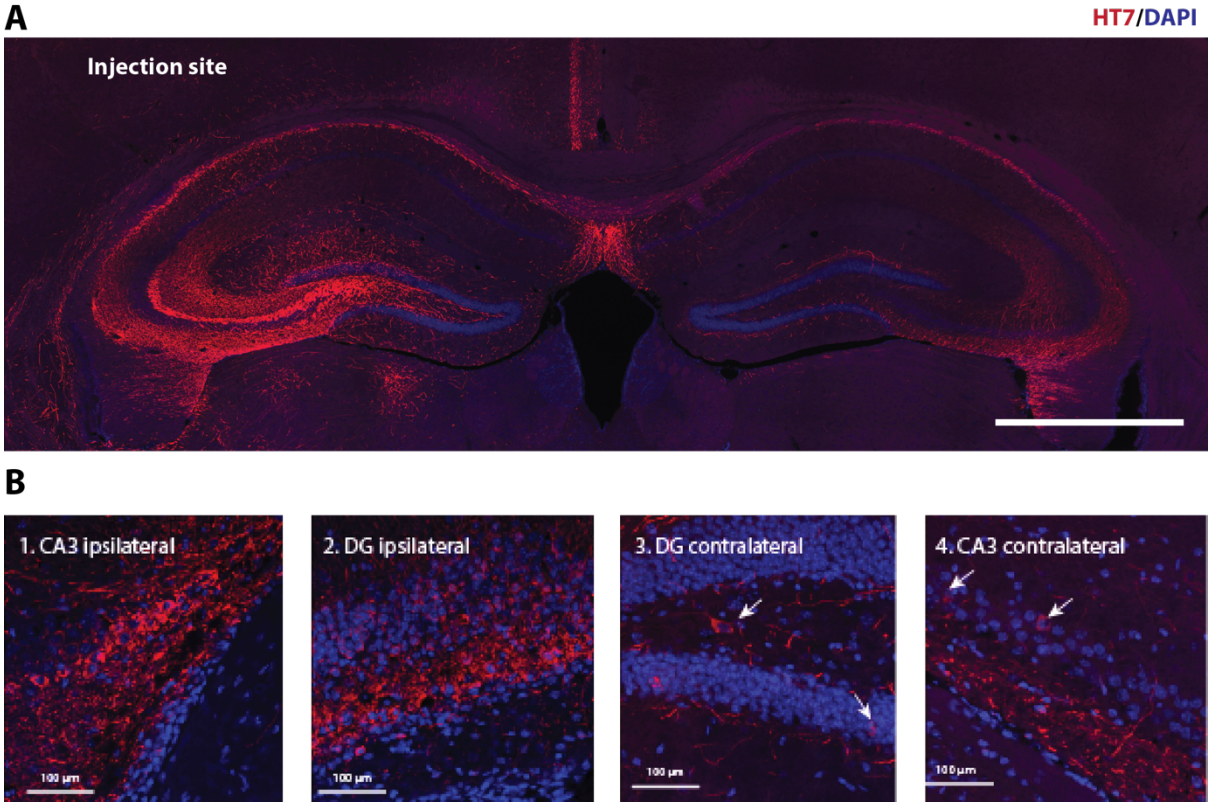



*Figure 35: Overexpression of human tau by AAV leads to consistent spread of HT7 positive signal to the contralateral hippocampus*

**(A)** Representative picture of a coronal section of the mouse hippocampus showing overexpression of human tau (HT7 immunostaining) at the site of AAV8-PGK-4R0N Tau WT injection, mainly in the CA3 and dentate gyrus (DG) areas. Human tau positive axons are seen crossing the midline towards the contralateral hippocampus. Scale bar: 1 mm.
**(B)** HT7 positive neuronal cell bodies are observed in the CA3 area of the injected hippocampus **(1)** as well as in the ipsilateral dentate gyrus **(2)**. HT7-positive neurons are also observed in the contralateral hippocampus: in the granule cell layer and/or in the hilus area of the dentate gyrus **(3)**, as well as in the contralateral CA3 **(4)**. Scale bars: 100 µm.

## Effect of two tau isoforms on the transfer of tau from neuron to neuron

In order to develop a reliable model of tau propagation, we tested two different isoforms of tau for their rate of transfer from neuron to neuron. Indeed, tau isoforms have been described to have different properties, such as aggregation propensity[112], which may affect their propagation. We compared the 4R2N and 4R0N tau isoforms, which are considered the most abundant in the axonal compartment[110], as opposed to 4R1N which is mainly located in the somatodendritic and nuclear compartments.

We unilaterally injected 1.5E10 vg of either AAV8-PGK-4R0N tau WT or AAV8-PGK-4R2N tau WT in the CA3 hippocampus of C57BL/6 mice. A group of mice was analyzed 1.5 months post-vector injection and another group 3 months post-injection. Using coronal brain sections covering the entire hippocampus and processed for HT7 immunohistochemistry, we quantified the number of neuronal cell bodies positive for human tau in the contralateral hippocampus (Fig. 36 A-B). We found a non-significant trend towards an effect of the isoforms (P = 0.06), with an increased number of neuronal cell bodies in the contralateral hippocampus with the 4R2N tau isoform compared to the 4R0N tau isoform (Fig. 36 C).

Because the difference in the rate of propagation was not significant between the 4R2N tau and the 4R0N tau isoforms, and because the 4R0N tau has been reported to be more abundant in AD compared to the 4R2N tau[236], we decided to use the 4R0N tau isoform for further experiments.

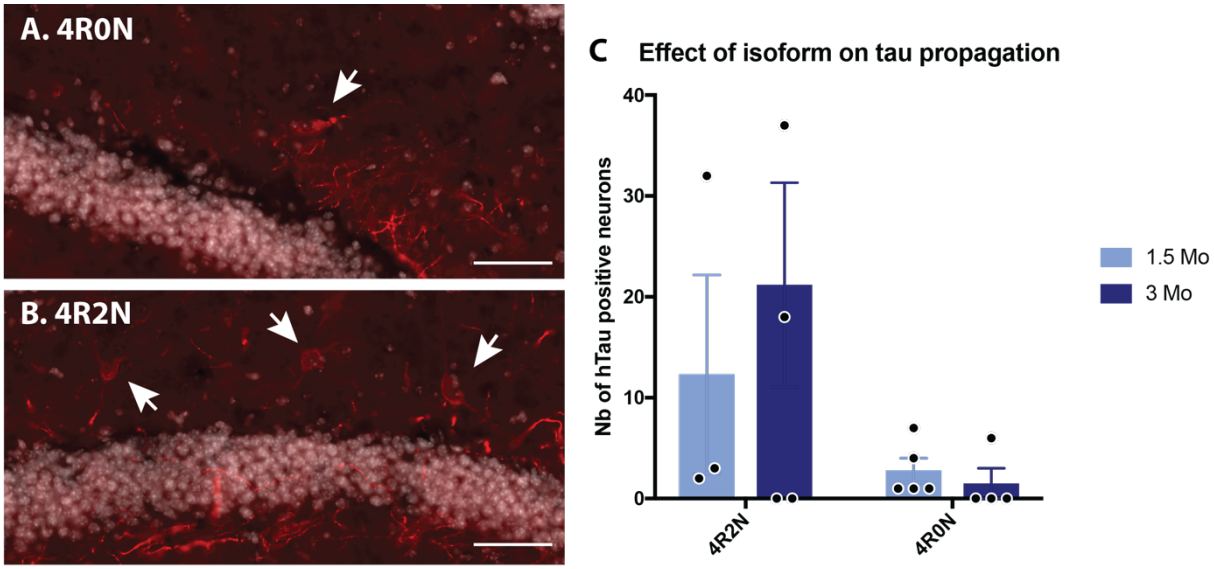



*Figure 36: Effect of the isoforms on tau propagation*

**(A-B)** Representative images of HT7 positive neurons in the hilus area of the dentate gyrus in the contralateral hippocampus of mice injected with 4R0N **(A)** and 4R2N **(B)**. Neuronal cell bodies are indicated with arrowheads. Scale bar: 100µm

**(C)** Quantification of HT7 positive neurons in the contralateral hippocampus. Results show a trend towards an increased number of HT7 positive neurons in mice expressing 4R0N human tau compared to mice expressing 4R2N human tau (P = 0.06 effect of the isoforms by 2-way ANOVA).

## The presence of the amyloid pathology increases the expression level of markers of the tau pathology

Alzheimer's Disease is characterized by both tau and amyloid beta pathologies, which are thought to synergistically potentiate each other[81,82]. Therefore, we sought to characterize the effect of the amyloid pathology on the accumulation and aggregation of pathological forms of tau in our model. To address this question, we used 5xFAD transgenic B6SJL mice, harboring three APP mutations and two PS1 mutations related to familial Alzheimer's Disease. This mouse line shows rapid development of amyloid beta plaques throughout the brain[237]. To induce overexpression of human tau in the CA3 hippocampus, we injected the AAV8-PGK-4R0N Tau WT vector at a dose of 2.8E10 vg in 5xFAD mice and their WT littermates that do not have the amyloid pathology. To determine qualitatively the expression of pathological tau markers, we performed DAB immunohistochemistry on brain sections of 5xFAD mice and their WT littermates sacrificed 6 months after vector injection. We used the PHF-1 and AT8 antibodies for hyperphosphorylated tau associated with NFTs in AD, and the MC1 conformation-specific antibody for misfolded tau, to reveal the presence of aggregated tau. For all three markers of tau pathology, we observed a consistent immunoreactivity in the CA3 area of the hippocampus at the site of vector injection (Fig. 37 A-F).

To quantitatively assess the levels of MC1, PHF-1 and AT8 markers, we performed an AlphaLISA analysis in protein extracts from the whole hippocampus in 5xFAD mice and WT littermates, 3 months after injection of 2.8E10 vg of AAV8-PGK-4R0N tau WT vector. When we normalized the levels of MC1 and PHF1 signals to total human tau levels, we found a significant increase in the level of the MC1 and PHF-1 markers in 5xFAD mice as compared to WT littermates. For AT8 however, there was no significant difference between groups, albeit this result would need to be confirmed with a higher number of replicates (Fig. 37 G-I).

Overall, this result shows that the presence of amyloid pathology enhances the level of markers of tau pathology, in particular MC1 and PHF-1.

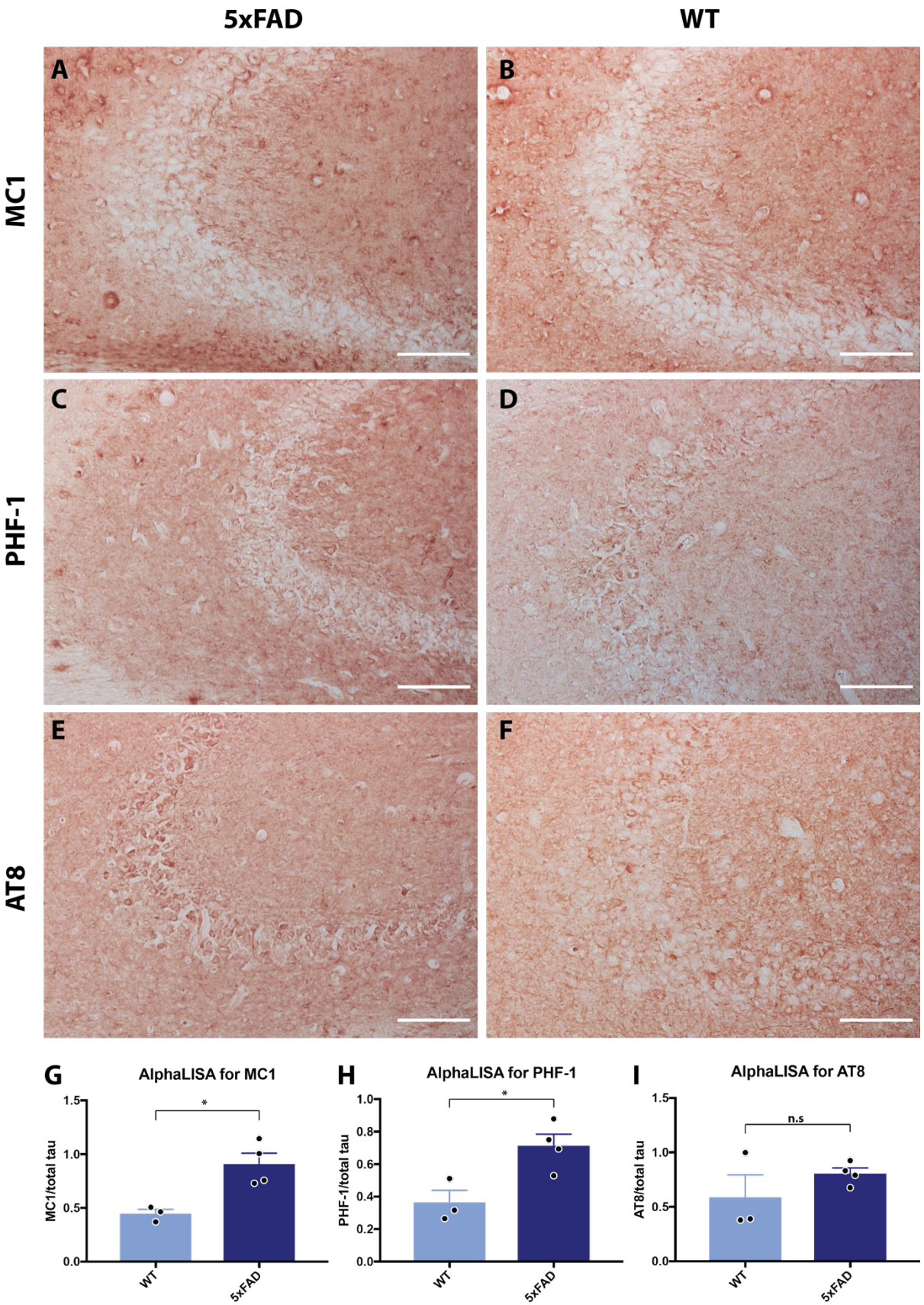

5xFAD
WT
MC1
PHF-1
AT8
A
B
C
D
E
F
G
AlphaLISA for MC1
MC1/total tau
1.5
1.0
0.5
0.0
*
WT
5xFAD
H
AlphaLISA for PHF-1
PHF-1/total tau
1.0
0.8
0.6
0.4
0.2
0.0
*
WT
5xFAD
I
AlphaLISA for AT8
AT8/total tau
1.5
1.0
0.5
0.0
n.s
WT
5xFAD

*Figure 37: The presence of the amyloid pathology enhances tau pathology*

**(A-F):** Representative pictures of the ipsilateral CA3 area of the hippocampus of 5xFAD mice **(A, C, E)** and WT littermates **(B, D, F)** sacrificed 6 months after injection with a dose of 2.8E10 vg of AAV8-PGK-4R0N Tau WT vector. Consistent pathology is observed in the ipsilateral CA3 near the site of vector injection with common markers of tau pathology found in AD: MC1, PHF-1 and AT8. Scale bar: 100 μm.
**(G, H, I):** MC1, PHF-1 and AT8 signal values measured by alphaLISA and normalized to total human tau values, in protein extracts from the hippocampus, 3 months post-injection of the AAV-PGK-4R0N Tau WT vector (2.8E10 vg). Note the significant increase in the MC1 ($P = 0.013$) and PHF-1 ($P = 0.022$) normalized signals in 5xFAD mice compared to WT littermates. Statistical analysis: Student's T-test, WT mice N = 3, 5xFAD mice N = 4. * $P < 0.05$, n.s: not significant.
AlphaLISA analysis was performed by collaborators.

## Tau propagation from neuron to neuron is increased in mice carrying the amyloid pathology

Next, we sought to determine whether the synergistic interaction between the amyloid pathology and the tau pathology also exerts its effect on the transfer of the tau protein from neuron to neuron in our animal model. To induce overexpression of human tau in the CA3 hippocampus, we injected 1.4E10 vg of the AAV8-PGK-4R0N human tau WT vector in a group of 5xFAD mice and a control group of WT littermate mice. To determine whether we have a dose effect of the injected vector on tau propagation, we also included another cohort of mice injected with a double dose of AAV8-PGK-4R0N human tau WT vector (2.8E10 vg).

We analyzed the distribution of human tau in the mouse hippocampus at two time points, either 1.5 months or 6 months post-vector injection. Vector injection induced consistent overexpression of human tau in the ipsilateral hippocampus. The protein was detected using the HT7 immunostaining in neuronal cell bodies and neurites of the CA3 and dentate gyrus in the ipsilateral hippocampus, as well as in axonal projections to the contralateral hippocampus. The spread of tau along the axons towards the contralateral side was increased when we compared mice sacrificed 1.5 months post-vector injection and those sacrificed 6 months after (Fig. 38 A-D), suggesting that tau spreads along the axons in a time dependent manner. We also observed the presence of human tau-positive neurons in the contralateral hippocampus, at both 1.5 and 6 months after vector injection. These neurons were located near hippocampal regions rich in human tau-positive afferents, mainly in the CA3 and hilar region of the dentate gyrus (Fig. 38 E-F).

Next, to assess the effect of the amyloid pathology on the transfer of the human tau protein to neurons located distally to the site of vector injection, we determined the number of neurons positive for human tau in the contralateral hippocampus. We found out a significant effect of the amyloid pathology ($P = 0.005$) and a significant effect of the vector dose ($P = 0.00013$), towards an increase of the number of neuronal cell bodies positive for human tau in the contralateral hippocampus (Fig. 38 G). This result showed that the presence of the amyloid pathology increases the propagation of human tau from neuron to neuron. Furthermore, the propagation of tau from neuron to neuron in our model was strongly

dependent on the injected dose of the vector. Therefore, we decided to use the high dose of AAV8-PGK-4R0N human tau WT (2.8E10 vg) for further experiments.

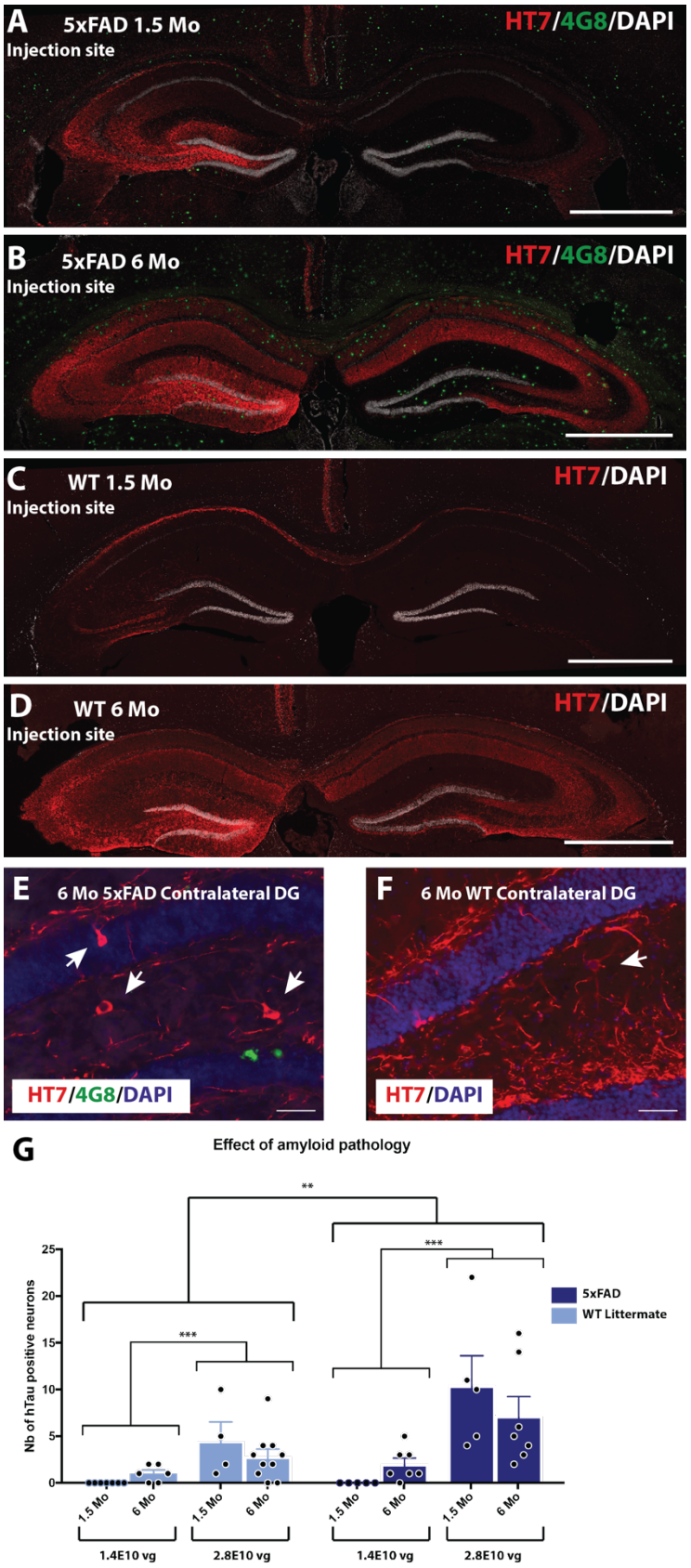


*Figure 38: Effect of the amyloid beta pathology on tau propagation*

**(A-D)** Injection of AAV8-PGK-4R0N Tau WT in 5xFAD mice and their WT littermate leads to consistent expression of tau in the ipsilateral hippocampus with HT7 positive axons projecting towards the contralateral hippocampus. The spread of tau along the axons increases overtime when we compare mice sacrificed 1.5 months post-vector injection to mice sacrificed 6 months after vector injection. Note the presence of amyloid beta plaques (4G8 staining) in the 5xFAD mice **(A-B)**. Scale bar: 1 mm.
**(E-F)** Representative images of the contralateral dentate gyrus showing HT7-positive neurons containing human tau (arrows). Scale bar: 50 μm.
**(G)** Quantification of HT7 positive neurons in the contralateral hippocampus of brain sections covering the whole hippocampus from anterior to posterior with an interval of 150 μm between sections. Note the significant effect of the amyloid pathology on the number of HT7 positive neurons in 5xFAD mice expressing 4R0N human tau compared to their WT littermate mice ($P = 0.005$ for an effect of the amyloid pathology). Note also the significant effect of the vector dose on the number of HT7-positive neurons ($P = 0.00013$ for an effect of the vector dose). Statistical analysis: 3-way ANOVA to examine the effect of vector dose, effect of time point and effect of amyloid pathology.

## Active immunization against tau phosphorylated at residues Ser396/404 in mice overexpressing 4R0N human tau induces a robust anti-tau antibody response

Next, we sought to explore the effects of anti-tau active immunization in mice locally overexpressing human 4R0N tau in the hippocampus. Nine-weeks old wild-type C57BL/6 mice and 5xFAD B6SJL mice were unilaterally injected in the CA3 hippocampus with 2.8E10 vg of AAV8-PGK-4R0N human tau vector (Fig. 39 A). Two weeks after vector injection, each of the two mouse cohorts were separated in two groups. One group was subcutaneously injected with a vaccine against Ser396/404-phosphorylated tau whereas the control group received PBS injections. In both wild-type and 5xFAD mice, the vaccination procedure was repeated four times during the whole experiment in order to boost the production of anti-tau antibodies. An additional control group of wild-type C57BL/6 mice was included, which was injected with the non-coding AAV8-PGK-MCS vector and received the same vaccine treatment as the mice injected with the tau-expressing vector.

Plasma was regularly collected to verify the efficacy of the vaccination procedure against Ser396/404-phosphorylated tau. First, we measured by ELISA the titers of the antibodies produced against the vaccine epitope after 63 days of treatment. As expected, in the cohort of C57BL/6 WT mice, we observed a clear increase of the antibody titers in the vaccinated group as compared to the PBS-injected group ($P < 0.0001$) (Fig. 39 B), demonstrating a robust anti-phospho-tau immune response. Of note, anti-tau antibody titers were significantly higher in the AAV8-PGK-4R0N human tau injected group as compared to the mice injected

with the non-coding vector and vaccinated with the same procedure (P = 0.009). This effect indicated that the overexpression of human tau may have further increased the immune response against the tau epitope in the vaccine-treated animals.

When we measured the antibody titers in the 5xFAD cohort (Fig. 39 C), we again found a significant increase in the vaccinated group compared to the PBS-injected group (P = 0.025). however, the antibody response remained lower in the 5xFAD mice than in the previous cohort of vaccinated C57BL/6 mice.

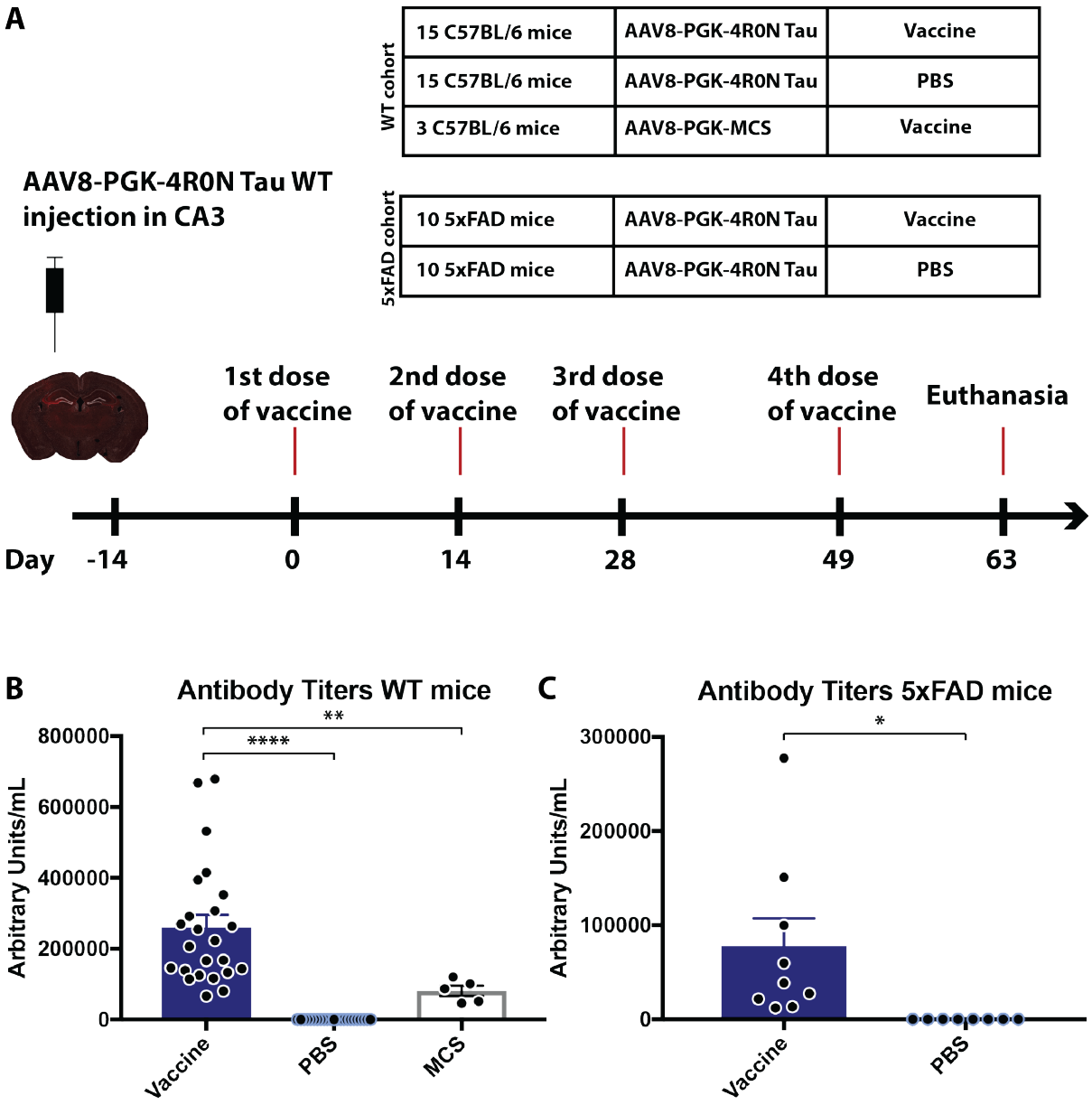



*Figure 39: Experimental design of the vaccination study and antibody titers*

**(A)** Experimental paradigm of the active immunization study. Two cohorts of mice are used for the study: C57BL/6 WT mice and 5xFAD B6SJL. In each cohort, two groups are injected in the CA3 with 2.8E10 vg of AAV8-PGK-4R0N WT tau, to compare control PBS-injected mice with animals administered with the active vaccine. The vaccination protocol is based on 4 doses of vaccine. An additional group of vaccine-treated WT mice is injected with 2.8E10 vg of the non-coding AAV8-PGK-MCS vector in the WT cohort.
**(B)** Titers of antibodies directed against the phospho-Ser396/404 epitope measured in the plasma of wild-type C57BL/6 mice, 63 days after the first dose of vaccine. The group treated with the anti-tau vaccination protocol shows a significant increase in antibody titers compared to the group which received PBS ($P < 0.0001$). Of note, C57BL/6 mice injected with the AAV8-PGK-4R0N WT tau vector have significantly higher antibody titers than the mice injected with the non-coding vector ($P = 0.009$). Statistical analysis: one-way ANOVA with Tukey's multiple comparison test.
**(C)** Titers of antibodies directed against the phospho-Ser396/404 epitope measured in the plasma of 5xFAD mice, 63 days after the first dose of vaccine. The vaccine-treated group developed a significant increase in antibody titer compared to the group injected with PBS ($P = 0.025$), although the response is milder than in the C57BL/6 cohort. Statistical analysis: Student's T-test. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

## Microglia area coverage is not changed between vaccine treated and PBS injected group

All the mice injected with the AAV8-PGK-4R0N tau WT vector showed robust expression of human tau in the CA3 and dentate gyrus regions of the hippocampus (Fig. 40 A-D). Additionally, an atrophy of the ipsilateral hippocampus, with a neurodegeneration in the CA3 area and the dentate gyrus was visible in mice injected with the tau vector. This observation will be discussed more in details later in this chapter.

Next, to determine whether the microglial response was changed in the hippocampus of the vaccine-injected mice, we performed immunohistochemistry for Iba1 (Fig. 40 E-H), a marker of microglial cells, and measured the area coverage of the Iba1 signal in the ipsilateral hippocampus (Fig. 40 I-J). No difference in the density of microglial cells was observed between the vaccine-treated and the PBS-injected groups, in both wild-type and 5xFAD mouse cohorts, suggesting that there were no major changes in the local microglial response following vaccination.

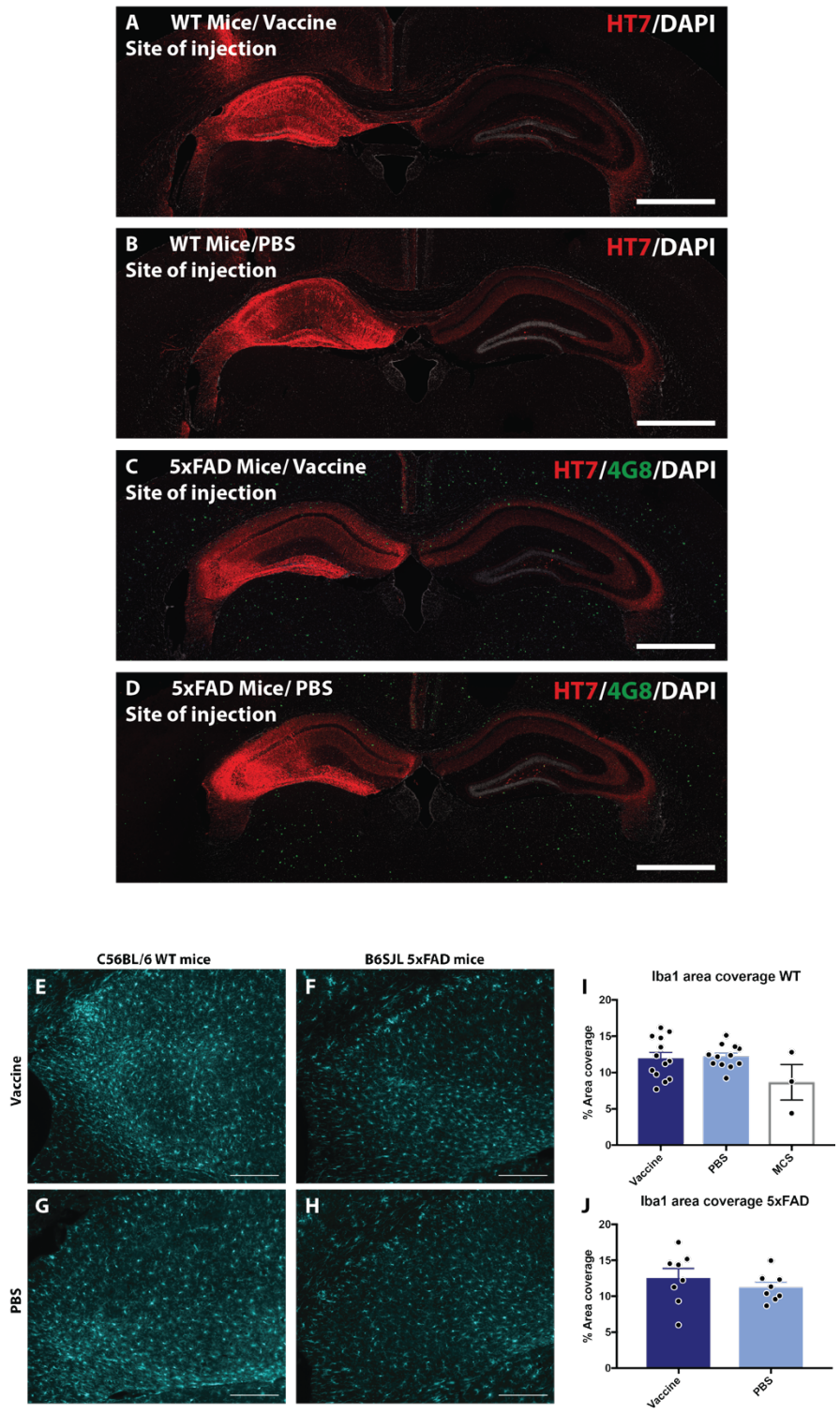



*Figure 40: Human tau overexpression in the hippocampus and effect of the vaccine on microglia*

**(A-D):** Representative pictures of coronal brain sections at the site of vector injection in the CA3 area of the hippocampus which shows consistent expression of total human tau 4R0N WT. Scale bar: 1 mm
**(E-H)** Representative pictures of the presence of microglia in the ipsilateral CA3 area of the hippocampus. Scale bar: 200 µm.
**(I-J)** Quantification of the area coverage by the Iba1 signal in the ipsilateral hippocampus does not show any difference between the vaccine group and the PBS group in both the WT cohort and the 5xFAD cohort.

## The vaccine against hyperphosphorylated Ser396/404 tau decreases the PHF-1 signal in both ipsilateral and contralateral hippocampi in the cohort of C57BL/6 mice

The vaccine contains the peptide sequence found in the protein tau comprising the phosphorylated Ser396/404 residues that are specifically recognized by the PHF-1 antibody.
To determine the effect of the vaccine on the level of PHF-1 hyperphosphorylated tau, we performed IHC on coronal brain sections (Fig. 41 A-H). We quantified the mean intensity of the PHF-1 signal on three brain sections around the site of injection in the ipsilateral hippocampus, in both the C57BL/6 WT and 5xFAD B6SJL mouse cohorts.
In the cohort of C57BL/6 WT mice which developed high antibody titers, we observed a significant decrease ($P = 0.027$) in the mean PHF-1 signal intensity in the vaccinated group as compared to the PBS-injected group. Therefore, the level of tau hyperphosphorylated at the Ser396/404 sites appears to be decreased following mouse vaccination (Fig. 41 I). In the contralateral hippocampus, we also observed a significant decrease ($P = 0.022$) of the PHF-1 signal intensity in the vaccinated group as compared to the PBS-injected group (Fig.41 J), which indicates that the vaccine also reduces the expression of PHF-1 hyperphosphorylated tau in the axons projecting from the vector-injected CA3 hippocampus.
We performed a similar immunostaining with the PHF-1 antibody in the cohort of 5xFAD mice carrying the amyloid pathology and which displayed only low antibody titers (Fig. 42 A-H). In these mice however, we did not find any difference in the level of PHF-1 immunofluorescence between the vaccinated and PBS-injected groups, neither in the ipsilateral (Fig. 42 I), nor in the contralateral hippocampus (Fig. 42 J).
Overall, these results show an effect of immunotherapy on the level of phospho-Ser396/404 tau immunoreactivity in mice overexpressing human tau. The decrease in the level of PHF-1-labelled tau however appears to depend on the presence of high antibody titers following vaccination against phospho-tau.

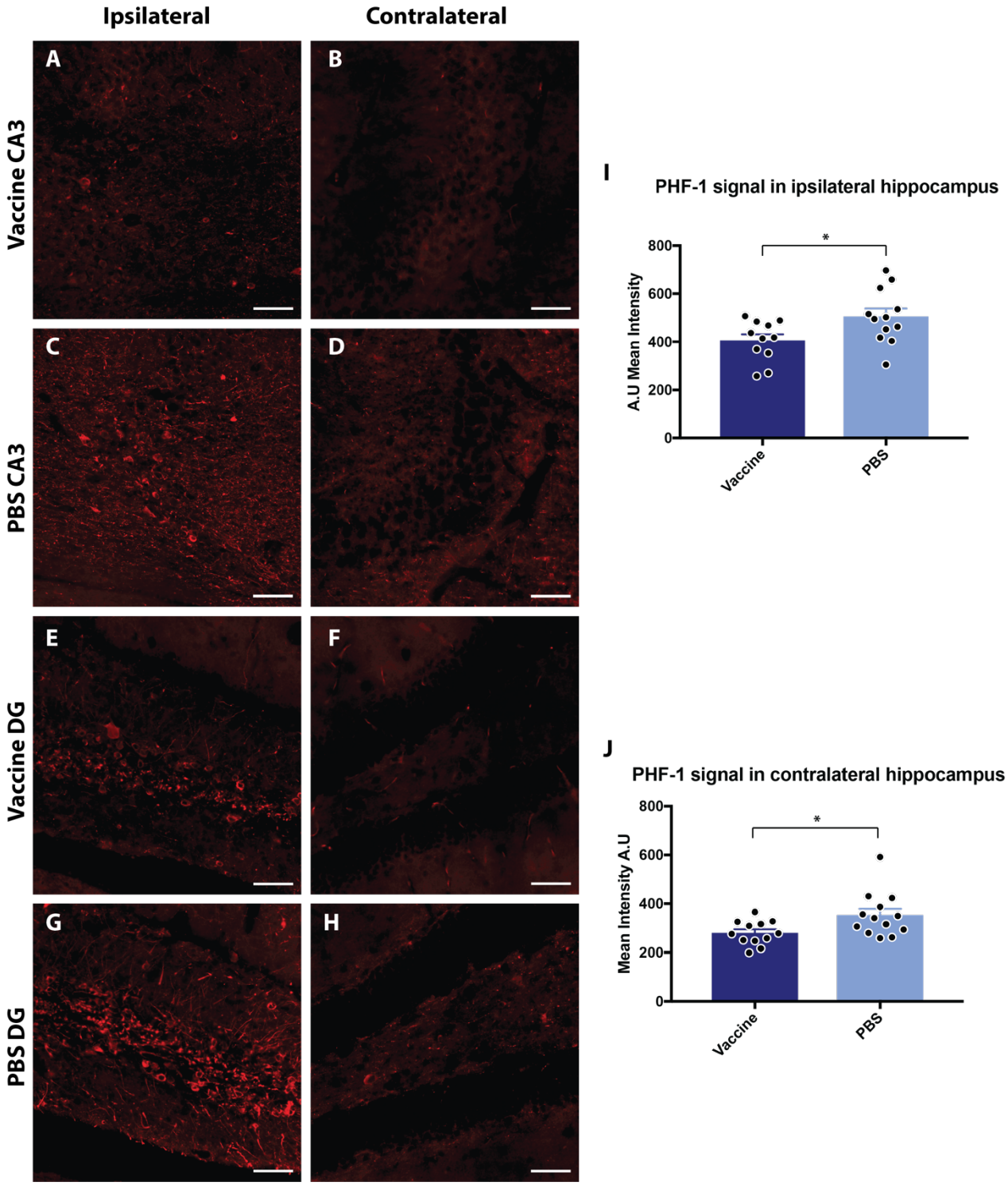



*Figure 41: Effect of the vaccine on the level of PHF-1-labelled phospho-tau in the cohort of WT mice*

**(A, C, E, G)** Ipsilateral to the site of AAV8-PGK-4R0N WT tau injection, PHF-1 immunohistochemistry shows the presence of hyperphosphorylated tau in neuronal cell bodies and neurites in the CA3 and dentate gyrus regions of the hippocampus. **(B, D, F, H)** PHF-1 positive neurites are also visible in the contralateral hippocampus. Scale bar A-H: 50 μm.

**(I)** Quantification of the mean PHF-1 intensity signal by immunohistochemistry in the AAV8-PGK-4R0N WT tau vector-injected hippocampus. Note the significant decrease in the PHF-1 signal in the vaccine-treated group compared to the PBS-injected group. **(J)** The same analysis in the contralateral hippocampus shows a similar effect of the anti-phospho-tau vaccine on PHF-1 immunoreactivity.

Statistical analysis: Student's T-Test. * $P < 0.05$.

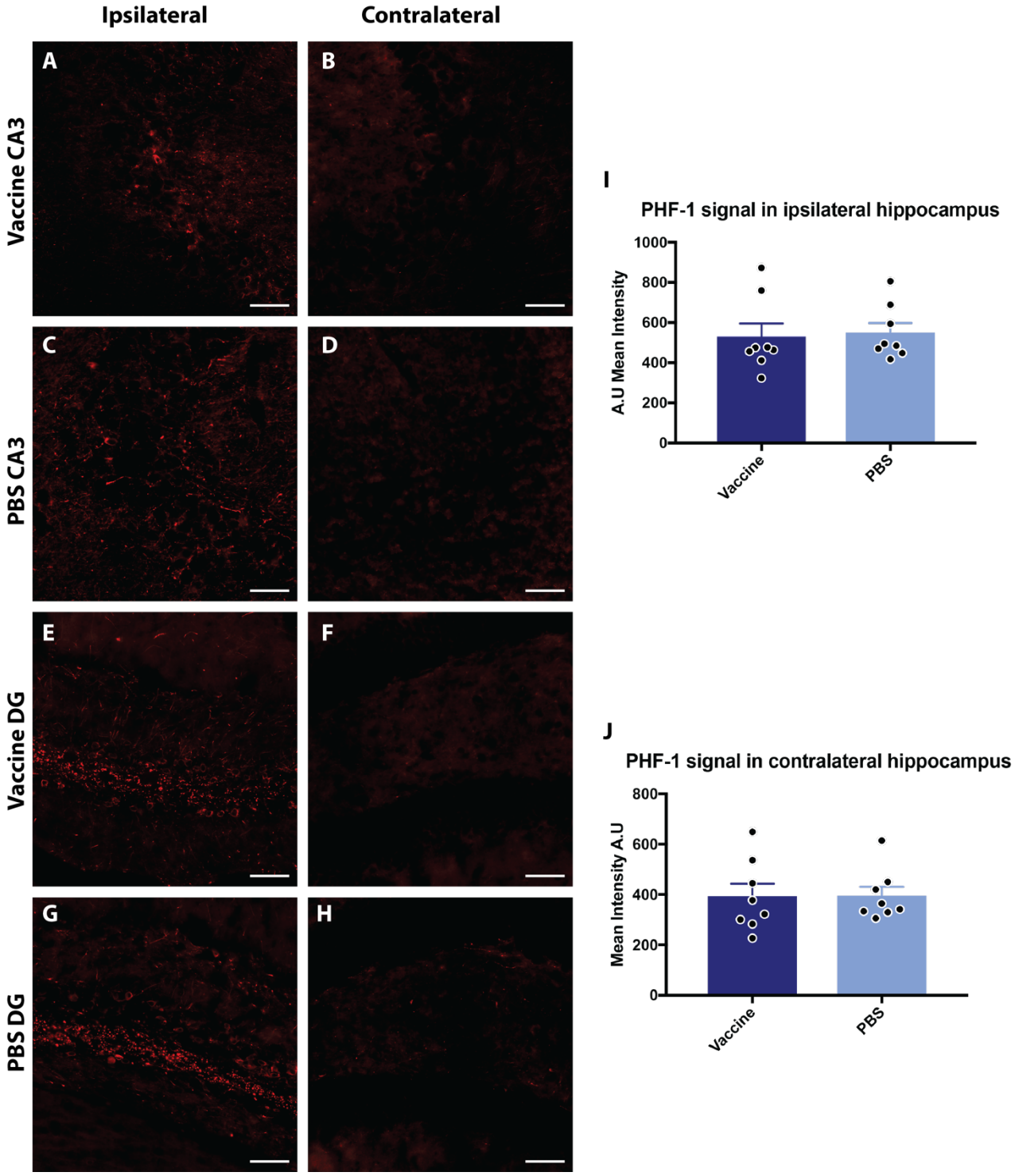



*Figure 42: Effect of the vaccine on the level of PHF-1-labelled phospho-tau in the cohort of 5xFAD mice*

**(A, C, E, G)** PHF-1 immunohistochemistry shows the presence of Ser396/404 phosphorylated tau in neuronal cell bodies and neurites in the CA3 and dentate gyrus regions of the ipsilateral hippocampus. **(B, D, F, H)** Some PHF-1 positive neurites are also visible in the contralateral hippocampus. Scale bar A-H: 100 μm.
**(I, J)** Quantification of the mean PHF-1 intensity signal shows no significant effects of the anti-phospho-tau vaccine in 5xFAD mice, neither in the AAV8-PGK-4R0N WT tau vector-injected hippocampus **(I)**, nor in the contralateral hippocampus **(J)**. Statistical analysis: Student's T-Test. * $P < 0.05$.

## The anti-phospho-tau vaccine decreases the transfer of tau from neuron to neuron

By inducing the production of anti-tau antibodies, active immunotherapy may block tau transfer from neuron to neuron, and thereby possibly prevent the spread of pathological tau in the diseased brain. Once we had determined that the vaccine induced a decrease in the level of PHF-1 hyperphosphorylated tau, we next sought to assess if the vaccine could decrease the propagation of human tau to neurons located in the contralateral hippocampus. Therefore, the number of neuronal cell bodies positive for human tau was quantified in the contralateral hippocampus using HT7 immunohistochemistry (Fig.43 A-D).

Although high variability was observed between animals, the average number of HT7-positive neurons present in the CA3 and dentate gyrus of the contralateral hippocampus was found to be reduced in the mice injected with the anti-phospho Ser396/404 tau vaccine, as compared to PBS-injected mice (Fig. 43 E). Remarkably, this effect was consistently observed in both the C57BL/6 wild-type and the 5xFAD B6SJL mouse cohorts. (Fig. 43 E). Overall, the effect of the vaccine on the total number of neurons positive for HT7 was statistically significant ($P = 0.023$).

To determine if the inter-animal variability was at least in part due to the observed differences in the response to the anti-phospho-tau vaccine, we sought to determine whether there was any correlation between the number of HT7-positive neurons in the contralateral hippocampus and the titers of anti-tau antibodies measured in each immunized C57BL/6 wild-type mouse. We indeed found a trend towards a negative correlation (Fig.43 F). However, this correlation reached statistical significance ($P = 0.008$) only when excluding an outlier animal, which displayed a high number of HT7-positive cells in the contralateral hippocampus despite the presence of a high antibody titer.

These results show that the anti-phospho-tau vaccine reduces the propagation of the human tau protein from neuron to neuron and may thereby limit the spread of the tau pathology.

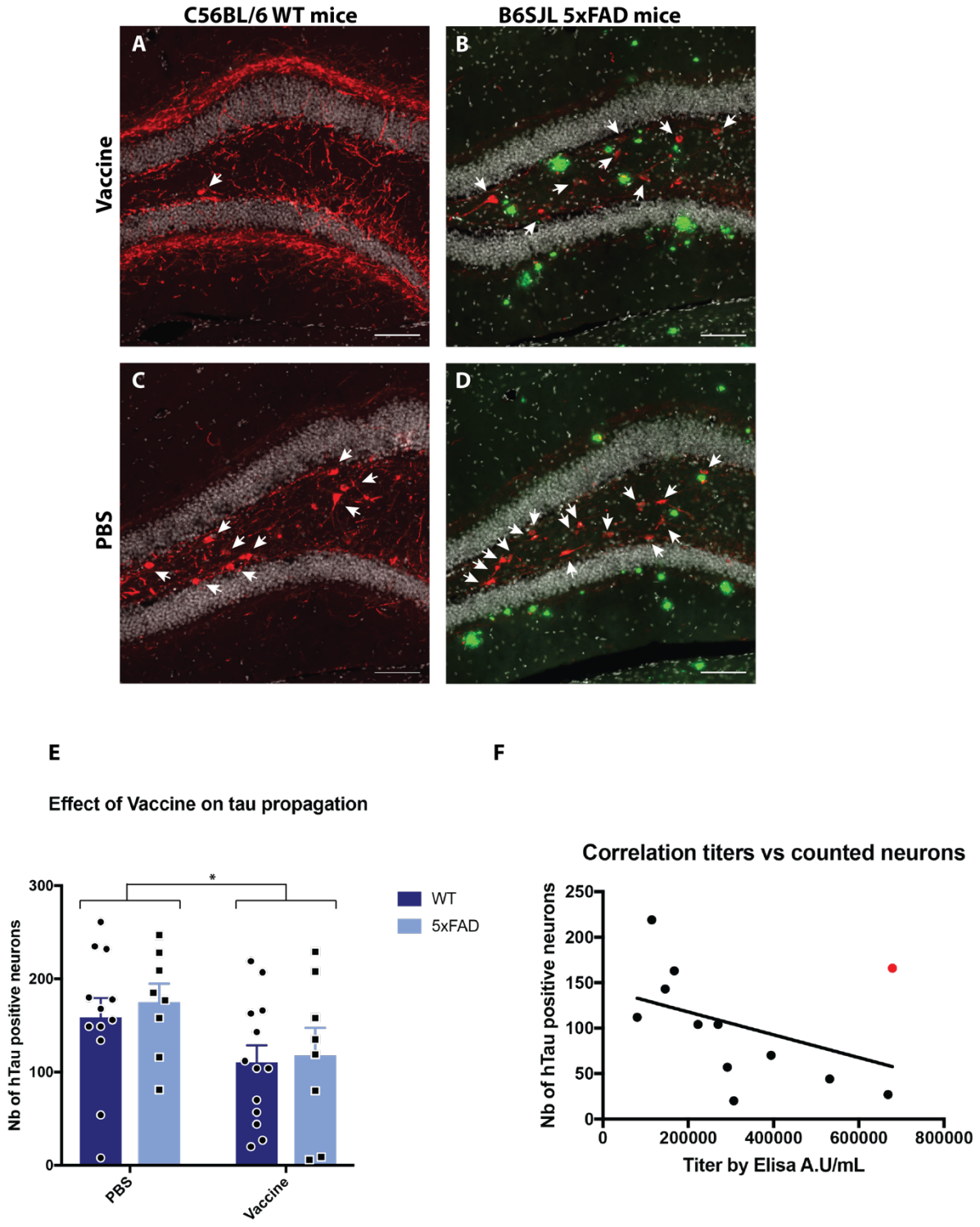



*Figure 43: The anti-phospho-tau vaccine reduces the number of neurons positive for human tau in the contralateral hippocampus*

**(A-D)** Representative pictures of the contralateral dentate gyrus stained with HT7 by immunohistochemistry (red). Note the presence of human tau-positive neuronal cell bodies (arrows). **(A)** WT mouse treated with vaccine. **(B)** 5xFAD mouse treated with vaccine. Note the presence of amyloid plaques stained with 4G8 antibody (green). **(C)** WT mouse injected with PBS. **(D)** 5xFAD mouse injected with PBS. Nuclei are stained with DAPI (grey). Scale bars A-D: 100 μm.

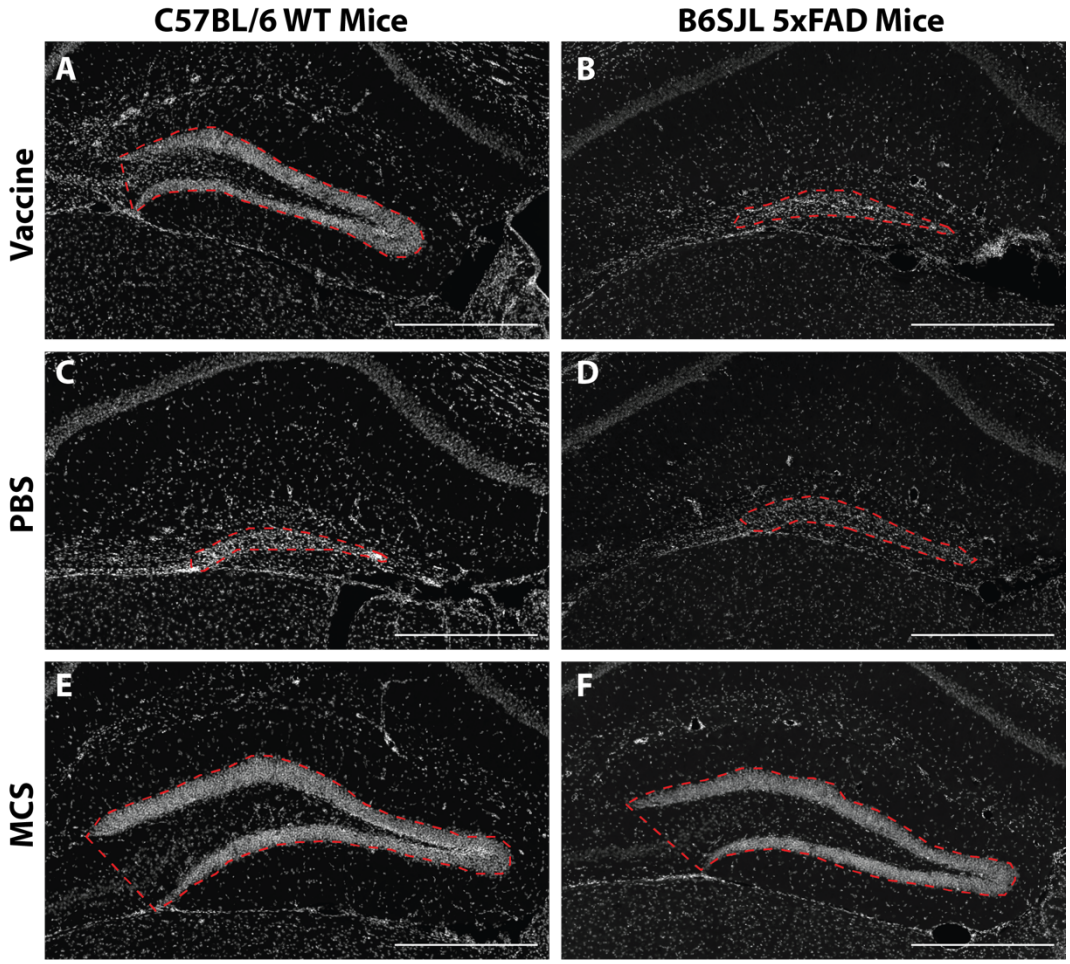
**(E)** Quantification of the total number of HT7-positive neurons in the contralateral hippocampus. Statistical analysis: two-way ANOVA, vaccine effect: *P < 0.05. WT mice N = 12-13. 5xFAD mice N = 8.
**(F)** In the WT cohort, correlative analysis between anti-phospho-tau antibody titers measured in the plasma and the number of HT7-positive neurons in the contralateral hippocampus, distal to the site of human tau overexpression. One outlier data point is indicated in red. When removing this outlier from the analysis, the correlation is significant (P = 0.008). Statistical analysis: Linear regression.

## Mice with high antibody titers show a neuroprotective effect of the vaccine against tau-induced degeneration in the dentate gyrus

Furthermore, we found that AAV-mediated overexpression of human 4R0N tau in the CA3 area of the hippocampus led to neurodegeneration in the ipsilateral hippocampus, mainly in the CA3 and dentate gyrus, observed both in the WT as well as 5xFAD mouse cohorts. In particular, human tau overexpression severely decreased the size of the hilus area and reduced the thickness of the granule cell layer in the dentate gyrus (Fig. 44 A-D). This effect of AAV-mediated tau overexpression has previously been reported in mice following local vector injection in the hippocampal CA1/2 area, and in the cortex of mice injected at neonatal stage[207,238]. Here, we sought to determine if there was any neuroprotective effects of anti-phospho-tau active immunization against neuronal degeneration.
To address this question, we measured in the brain section near the site of AAV8 vector injection the thickness of the dentate gyrus area including the granule cell layer (as outlined in red in Fig. 44 A-F) in the cohort of WT mice. We further divided the vaccinated group of the WT cohort into mice having titers lower than 250,000 A.U/mL and those having titers higher than 250,000 A.U/mL, to highlight the effect of the immune response. When treated and non-treated mice injected with the AAV8-PGK-4R0N human tau vector were compared to the group injected with the non-coding AAV8-PGK-MCS vector, we found, as expected, a significant decrease in the thickness of the dentate gyrus in mice overexpressing tau (Fig. 44 G). When the low-titer (< 250,000 A.U/mL) vaccine-treated group is compared to the control PBS-injected mice, no difference was observed. Remarkably, when the high-titer (> 250,000 A.U/mL) vaccine-treated group is compared to the PBS-injected group, we found that the thickness of the dentate gyrus in the vaccine-treated animals was significantly increased (P = 0.036) as compared to the group of PBS-injected wild-type mice (Fig. 44 G). The thickness of

the dentate gyrus was also significantly increased when the high-titer group was compared to the low-titer group (P = 0.036).

Overall these results indicate a potential role of the anti-phospho-tau vaccine in preventing hippocampal neurodegeneration caused by tau overexpression.



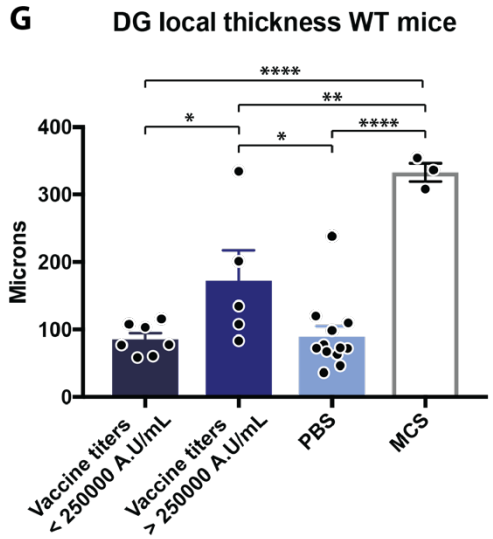



*Figure 44: Effect of the vaccine on neurodegeneration observed in the dentate gyrus near the site of AAV8-PGK-4R0N human tau vector*

**(A-F)** WT and 5xFAD mice injected with the tau-expressing vector **(A-D)** develop a severe degeneration of the dentate gyrus and an overall shrinkage of the hippocampus compared to mice injected with the non-coding vector **(E-F)**. Scale bar **(A-F)**: 500 μm.

**(G)** Analysis shows the comparison in dentate gyrus local thickness measured in vaccinated wild-type mice overexpressing human tau and with anti-phospho-tau antibody titers < 250,000 A.U./mL (N=7), in vaccinated wild-type mice overexpressing human tau and with anti-phospho-tau antibody titers > 250,000 A.U./mL (N=5), in PBS-injected mice overexpressing human tau (N=12) and in the control group injected with the non-coding vector (N=3). Statistical analysis: 1-way ANOVA with Holm-Sidak post-hoc test. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

# Discussion

Halting the propagation of pathological tau species that are capable of seeding tau aggregation in recipient neurons is one among the proposed strategies to develop disease-modifying treatments that may slow down the progression of tauopathies[194,228].
In this study we used an AAV8 model of human tau overexpression in the CA3 area of the hippocampus to quantify the propagation of tau from neuron to neuron to the heavily interconnected contralateral hippocampus. We used this model to assess if a vaccine which targets tau phosphorylated at Ser396/404 residues is able to limit human tau propagation across neuronal connections and prevent neurodegeneration.

## An AAV-based model to induce neuron to neuron transfer of the tau protein

Other animal models have been proposed to study the propagation of the tau protein and associated pathologies in the mammalian brain. These models include transgenic mice that are based on a region-specific promoter to selectively overexpress human tau in the entorhinal cortex[176], with the aim to replicate the early stage of Alzheimer's disease during which the tau pathology spreads from the entorhinal cortex to hippocampal CA1. However, some concerns have been raised about this approach, as the transgene was shown to have leaky expression in other areas of the brain, including the hippocampus[239,240]. Another model is based on the local injection of either brain homogenates containing pathogenic forms of tau derived from tauopathy patients or transgenic mice[175,182], or recombinant preformed fibrils[179]. However, these models do not faithfully reproduce the full sequence of pathogenic events, from the transfer of the tau protein occurring in physiological conditions, to the spreading of abnormal forms of tau which lead to persistent hyperphosphorylation or aggregation of the protein[240]. The injection of pathological tau fibrils most likely models already advanced stage of tauopathy, when the conversion to tau aggregates has already happened. In the model applied here, the local overexpression of tau by adeno-associated viral vector injection takes into account the early stages of tau pathology and the progressive formation of toxic oligomeric tau caused by high tau levels. This is of particular importance when studying the effects of disease-modifying treatments which may target initial conditions leading to the formation and spreading of pathological tau. By locally overexpressing the wild-

type 4R0N form of human tau, our model aims at reproducing the situation of a sporadic tauopathy, with the propagation of both physiological and pathogenic forms of tau.

To avoid any spread of AAV-mediated transduction of neurons, in particular via retrograde or anterograde infection which has been reported with some serotypes[241], we used serotype 8 AAV which has been described to have a low capacity for retrograde transport[235]. Injection of an AAV8 vector encoding GFP expression confirmed that no retrograde transport of the vector had occurred in the contralateral hippocampus. Furthermore, we used a WPRE probe to show that no evident expression of the viral mRNA is observed in the contralateral hippocampus, in contrast to the injected hippocampus. These results further indicate that the presence of the human tau detected in specific populations of neurons in the contralateral hippocampus is most likely due to the transfer of the protein from the pool of neurons locally overexpressing human tau at the site of vector injection.

Here, we show that injection of an AAV8 vector in the hippocampal CA3 induces overexpression of human tau in projections that connect both hippocampi, and thereby leads to the transfer of the human tau protein to recipient neurons. We chose to target the CA3 area of the hippocampus for overexpression of tau based on the well-described inter-hemispheric projections stemming from this region, mainly via the axonal projections of pyramidal neurons[232–234]. Human tau-positive neurons visible in the contralateral hippocampus are observed in the dentate gyrus and the CA3 areas. In the dentate gyrus, these neurons are mainly located in the granule cell layer and the hilus area, which are regions involved in memory encoding, and they have interhemispheric axonal projections towards the contralateral dentate gyrus[242–244]. Further characterization is warranted to identify the neurons that take up human tau in the hilus, especially as this region is involved in neurogenesis and memory formation.

## Parameters influencing the propagation of tau

In our study we found a trend towards an increased cell to cell transfer of tau with the 4R2N isoform compared to the 4R0N isoform. Although the difference did not reach statistical significance, this result gives an insight into the potential differences in the pathogenic contribution of the various tau isoforms. Indeed, studies show a clear difference in terms of release in the extracellular space and propensity to aggregate between 3R and 4R

isoforms[112,209,245]. In particular, 3R tau isoforms have been described to be more efficiently secreted than 4R tau isoforms. Whereas 4R isoforms have been described to have a higher propensity to aggregate, most likely because of the presence in the microtubule-binding domain of the protein of an additional repeat (R2) which is prone to convert into beta-sheet-forming structures. In addition, the 4R0N isoform has been described to be more prone to aggregation than the 4R2N isoform, as the second insert in the N-terminal domain that has an inhibitory effect on aggregation[112]. Therefore, 4R2N being less prone to aggregate might be more available for extracellular secretion and uptake in recipient neurons.

Next, when comparing 5xFAD mice to their WT littermates, we have found an increased signal for MC1 and PHF-1 by AlphaLISA, in addition to an increased propagation of tau from neuron to neuron. This finding is in line with the notion that amyloid beta pathology synergistically enhances and accelerates tau pathology[82,246–249]. Furthermore, the increase in PHF-1 signal we observed by AlphaLISA goes in line with *in vitro* findings by Frandemiche et al. that oligomeric amyloid beta induces a change in the phosphorylation pattern of tau, with an enhanced phosphorylation at the Serine 404 residue, a site that is recognized by the PHF-1 antibody[217]. In addition, another study by Dunning et al. showed that Aβ42 peptide may interact with the GSK3α kinase, which stimulates the phosphorylation of tau at the Serine 396 residue[250], which is another site recognized by the PHF-1 antibody.

Interestingly, Pooler et al. made a similar finding to ours regarding the increased propagation of tau from neuron to neuron in presence of the amyloid pathology, observed in their transgenic model[251]. Although it is unclear by which mechanism this effect might occur, it has been shown that oligomeric amyloid beta induces the translocation of tau into the synaptic compartment[217], which may facilitate transsynaptic transfer of the protein. Another contributing factor might be related to the local enhancement of neuronal activity in presence of the amyloid beta pathology[252–254], which may further enhance tau secretion, as previously reported[168,170]. In addition, it has been shown that the presence of APP at cell membranes facilitates the internalization of tau into recipient cells[172]. Therefore, APP overexpression in 5xFAD mice may further increase the propagation of tau from neuron to neuron. Further work will be needed to address the respective contribution of these different factors in tau propagation.

## Anti-phospho-tau vaccine reduces the propagation of tau to the contralateral hippocampus

For the active immunization study, we used two different cohorts of mice, either C57BL/6 wild-type or transgenic 5xFAD B6SJL mice, which differ by their strain of origin. While wild-type mice developed a strong immune response against the phospho-tau epitope, the immune response remained mild in the 5xFAD cohort. This result might be explained by the inherent ability of each of these two strains to mount an effective immune response, and highlights the importance of the immune system status for active vaccination to be successful[228].

Nevertheless, vaccine treatment successfully induces the production of antibodies specifically recognizing the phosphorylated Ser396/404 site found in pathological tau in AD, which leads to decreased PHF-1 signal intensity in both the ipsilateral and the contralateral hippocampi of wild-type mice overexpressing human tau. The decrease in the PHF-1 mean intensity signal might be due to the clearance of the recognized Ser396/404 hyperphosphorylated tau, although we cannot exclude that reduced PHF-1 immunoreactivity is due to competitive occupation of the site by endogenous antibodies raised against the vaccine epitope. However, the same effect was not observed in the 5xFAD cohort, most likely because these mice display only a mild antibody response.

The vaccine effectively decreases the number of neuronal cell bodies immunoreactive for human tau in the contralateral hippocampus. This result suggests that active immunization against Ser396/404 phospho-tau can reduce the propagation of tau. The exact mechanism by which antibodies raised following immunization decrease tau propagation is unclear, but it is more likely that these antibodies bind phosphorylated tau released in the extracellular compartment. Thereby, they may either block the uptake of extracellular tau by recipient neurons and/or promote its clearance by microglia[255]. As we do not find any difference in the microglia-specific Iba1 signal in the hippocampus of the mice treated with the anti-phospho-tau vaccine, it is unlikely that immunization leads to major changes in microglial density. However, it will be important to assess if the vaccine induces more subtle effects on the phagocytic activity of microglia, possibly contributing to the clearance of Ser396/404 phospho-tau[256].

It has been shown that hyperphosphorylation and truncation of tau promotes its secretion in the extracellular space[257–259]. Therefore, hyperphosphorylated tau species are susceptible of being recognized by antibodies when reaching the extracellular milieu. However, Plouffe et al. also showed that the pool of extracellular tau tends to be less hyperphosphorylated than the protein that resides inside the cells, suggesting that extracellular tau might be de-phosphorylated at certain sites[258]. Accordingly, although Ser396/404-phosphorylated tau has been measured in the CSF and was found to be increased in Alzheimer's patients, it remains at very low levels that are difficult to quantify[260]. This suggests that the phosphorylation state of tau might be modified when transferred from neuron to neuron. This might explain why in our model, which is based on the overexpression of a wild-type form of tau with low propensity to aggregate, recipient neurons mainly contain a non-phosphorylated form of human tau with a diffuse cytoplasmic distribution.

Another important observation in our study is that in the animals with high antibody titers, the vaccine appears to have neuroprotective effects, preventing the loss of neurons and tissue shrinkage in the dentate gyrus area. We show that degeneration in the dentate gyrus is due to the overexpression of human tau, as it is not observed following injection of a control non-coding vector at the same dose. This result is in line with the finding that the administration of another vaccine, the AADVac-1, that was in phase I (currently in phase-II clinical trial) effectively decreased the shrinkage of the hippocampus in Alzheimer's patients[261]. Hence, it will be important to determine the effects of the anti-Ser396/404 phospho-tau vaccine used in the present study on cognitive and memory decline in models of tauopathies.

To better understand the mechanisms involved with the vaccine, future work could be done to characterize the immunoglobulin response and the distribution of antibodies in the brain. In particular, it will be important to explore whether the antibodies act in the brain after crossing the blood-brain barrier, or if they clear the Ser396/404 phospho-tau through the "sink effect". In this case, antibodies bind peripheral Ser396/404 phospho-tau in the blood, possibly increasing the clearance of the protein from the brain.

Overall, we show here that our model reliably induces the transfer of human tau to recipient neurons in the contralateral hippocampus and that this transfer is quantifiable. Using this model, we show that the vaccine effectively reduces the level of Ser396/404 hyperphosphorylated tau and decreases the propagation of tau from neuron to neuron.

Thereby, this approach has some potential to prevent the spreading of pathological forms of tau that may seed the formation of pre-fibrillar and fibrillary aggregates in neurodegenerative diseases associated with cognitive decline.

# Chapter 5: Materials and methods

## Preparation of AAV plasmids, viral vectors production and titration

AAV plasmids encoding GFP, human 4R0N wild-type tau fused to EGFP, human 4R0N WT tau and human 4R2N WT tau were derived from the pAAV-PGK-MCS-WPRE backbone following standard cloning procedures.

Production and titration of the amount of vector genomes (vg) of AAV8 vectors was done as previously described[235]. Briefly, pAAV plasmids were co-transfected with the pDP8rs.gck helper plasmid in HEK-293-AAV cells (Agilent Technologies). After 48 hours, the cells were lysed and recovered viral particles were purified on iodixanol density gradients and ion exchange chromatography. Particles were concentrated by centrifugation on Amicon columns and buffer exchanged for DPBS. The number of viral vector genomes was determined by real-time PCR (qPCR). Final titers of the vectors were for AAV8-PGK-GFP: 1.18E11 vg/μL; for AAV8-PGK-4R0N htau WT – EGFP: 6.1E10 vg/μL; for AAV8-PGK-4R0N htau WT: 7.37E10 vg/μL, 2.2E10 vg/μL, 3.1E10 vg/μL, 2.03E10 vg/μL and 2.99E11 vg/μL; for AAV8-PGK-4R2N htau WT: 8.9E9 vg/μL, and for AAV8-PGK-MCS: 5.1E10 vg/μL and 5.9E10 vg/μL.

## Animals

Female WT C57BL/6 mice were purchased at Charles River and housed in opti-MICE cages (Animal Care Systems, CO) at the animal facility, with standard 12h/12h light/dark cycles and ad libitum supply of food and water. Controlled temperature and humidity conditions were maintained throughout the whole studies.

Male and female balanced groups of transgenic B6SJL Tg(APPSwFlLon,PSEN1*M146L*L286V) 5xFAD mice (6799Vas/Mmjax) and their WT littermates were bred in house. 5xFAD mice overexpress the APP and PS1 human genes, with 3 familial Alzheimer's Disease (FAD) mutations on the human APP gene (Swedish (K670N, M671L), Florida (I716V) and London (V717I) mutations) and 2 FAD mutations on the human PS1 gene (M146L and L286V)[237].

For vector injection, mice underwent surgery at the age of 11 weeks by stereotaxy. For the vaccine study, mice underwent surgery at the age of 9 weeks. Animals were accustomed for

at least 2 weeks prior to stereotactic surgery. All procedures were in compliance with the Swiss legislation and European Community Council directive (86/609/EEC) that oversees care and use of laboratory animals. Authorization for animal experimentation was delivered by the Veterinarian Office of the Canton of Vaud and approved by a local ethics committee.

## Stereotactic surgery

Mice were anesthetized with a 2:3 mix of ketamine (100 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally (i.p). Following anesthesia, the top of the skull of mice was shaven and disinfected using Betadin® (povidone-iodine) to prepare for surgery. The eyes of the mice were covered in Viscotears® liquid gel. Next, mice were placed on stereotaxic frames (David Kopf Instruments) and injected with the viral vector suspension diluted in Phosphate-Buffered Saline (PBS) at a concentration of either 1.4E10 vg/μL or 2.8E10 vg/μL, using a Hamilton syringe with a 34-gauge blunt tip needle, either in the CA3 area of the hippocampus or the lateral entorhinal cortex. Injection of 1 μL (2 μL for the experiment comparing 4R0N and 4R2N tau isoforms) of the viral vector solution was performed by an automatic injection pump (CMA Microdialysis) at a rate of 0.2 μL/min. Following the completion of the injection, the needle was left in place for 5 min before slow withdrawal from the injection site. Coordinates used to target the CA3 area of the hippocampus were: -2.1 mm (anteroposterior), -2.25 mm (lateral) and -2.25 mm (dorsoventral) from the bregma.
Coordinates used to target the entorhinal cortex were: -3 mm (anteroposterior), -3.7 mm (lateral) and -4 mm (dorsoventral) from the bregma.

## Pan-protein aggregation inhibitor compound preparation and administration

Pan-protein aggregation inhibitor compounds 1 and 2 were provided by collaborators as powders. Compounds were weighed and freshly prepared weekly as a suspension in the vehicle solution at 2 mg/mL. The vehicle solution was made of 0.5% w/v HPMC (Hydroxypropylmethylcellulose) 4000 cps, 0.5% w/v Polysorbate 80 (Tween 80) in sterile water. For the preparation of the compounds, the powders were crushed with a spatula

before suspension in the vehicle, followed by vortexing and sonication in ultrasonic bath for no longer than 2 min until homogeneous suspension was obtained. The stock of vehicle and of the solution of each compound was stored at 4°C, until administration to animals. Solutions were vortexed regularly before and during administration.

Treatment with aggregation inhibitor compounds and vehicle started 1 week after vector injection. Both compounds and vehicle were administered once daily from Monday to Thursday by oral gavage at a dose of 10 mg/kg with a 24-hour interval between administrations. On Fridays, mice were administered with a double dose of 20 mg/kg. No administrations were performed on week-ends. Mice were weighed once every week to adapt the quantity of treatment administered according to body weight. Volume of the vehicle control solution was matched to the volume of treatment.

To measure the exposure levels of the compounds at steady state, blood plasma was collected 6 weeks after the start of the treatment, 4 hours before the daily gavage, in 5 mice of the compound 1 treated group and 5 mice of the compound 2 treated group. A second blood plasma collection was done 3 days before the end of the treatment also 4 hours before the daily gavage, in 5 different mice treated with compound 1 and 5 different mice treated with compound 2.

Compounds and vehicle administration were performed for 3 months until euthanasia. On the day of euthanasia, mice received the final dose of treatment or vehicle 2 hours before sacrifice.

## Pan-protein aggregation inhibitor exposure analysis

From collected blood samples, an aliquot of 10 μL was protein precipitated with 200 μL of Tolbutamide, the mixture was well vortex-mixed and centrifuged at 13000 RPM for 10 min at 4°C. 30 μL of supernatant was then mixed with 60 μL of water, well vortex-mixed and centrifuged at 13000 RPM for 10 min at 4°C. 3 μL of the sample was used for blood exposure analysis by Liquid Chromatography – Mass Spectrometry (LC-MS) (QTRAP6500 LC-MS/MS-AK, AB Sciex Instruments).

For brain samples, brain homogenates were prepared by homogenizing brain tissue with 9 volumes (w/v) of homogenizing solution (ACN/water (1:1 w/v)). An aliquot of 20 μL sample

was protein precipitated with 400 μL of Tolbutamide, the mixture was well vortex-mixed and centrifuged at 13000 RPM for 10 min at 4°C. 30 μL of supernatant was then mixed with 60 μL of water, well vortex-mixed and centrifuged at 13000 RPM for 10 min at 4°C. 3 μL of the sample was used for brain exposure analysis by LC-MS. (QTRAP6500 LC-MS/MS-AK). All exposure analyses were performed by collaborators.

## Vaccine preparation and administration

Vaccine was provided by collaborators each day of injection, at the concentration of 400 μg/mL in a PBS solution and ready to use. A PBS solution was also provided by collaborators for the control group. Vaccine was stored at 4°C, protected from light until the moment of injection. Before injection, the vaccine was brought at room temperature and gently shaken. Vaccine or PBS control were administered in a single day by subcutaneous injection and all mice were treated with 80 μg/dose of vaccine with a maximum of 200 μL/dose. Volume injected in the PBS group was matched to the volume of the vaccine.

11-week old mice were treated 14 days after vector injection, with a total of 4 doses of either vaccine or PBS on day 0, 14, 28 and 49 after the start of the treatment. To monitor the level of antibodies over the course of the study, blood plasma was collected from all the mice at day 7 before the start of the treatment (day -7), and then on days 7, 21, 35, 42, 56 and 63 after vaccination. Animals were euthanized on day 63 after the first vaccine injection.

## ELISA for antibodies raised against the phospho-Ser396/404 tau vaccine

Serum IgG titers were determined by ELISA on plates coated with the phosphorylated Tau peptide. The 96-well plates were coated with peptides (10 μg/ml) by overnight incubation at 4°C. After washing (0.05% Tween-20 in PBS) non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in the same buffer. Subsequently, serial dilutions of mouse antisera were incubated for 2 hrs at 37°C and after extensive washing, the immune complexes

were quantified by reaction with secondary anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson Labs, USA) for 2 hrs at 37°C.

After incubation with para-nitrophenylphosphate for 1 hr at RT in the dark, the reaction was stopped and the optical density was recorded at 405 nm. All ELISA assays were performed by collaborators.

## Euthanasia, sample collection and sample processing

At the completion of each study, mice were terminally anesthetized by intraperitoneal injection of an overdose of pentobarbital (150 mg/kg). For mice in the aggregation inhibitor compounds and vaccine studies, CSF was collected by inserting an insulin syringe (BD Micro-Fine) in the CSF space of the foramen magnum. Then, following the CSF collection, the maximum amount of blood was collected by intracardial puncture.

For all the mice, we performed perfusion before brain collection. An incision in the abdominal cavity was made to access the chest cavity and the heart. Following the insertion of the perfusion needle in the left ventricle, an incision in the right atria was made. Then, mice designated for biochemical analysis of the brain were perfused with PBS and their brains collected and dissected into entorhinal cortex on the ipsilateral side of vector injection, ipsilateral hippocampus, contralateral entorhinal cortex and contralateral hippocampus. Samples were kept at -80°C until further analysis.

Mice designated for immunohistochemistry were perfused with 4% paraformaldehyde (PFA), their brains collected and post-fixed in 4% PFA overnight. The next day, the brains were placed in 25% sucrose solution and kept at 4°C until processing.

Brains were cut coronally in 25 μm-thick sections, using a cryostat (Leica CM3050S) and collected in 96-well plates containing PBS-Azide. Brain sections were then mounted on Superfrost Plus microscope slides (Thermofischer Scientific) with an interval between sections of 150 μm for the HT7 staining, or a 300 μm interval for all other stainings.

## AlphaLISA assays to characterize tau pathology

To prepare brain homogenates, frozen brains parts were resuspended in 9 volumes/weight of ice-cold homogenization buffer [25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA containing phosphatase inhibitors (30 mM NaF, 0.2 mM Na3VO4, 1 nM Okadaic acid, 1 mM PMSF, 5 mM Na4P2O7) and protease inhibitor cocktail (Complete™, Roche)] and homogenated in eppendorf tubes with a VWR pellet mixer (47747-370). Samples were then aliquoted and stored at -80°C.

For AlphaLISA assay, total human Tau as well as aggregated, misfolded and phosphorylated Tau were quantified using the following antibody pairs:

Total human Tau: HT7-Acceptor beads + biotin (BT)-Tau13-Donor beads; Multimerized human Tau: HT7-Acceptor beads + biotin (BT)-HT7-Donor beads; Misfolded human Tau: HT7-Acceptor beads + BT-MC1-Donor beads; pS202 and pT205 human Tau: HT7-Acceptor beads + BT-AT8-Donor beads; pS396 and pS404 human Tau: HT7-Acceptor beads + BT-PHF-1-Donor beads.

The Tau13 (Abcam), $MC1^{262}$ and PHF-1 (Prof. Peter Davis, Albert Einstein College of Medicine, NY, USA) antibodies were biotinylated using EZ-Link® NHS-PEO Solid Phase biotinylation kit (Thermo Scientific), while the HT7-biotin and AT8-BT were acquired from a commercial source (Thermo Scientific).

To perform the assay, the following reagents were added in a 384-well white OptiPlate (PerkinElmer): 5 µL of test diluted sample; 20 µL of the following mix: HT7-BT, MC1-BT, PHF-1-BT or AT8-BT antibodies at 1.25 nM in combination with HT7-Acc beads at 10 µg/ml and Tau13-BT antibodies at 0.6 nM in combination with HT7-Acc beads at 2.5 µg/ml

After incubation at room temperature for 1 h, 25 µL of Streptavidin Donor beads (Perkin Elmer) at 25 µg/mL were added in the dark. Plates were analyzed after 30 min incubation (RT, dark) using the EnSpire Alpha instrument and EnSpire Workstation version 3.00. In case of low total tau signal (Alpha-units lower than 2 times the level measured in control samples not expressing human tau), samples were removed from the analysis. All AlphaLISA assays were performed by collaborators.

## Measurement of total tau in CSF by GyroLab technology

2 µL of CSF was diluted in buffer F (GyroLab®, P0004825) to a total volume of 8 µl. Additionally, full-length Tau441 protein (rPeptide, T-1001-1) was used to generate a standard curve, with a dilution to 25,000 ng/ml followed by 2-fold serial dilutions. MW96 PCR plate (GyroLab®, P0004861) was prepared with 6.25 µg/ml of HT7 biotinylated ligand (Thermofisher scientific MN 1000B) diluted in buffer A (GyroLab®, P0004820), CSF samples, full-length Tau441 protein standard curve and 5 µg/ml of Tau 13 antibody (Santa Cruz, sc-21796) labelled with Alexa Fluor 674 (Pierce A20186). Finally, a GyroLab® CD Bioaffy 1000 (GyroLab®, P0004253) and the prepared MW96 PCR plates were placed in the GyroLab® Xplore instrument and a standard three step protocol from the template method list was selected. Data was saved on an excel file format. CSF analysis was performed by collaborators.

## RNAscope

Brain sections were cut at 25 µm on cryostat (Leica CM3050S) and immediately mounted on SuperFrost microscope slides and kept at -80°C until the RNAscope assay. Sections were then directly taken from -80°C and fixed for 30 min at 4°C in 4% PFA. After one PBS wash, they were dehydrated through 50%, 70% and twice 100% ethanol incubation and dried for 5 min at RT and 30 min at 60°C. After a wash in PBS, tissues were pretreated with H2O2 for 10 min at room temperature, target retrieval was performed at 95°C for 5 min and Protease III was incubated for 20 min at 40°C.

RNAscope Multiplex Fluorescent V2 assay (Bio-techne, Cat. No. 323110) was performed according to manufacturer's protocol. 25 um-thick fixed frozen sections were hybridized with the probes WPRE-O1-C2 (Bio-techne, Cat. No. 450261), positive control Mm-Ppib-C1 (Bio-techne, Cat. No. 313911) and negative control DapB (Bio-techne, Cat. No. 310043) at 40°C for 2 h and revealed with TSA Opal570 (Perkin Elmer, Cat. No. FP1488001KT). After blocking with 1 %BSA, tissues were incubated with a mouse anti-Tau-biotin antibody overnight at 4°C followed by an incubation with streptavidin HRP and revelation using TSA-Alexa488 (Thermo Fisher, T20949). Tissues were counterstained with DAPI and mounted with FluoromountG (Southern Biotech, 100.01).

The RNAscope assay was performed at the Histology Core Facility of EPFL.

# Immunohistochemistry

*Immunofluorescence staining*

The following primary antibodies were used with the indicated dilutions for immunofluorescence stainings: mouse anti-human tau Biotinylated HT7 (1:1000) (ThermoFisher Scientific, MN1000B), mouse anti-beta-amyloid 4G8 clone (1:500) (BioLegend, SIG-39220), mouse anti-hyperphosphorylated PHF-1 tau (1:1000) (phosphoSer396/404) (kindly provided by Dr. Peter Davies), rabbit anti-GFP (1:1000) (Invitrogen, G10362), rabbit anti-Iba1 (1:500) (Abcam, ab178846), rat anti-mouse CD68 (1/500) (Bio-Rad, MCA1957GA), mouse anti-GFAP (1/500) (Sigma-Aldrich, G3893).
Secondary antibodies with dilutions: Alexa Fluor 555 Tyramide SuperBoost™ kit (Invitrogen, B40933), Alexa Fluor 488 Goat Anti-Mouse IgG2b, Fcγ fragment specific (1:500) (Jackson ImmunoResearch, 115-547-187), Cy3 Goat Anti-mouse, Fcγ subclass 1 specific (1:500) (Jackson ImmunoResearch, 115-165-205), Alexa Fluor 488 goat anti-rabbit (1:500), Alexa Fluor 647 Donkey anti-rabbit (1:250) (ThermoFisher Scientific, A-31573), Cy3 Donkey anti-rat (1:1000) (Jackson ImmunoResearch, 712-165-153), Alexa Fluor 488 Donkey anti-mouse (1:500) (Invitrogen, A21202).

Mounted sections on slides were washed with PBS. Then, slides for 4G8 staining were treated for 30 min with formic acid 70%. Slides for HT7 and PHF-1 stainings were treated with an antigen retrieval procedure with citrate buffer at pH 6. Following those initial steps, slides were washed 3 times in PBS for 10 min, then incubated in blocking solution for 2 hrs at room temperature. The blocking solution contained serum (depending on the secondary antibody used, either 10% normal goat serum or 5% normal goat serum and 5% normal donkey serum) and 0.1% Triton X in PBS. Following the incubation in blocking solution, the corresponding primary antibodies diluted in blocking solution were applied and left for incubation overnight at 4°C. Subsequently, the slides were washed 3 times for 10 min with PBS. Then, for HT7 staining, slides were incubated for 1h in horseradish peroxidase (HRP) - conjugated streptavidin, then washed again in PBS as previously described, followed by amplification with the tyramide signal amplification kit with a revelation time of 10 min.

Afterwards, slides for HT7 staining and slides for other stainings without the amplification step were incubated for 2 hrs at RT with the corresponding secondary antibody diluted in PBS. Slides were then washed again with PBS 1x, counterstained with DAPI staining (1:1000) and covered with cover slips in Mowiol mounting medium.

*DAB staining*

The following primary antibodies with dilutions were used for DAB stainings: mouse anti-PHF conformation-specific MC1 (1:500) (kindly provided by Dr. Peter Davies), mouse anti-hyperphosphorylated PHF-1 tau (1:1000) (phosphoSer396/404) (kindly provided by Dr. Peter Davies), mouse anti-hyperphosphorylated AT8 tau biotinylated (phosphoSer202/Thr205) (1:500) (ThermoFisher Scientific, MN1020B).
Secondary antibody used with dilution: Biotinylated Goat anti-mouse IgG (1:200) (Vector Laboratories, BA-9200).

Mounted sections on slides were first washed in PBS. Next, slides were incubated at 37°C for 30 min in 0.1% phenylhydrazine to quench endogenous peroxidase activity. Following this initial step, slides were washed 3 times in PBS for 10 min, and incubated in blocking solution for 2 hrs at RT. The blocking solution was made of 10% normal goat serum and 0.1% Triton-X-100 in PBS. Following incubation in blocking solution, the corresponding primary antibodies diluted in blocking solution were applied and left for incubation overnight at 4°C. Afterwards, the slides were washed 3 times for 10 min with PBS, incubated with the secondary antibody diluted in PBS for 2 hrs at RT. After washing with PBS, the slides were incubated with the ABC solution (Vector Laboratories, PK-6100) for 30 min at RT. The ABC solution was freshly prepared 30 min prior to incubation with the slides by mixing 10 μL of solution A with 10 μL of solution B per 980 μL of PBS. After washing with PBS, we performed the DAB revelation with the DAB substrate (ThermoFisher Scientific, 34065). Slides were then washed again with PBS, and covered with cover slips in Eukitt mounting medium.

## CLARITY

After perfusion with 4% PFA, brains were collected and post-fixed in 4% PFA at 4°C for 24 hrs. Then, after another wash in PBS, brains were immersed in a bis-free hydrogel solution with 4% bis-free acrylamide and 0.25% VA-044 polymerization thermal initiator in water for 24 hrs at 4°C. Afterwards, for the polymerization step, brains were incubated in hydrogel solution at 37°C for 3 hrs in an oxygen-free condition. Samples were then washed and kept in PBS at 4°C until clearing step. For the clearing step, we used the X-Clarity machine (Logos Biosystems) to clear the brains in an SDS clearing solution (0.2 M boric acid and 4% SDS in water). Parameters for clarification were set as 37°C for temperature, 100 RPM for pump speed and 1.2 A for the current. Brains were cleared for up to 24 hrs, with regular checks for optimum clearing. After this procedure, brains were washed for 24 hrs in 0.1% Triton X-100 and PBS, and kept at 4°C in PBS until imaging.

For imaging, samples were soaked in the Histodenz (Sigma-Aldrich, D2158) imaging solution for 5 hrs. Imaging was performed using a custom-built lightsheet microscope, similar to the one described by Tomer et al[263]. Images were acquired with a 4x objective and were reconstructed using the IMARIS software, with a voxel size of 1.4 μm x 1.4 μm x 5 μm. Clearing and imaging were performed at the Microscopy Facility at the Wyss Center for bio- and neuroengineering in Geneva.

## Image analysis

Slides with MC1, AT8 and PHF-1 DAB stainings were scanned with the Olympus Slide scanner VS120-L100, using a 10x objective. Following segmentation of regions of interest in three brain sections around the site of vector injection, the mean intensity signal corrected to background was measured using Fiji Software.

In the study on aggregation inhibitor compounds, slides for Iba1, CD68 and GFAP fluorescent stainings were scanned with the Olympus Slide scanner VS120-L100, using a 10x objective. Following segmentation of regions of interest in three brain sections around the site of vector injection, automated threshold masks were applied using the Default (for Iba1) and the Triangle (for CD68 and GFAP) thresholding algorithms on Fiji Software to determine the area coverage of each fluorescence signal.

Slides for HT7 positive neuronal quantification were manually counted using an Olympus AX70 microscope under a 20x objective. Neuronal cell bodies were counted in the hippocampus contralateral to the site of vector injection on coronal brain sections covering the entire hippocampus from anterior to posterior, with an interval of 150 μm between sections.

Slides for PHF-1-labelled fluorescent staining were scanned with the Olympus Slide scanner VS120-L100, using a 40x objective. Following segmentation of the regions of interest in three brain sections around the site of vector injection, the mean intensity signal corrected to fluorescence background was measured using Fiji Software.

Slides for Iba1 fluorescent staining for the vaccine study were scanned with the Olympus Slide scanner VS120-L100, using a 10x objective. Following segmentation of regions of interest in three brain sections around the site of vector injection, automated threshold masks were applied using the Otsu (for the C57BL/6 mice cohort) and the Default (for 5xFAD mice cohort) thresholding algorithms on Fiji Software to determine the area coverage of the Iba1 fluorescence signal.

To determine the thickness of the dentate gyrus in the vaccine study, we used the DAPI channel of the images captured with the Olympus Slide scanner VS120-L100, using 10x objective. Following segmentation of the region of interest in one brain section per animal close to the site of vector injection, we measured the thickness of the dentate gyrus using the local thickness feature of Fiji Software.

## Statistical analysis

With the exception of 3-way ANOVA, all statistical assays were performed using GraphPad Prism version 7 (GraphPad Software, CA, USA). Three-way ANOVA was performed using the software Statistica (Statsoft). All data are presented as mean ± SEM. Alpha level of significance was set at 0.05. The statistical tests used in the different studies are indicated in the legend of the figures.

# Chapter 6: General Discussion

The abnormal intraneuronal deposition of the protein tau is a common pathological feature of several debilitating diseases, generalized as tauopathies, which cause a wide spectrum of clinical symptoms, spanning from dementia to motor impairments. Tau-related neurodegenerative diseases including Alzheimer's Disease, Frontotemporal Dementia or Chronic Traumatic Encephalopathy have high prevalence and hence represent a global challenge to society. In particular, they pose a threat to development considering the current ageing of population. Patients and their relatives alike are both affected and powerlessly face the consequences of the debilitating symptoms of these dreadful diseases that do not have any curative treatment. These facts highlight the urgency of understanding the pathophysiology of tauopathies in order to develop therapeutic strategies that are able to slow the progression of the disease, but also to develop biomarkers and diagnostic tools to better detect and manage those diseases.

Different tauopathies give us valuable information on the pathophysiology of tau protein. We saw the example of three tauopathies: Alzheimer's Disease, the class of frontotemporal dementias caused by tau, such as FTDP-17 and chronic traumatic encephalopathy. From Alzheimer's disease, we learned that the progression of the tau neurofibrillary tangles is correlated with cognitive decline and that the presence of another pathological protein, amyloid beta peptide, can synergistically enhance its pathology. With FTDP-17, we learned that a mutation of tau is sufficient to induce and drive tau pathology, resulting in cognitive impairment and other symptoms. Last, with chronic traumatic encephalopathy, we learned that an external injury can trigger tau pathology, the deleterious effects of which progressively appear over time.

Understanding the biology of the formation of pathologic tau species that lead to the deposition of tau aggregates and their propagation to synaptically connected regions is key to identify targets for therapeutic agents. The initial step of the pathogenic cascade involves an unusual pattern of posttranslational modifications of the tau protein. Acetylation, ubiquitination, truncation, nitration, glycosylation or most notably phosphorylation are among the many post-translational modifications that tau can undergo. Some of these modifications, such as hyperphosphorylation at certain sites, are thought to change molecular

interactions in the tau protein that render it more prone to form beta-sheets and interact with other tau proteins through the hexapeptide motifs present near the R2 and R3 repeats in the microtubule-binding domain. This process prompts the transition from tau monomers into dimers, oligomers and insoluble multimers to form paired helical filaments or straight filaments, which are at the core of the pathophysiology of tauopathies. Although the role of these molecular species in tauopathies remains unclear, it is believed that soluble pathological oligomeric tau might be an important neurotoxic actor, able to propagate from neuron to neuron and seed the pathology in recipient cells. Therefore, halting the aggregation of tau and blocking the propagation of pathogenic tau seeds are two promising approaches for therapeutic intervention, on which the present thesis work is focused.

In the first part of the thesis, we explored the approach of blocking the aggregation of tau by determining the efficacy of two pan protein aggregation inhibitor compounds in a mouse model based on AAV8-mediated overexpression of human 4R0N tau WT in the entorhinal cortex, a region which is described as the initial location where neurofibrillary tangles start to appear in Alzheimer's Disease. Although, we could not highlight an effect of the compounds on decreasing tau multimers, most likely because of the mild pathology induced in the model, we discovered some interesting insights on possible mechanisms of action of the aggregation inhibitor compounds. We showed with both immunohistochemical and biochemical analyses that one of those compounds had an effect on the phosphorylation state of tau, specifically at the Ser202/Thr205 sites recognized by the AT8 antibody. This hyperphosphorylation was also detected in the distal synaptically connected hippocampus. In addition, we discovered that the density of microglia was increased in the hippocampus, while there was a trend towards an increase in the entorhinal cortex. Lastly, we could detect the presence of human tau in the mouse CSF of the mice overexpressing tau, similar to the increased presence of the tau protein in the CSF of AD patients. But more interestingly, the levels of total tau in the CSF of the mice treated with aggregation inhibitor compounds were decreased, suggesting that the compounds had an effect on the release of tau and/or its clearance.

Overall, these findings indicate that the treatment compounds may interact with tau in the central nervous system and induce a change in the conformation of the protein that leads to phosphorylation at the Ser202/Thr205 residues. The most evident effects of the treatment are seen at the level of microglial cells which appear to be more activated in the treated mice,

possibly contributing via clearance mechanisms to the decreased level of tau in the CSF. In AD, the increase in total tau in the CSF is an indicator of the pathology. Therefore, the decrease of tau in the CSF observed with the compounds could highlight an effect of the treatment in decreasing tau pathology.

In the second part of this thesis, we explored the therapeutic approach of interfering on the neuron to neuron propagation of tau with a liposomal based vaccine against the hyperphosphorylated Ser396/404 sites of tau in a mouse model overexpressing 4R0N human tau WT following injection of an AAV8 vector in the hippocampus.

The vaccine was tested in a cohort of C57BL/6 wild-type mice and another cohort of 5xFAD B6SJL mice to more closely model the pathological condition of AD, by inducing the progressive accumulation of amyloid beta plaques. Our first finding showed that the antibody response against the epitope of the vaccine was different between both cohorts. Indeed, the cohort of wild-type mice had a robust response against the vaccine with high titers of antibodies measured by ELISA in the plasma. Whereas in the 5xFAD mice cohort, the immune response was only mild. This difference in immune response might be due to the inherent characteristics of the strains of mice, or could be possibly linked to the expression of mutated Presenilin 1 in the 5xFAD mice, which has been reported to affect the immune response[264]. Nevertheless, this difference in the ability of the immune system to mount a response against an antigen reminds us that this might also occur in patients, in particular in elderly people whose immune system's ability to respond is decreased[265]. Therefore, future clinical trials should take this factor into account when applying vaccine regimens.

Next, we observed that the antibodies raised against the vaccine were indeed recognizing the hyperphosphorylated Ser396/404 tau, as assessed by the decrease in the signal by the PHF-1 antibody. Although it remains unclear whether the decrease in the hyperphosphorylated Ser396/404 tau immunoreactivity is actually due to the clearance of the protein, we found out that the vaccine was decreasing the propagation of human tau to the contralateral hippocampus. This result indicates that the vaccine could decrease the progression of tau pathology by limiting the propagation of PHF-1 hyperphosphorylated tau, which is notoriously associated to the tau pathology in Alzheimer's Disease.

Encouragingly, our last finding shows that the vaccine has a dose-dependent effect on local neurodegeneration induced by the overexpression of human tau in the mouse hippocampus.

## Importance of the animal model for efficacy studies of treatments

The two studies of this work highlight the importance and the challenges of developing animal models well adapted to the scientific question at hand. Here, to target the initial step of tau aggregation, we established a model based on wild-type tau overexpression using AAV vectors injected in the entorhinal cortex. The wild-type form of tau was chosen to model the case of a progressive sporadic tauopathy. Hence, the degree of pathology observed in our model corresponds to the initial stages of tau pathology. The question that we addressed using our model was "what are the effects of the aggregation inhibitor compounds in a sporadic model of tauopathy?". We showed the effects of the compounds on tau hyperphosphorylation, on microglial activation and the clearance of tau from the CSF. However, we could not answer the question of whether these compounds can prevent the formation of tau aggregates at later stages of the disease. To answer that question, an aggressive model of tau pathology that leads to the rapid formation of tau aggregates might be more appropriate, for example by overexpressing the mutated P301S form of tau associated to frontotemporal dementia due to FTDP-17, a familial tauopathy[207,209,266].

As for the vaccine study, we used a model of wild-type tau overexpressed by AAV following unilateral injection in the CA3 area of the hippocampus, again to model the case of a progressive sporadic tauopathy that reflects the slow progression of the disease, starting with the tau protein being overexpressed, then accumulating in the neuron and then undergoing changes that lead to soluble pathological forms of tau, favoring their subsequent propagation to other neurons. Whereas a more aggressive model of tauopathy that leads to rapid formation of aggregates might jeopardize the subtle pathological transformations of tau before the formation of insoluble aggregates, which may not have the same propensity to transfer from neuron to neuron. The question we wanted to address was "what are the effects of the vaccine on tau propagation and on the level of PHF-1 hyperphosphorylated tau?". Therefore, our model of unilateral tau overexpression by AAV8 in the hippocampus to assess the transfer of tau to neurons in the contralateral hippocampus allowed us to answer that question.

## Importance of understanding the mechanisms of action of treatments

By using the models described earlier, we showed that the treatments tested in this work had a beneficial effect on some features of tau pathology that are found in patients with tauopathies, typically the level of total human tau in the CSF with the aggregation inhibitor compounds, and the decrease in PHF-1 immunoreactivity, with a rescue effect on neurodegeneration of the dentate gyrus in the hippocampus observed with the vaccine. However, as promising those observations can be for the perspective of using these treatments in the clinic, further work needs to be done to understand how these therapeutic agents may exert therapeutic effects.

In the case of vaccines for example, it will be important to explore how antibodies produced in the periphery interact with the phosphorylated Serine 396/404 epitope present on human tau expressed in diseased neuronal cells within the central nervous system. Indeed, we can envision three possibilities: (1) antibodies could bind the epitope in the peripheral blood circulation without crossing the blood-brain barrier, favoring the clearance of tau through a "sink effect"[267]; (2) blood plasma antibodies have been shown to cross the blood-brain barrier at rate of 0.1% of penetration and bind to the epitope when tau is in the extracellular space[268,269]; and (3) antibodies can be internalized inside neurons and bind to the epitope inside the cell[270–273].

In the case of the aggregation inhibitors, one intriguing observation was the increase in the phosphorylation of the Ser202/Thr205 residues on tau recognized by the AT8 antibody. It would be important to understand how mechanistically the compounds act on tau to result in this particular state of phosphorylation and also to explore whether other residues are phosphorylated in addition to the Ser202/205, which would give us valuable information on a possible conformation of tau that would inhibit the propensity to aggregate. This is a complicated question to answer, because tau has at least 85 identified sites of phosphorylation and different kinases have been identified to phosphorylate particular sites. The role of phosphorylation is not fully understood and studies suggest that depending on which residue is phosphorylated, it can either promote or inhibit tau aggregation. In our case, we hypothesized that the compounds might interact with tau in a pre-aggregated form, possibly through the exposed beta-sheet prone hexapeptides in R2 and R3 repeats following extensive post-translational modifications[274]. Then, the interaction compound-tau might

affect the conformation of tau, which could in turn expose a specific combination of residues to kinases that phosphorylate the protein at those specific residues that might inhibit aggregation. Therefore, further effort is needed to characterize the combination of phosphorylated residues induced by the compounds, which leads to the inhibition of aggregation, eventually leading to the clearance from the CSF.

## Perspectives for the translation of treatments into the clinic

Even though the exact mechanisms of action are not fully presented here, we showed that the *in vivo* results of these treatments on certain pathological features of tau are promising. However, their effect on cognitive impairment and symptoms of tauopathies remain to be evaluated in patients. So far, therapeutical approaches against AD have focused on amyloid beta pathology and the vast majority failed in clinical trials[275,276]. Therapy against tau seems to be more promising because of the correlation between the progression of tau pathology and the progression of the symptoms in tauopathies, but it is evident that the mechanisms leading from tau monomers to tau aggregates capable of seeding the pathology are complex. Several post-translational modifications have been associated with the formation of such toxic species, therefore targeting only hyperphosphorylation or only truncation might not have clinical benefits, as tau aggregates can be formed through other mechanisms. We believe that the formation of tau aggregates that have toxic effects on the neuron and the mechanism of tau propagation that spreads the pathology to further brain regions are two of the most important targets for therapies, as they specifically address the core of the pathogenesis of tau pathology. One could even imagine a combination of those two approaches, where pharmacological treatments could be used to inhibit tau aggregation or even disaggregate fibrils into smaller, more soluble tau species, that might subsequently be secreted and captured by the antibodies raised against a specific epitope of tau. Such a combination of treatments may be used to decrease the intraneuronal load of tau while preventing tau propagation.

Therapies against tau are often developed with AD as the main disease to cure. However, tauopathies are not limited to AD and it remains to be seen in the clinic if a particular treatment against tau can be used in a generic way for all tauopathies. Aggregation of tau is

the common pathological hallmark used to define tauopathies. Therefore, treatments inhibiting aggregation could be used on principle for most tauopathies, whether tau pathology is caused either by a pro-aggregant mutation or by amyloid beta deposition in the case of AD.

However, in the case of the vaccine treatment, it might be preferable to select a vaccine epitope that is specific for a particular type of tauopathy. In this work, the targeted epitope was the phosphorylated Ser396/404 residues on tau, often found in tau aggregates in AD and which has been reported to be phosphorylated in other tauopathies, often confirmed by PHF-1 immunoreactivity. However, in the case of the R406W tau mutation associated to FTDP-17, tau has been described to have a decreased immunoreactivity with the PHF-1 marker, possibly through a mechanism of conformational change that prevents the access of Ser396/404 residue by kinases[277]. Therefore, the efficacy of the vaccine might not be optimal in that particular case, highlighting the importance of selecting the right epitope when targeting tauopathies.

In AD cases, the presence of the amyloid pathology should be taken into account for a therapy against tau pathology, as both pathologies have been described to synergistically interact. If amyloid beta pathology is considered to be the trigger of tau pathology, acting only on tau might therefore not be sufficient to halt disease progression. It would be promising to combine an anti-amyloid beta therapy with an anti-tau therapy to approach both aspects of AD physiopathology. The pan-protein aggregation inhibitor would therefore be an ideal drug candidate to address AD, as it might target the aggregation of both the amyloid beta peptide and the tau protein.

Furthermore, it is also important to determine at which stage of tau pathology each of these treatments should be applied to optimize therapeutic efficacy. Indeed, it is believed that one of the reasons why anti-amyloid beta immunotherapies have failed is because the stage of AD at which the therapy was applied was too advanced[275,278]. This question should also be raised for the tau pathology. In AD, the pathology is already well advanced before symptoms appear. Therefore, it is important to determine whether an anti-tau vaccine may have beneficial effects as a disease-modifying treatment provided after AD diagnosis, or if vaccination against AD should be administered as a preventive measure. In addition, the difficulty in AD is that the pathology is progressive and symptoms appear when it is already

well-developed. Therefore any curative treatment would be administered in already advanced stages of the disease. Whereas in chronic traumatic encephalopathy, the starting time point is quite often known, especially if it is a single traumatic brain injury. Therefore, it might be easier for therapeutic agents to be administered as soon as a head trauma occurs, limiting the long-term effects of dementia. Future management plans of traumatic brain injury cases could include a prophylactic tau immunotherapy as part of the protocol of treatment, in addition to other preventive therapies following head trauma, such as anti-seizures medications and antibiotics[279,280].

## Translation to other proteinopathies

Lastly, the understanding of the pathophysiology of tauopathies and the understanding of the mechanisms of action of treatments could be useful for other neurodegenerative diseases that involve an abnormal aggregation and seeding of a pathological protein, such as alpha-synuclein for Parkinson's Disease or huntingtin for Huntington's Disease[224,281]. Findings in the understanding of the mechanisms of one disease could be useful in the understanding of other pathological protein related disorders. Because of the common feature of cell-to-cell propagation of these pathological proteins, a similar approach of immunotherapy could be used to block the propagation of the pathological seeds. For example, immunotherapy against alpha-synuclein has already been tested in mouse models of synucleopathy and showed promising results in terms of reduction of alpha-synuclein in distal synaptically connected regions[282,283]. Therefore, understanding the biology of these pathogenic proteins, and elucidating the mechanisms of action of potential therapeutic approaches to specifically address these pathologies, are major efforts that are needed to devise effective disease-modifying treatments against neurodegenerative diseases.

# Chapter 7: Conclusion

Research and development of therapies against tauopathies is rapidly growing and we live in a very interesting time in this field. Rapid progress has been made by understanding the mechanisms of tau pathology, developing new animal models of tauopathies, and identifying new biomarkers and candidate therapeutic approaches. A few of those new potential therapies have already entered clinical trials in patients and are currently ongoing[191]. Although several challenges are standing in front of the scientific community who faced various drawbacks in drug development, notably with the anti-amyloid beta immunotherapies, it is important to stay on course and learn from past failures to improve future drugs. In 2013, health ministers from the G8 countries pledged to find a cure against dementia by 2025. Whether, this objective is feasible or not is speculative, however, should a drug be approved for market by 2025, then this drug is most likely already enrolled in one of the ongoing clinical trials. In the meantime, based on the guidelines of the World Health Organization, it is important to provide the appropriate care to dementia patients, by having national plans against dementia, by raising awareness in the population, by reducing the risk factors of dementia, by improving the diagnostic tools to correctly diagnose dementia and by providing appropriate support and information to the families and relatives of dementia patients.

# Chapter 8: References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# List of abbreviations

| | |
|---|---|
| AAV | Adeno-associated virus |
| APP | Amyloid Precursor Protein |
| bvFTD | Behavioural variant of FTD (see FTD) |
| CBD | Corticobasal Degeneration |
| CHIP | carboxyterminal of HSP70-interacting protein (see HSP) |
| CSF | Cerebrospinal Fluid |
| CTE | Chronic Traumatic Encephalopathy |
| DG | Dentate Gyrus |
| DSM | Diagnostic and Statistical Manual of mental disorder |
| EC | Entorhinal Cortex |
| FAD | Familial Alzheimer's Disease |
| FTD | Frontotemporal Dementia |
| FTD MND | FTD Motoneuron Disease variant |
| FTDP-17 | Frontotemporal Dementia and Parkinsonism linked to chromosome 17 |
| FTLD | Frontotemporal Lobar Degeneration |
| FUS | Fused in Sarcoma |
| GFP | Green Fluorescence Protein |
| HIV | Human Immunodeficiency Virus |
| HPC | Hippocampus |
| HSP | Heat Shock Protein |
| hTau | Human Tau |
| ICD | International statistical classification of diseases and related health problems |
| IHC | Immunohistochemistry |
| LBD | Lewy Body Dementia |

| | |
|---|---|
| LMTM | leuco-methylthioninium bis-hydromethanesulfonate |
| MCI | Mild Cognitive Impairment |
| MCS | Multiple Cloning Site |
| MMSE | Mini Mental State Exam |
| MoCA | Montreal Cognitive Assessment test |
| MT | Microtubule |
| MTBD | Microtubule Binding Domain |
| NFT | Neurofibrillary Tangle |
| PART | Primary Age-Related Tauopathy |
| PET | Positron Emission Tomography |
| PNFA | Progressive Non-Fluent Aphasia |
| PRD | Proline Rich Domain |
| Psen1 | Presenilin 1 |
| Psen 2 | Presenilin 2 |
| PSP | Progressive Supranuclear Palsy |
| PTM | Post-Translational Modification |
| SD | Semantic Dementia |
| TBI | Traumatic Brain Injury |
| TDP-43 | Transactive Response DNA-binding Protein-43 |
| UN | United Nations |
| WHO | World Health Organization |
| WT | Wild-type |

# Curriculum Vitae

**Last name:** Nazeeruddin
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**Education:**

| Study | School | From | To | Elective courses | Mentions |
|---|---|---|---|---|---|
| MD-PhD Doctoral Studies | EPFL<br>Brain Mind Institute Neurodegenerative Studies Laboratory<br><br>Thesis directors:<br>Bernard Schneider<br>Patrick Aebischer | November 2013 | Date | | |
| Medical School | Faculty of Biology and Medicine, University of Lausanne | September 2007 | July 2013 | - genes and vision (3rd year)<br><br>- Clinical neuroscience (4th year) | |
| "Ecole de maturité" (High School) | Gymnase cantonal du Bugnon, site de Sévelin | August 2004 | July 2007 | Section Mathematics and Physics Complementary course: Chemistry. | Total Points: 47.5/54 |
| Junior High School (7th to 9th) | Collège intercommunal de la Planta, Chavannes-près-Renens | August 2001 | July 2004 | Mathematics and Physics. | Total Points: 65/66 |

**Diplomas:**

- Swiss Federal Diploma of Medicine, 2013
- Master of Medicine, 2013
- Bachelor of Medicine, 2010
- Certificate of "Maturité Fédérale" (High school), 2007
- Junior High School Certificate, 2004

**Professional Experience:**

- Teaching Assistant for bachelor and master students in the school of Life Sciences at EPFL from February 2014 to 2016

- Member of the First Aid Team in EPFL and teacher of the first aid course for new collaborators in EPFL from 2015 to 2018

- Clinical internship in Internal medicine, Hôpital Riviera, Vevey, Switzerland, may 1st to june 30th 2012

- Clinical internship in Ophthalmology, Mount Sinai Medical Center, New York City, United States of America, August 1st to August 30th 2012

- Clinical internship in General Surgery, Mount Sinai Surgical Services, New York, United States of America, September 1st to September 30th 2012

- Clinical internship in Neurosurgery, Mount Sinai Medical Center, New York City, United States of America, October 1st to October 29th 2012

- Clinical internship in Neurosurgery, Lausanne University Hospital (CHUV), Switzerland, November 1st to December 31st 2012.

- Clinical internship in Crisis Psychiatric Emergency Department, Geneva University Hospital (HUG), January 1st to February 1st 2013

- Clinical internship in Neurosurgery, University College of London Hospital, London, United Kingdom, March 1st to March 30th 2013

**Representation and Association Activities:**

- President of the Life Sciences PhD students Association (Association des Doctorants en Science de la Vie, ADSV), 2016 – 2018
- PhD student's representative in the doctoral school of neuroscience (EDNE) at EPFL, 2016 – to date
- PhD representative in the Life Sciences (SV) Faculty Council in EPFL, 2016 – 2018
- President of the class of the Third Year of Master of Medicine, 2012-2013
- Class Representative of the Second Year of Master of Medicine, 2011-2012
- Class Representative of the First Year of Master of Medicine, 2010-2011

- Member of the Faculty Council of the Faculty of Biology and Medicine of the University of Lausanne, September 2009 to July 2011.

**Other activities:**

- Private Pilot Licence (PPL-A) passed on 17.11.2016
- Service member in the Swiss Civil Defence (Protection Civile) as "Collaborateur d'Etat Major" specialised in crisis/disaster management

**Languages:**

- French: Mother tongue
- English: fluent, written and oral (First Certificate in English of the University of Cambridge (grade A), 2007)
- German: good knowledge from school (written and oral)
- Urdu: fluent in oral only
- Spanish: Beginner (Level A1-2)

**Publications:**

Wojciech Bobela, **Sameer Nazeeruddin**, Graham Knott, Patrick Aebischer, Bernard L. Schneider; "Modulating the catalytic activity of AMPK has neuroprotective effects against α- synuclein toxicity"; Molecular Neurodegeneration. 2017

**S. Nazeeruddin et al.** , "Maltraitance infantile: l'école est-elle un "radar" sans faille?" (Child Abuse: is school a sensible screening tool?), Primary Care 2011; 11; n°4, p.56-57. (https://doi.org/10.4414/pc-d.2011.08796) (Publication as co-first author)

**Master in Medicine Research Project:**

"Doublecortin cells and neurodegenerative diseases", Department of Neurosurgery, CHUV, under the supervision of Dr. Jocelyne Bloch, MD and Dr. Jean-François Brunet, PhD

**Awards:**

- Leonhard Euler Award for best results in higher level mathematics, 2007
- High school class Award for best results of the class, 2007
- English Award for best results in English in High School, 2007
- Excellence Award for best results in Junior High School, 2004
- Junior High School Class Award for best results of the class, 2004

**Career plan:**

- MD-PhD thesis from 2013 to 2019
- General Surgery from 2019 to 2020
- Neurosurgery Residency from 2020

**Interests:**

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