

SYNAPTIC TAU PATHOLOGY IN ALZHEIMER'S DISEASE

ÉRIKA SÁNCHEZ ACED

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DOCTORAL THESIS

SYNAPTIC TAU PATHOLOGY IN

ALZHEIMER'S DISEASE

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NEUROSCIENCE PROGRAM

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Certify:

That the work **“Synaptic tau pathology in Alzheimer’s Disease”**, presented by Érika Sánchez Aced to qualify for Doctor in Neuroscience for the Universitat Autònoma de Barcelona has been done under our direction and meets all the requirements to be presented and defended in the presence of the corresponding Thesis Committee.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APOE ϵ4	allele ϵ 4 of apolipoprotein E
APP	Amyloid Precursor Protein
AT	Array Tomography
Aβ	Amyloid beta
BA	Broadmann area
BA17	Primary visual cortex
BA20/21	Temporal inferior cortex
BBN	Brain bank number
CFP	Cyan fluorescent protein
CSF	Cerebrospinal fluid
DNA-PAINT	DNA-based Points Accumulation for Imaging in Nanoscale Topography
dSTORM	Direct STORM
EM	Electron microscopy
ETC	Electron transport chain
EtOH	Ethanol
FRET	Förster Resonance Energy Transfer
GABA	γ -aminobutyric acid
GFAP	Glial fibrillary acidic protein
IHC	Immunohistochemistry
LOAD	Late-onset AD
LR	London Resin
MAPT	Microtubule associated protein tau
MCI	Mild cognitive impairment
NFTs	Neurofibrillary tangles

NMDA	N-methyl-D-aspartic acid
PALM	Photoactivated Localisation Microscopy
PBS	Phosphate-buffered saline
PET	Positron emission tomography
PMI	Post-mortem interval
PSD	Postsynaptic density
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PTMs	Post-translational modifications
ROIs	Regions of interest
ROS	Reactive oxygen species
SIM	Structured Illumination Microscopy
SMLM	Single molecule localisation microscopy
SRM	Super resolution microscopy
STED	Stimulated Emission Depletion
STORM	Stochastic Optical Reconstruction Microscopy
TBS	Tris buffered saline
TSA	Tau seeding activity
YFP	Yellow fluorescent protein

ABSTRACT

Alzheimer's Disease (AD) is neuropathologically characterised by the deposition of amyloid- β peptides in extracellular amyloid plaques, intracellular aggregates of pathological tau that form neurofibrillary tangles, and progressive synaptic loss. Growing evidence suggests that tau oligomers, small and soluble tau species, appear to be more toxic, disrupting synaptic function and impairing memory in animal models. However, data on synaptic tau in human brain are scarce.

This doctoral thesis focuses on the study of oligomeric tau at human synapses using high-resolution microscopy techniques. We demonstrate the presence of pathological forms of tau at both pre- and postsynaptic compartments of the synapse. In these compartments, oligomeric tau was consistently the most abundant form accumulated at the synapse, supporting the idea that this feature is an early event in AD.

By combining Array Tomography with direct Stochastic Optical Reconstruction Microscopy, we established a highly sensitive methodology to resolve individual synapses and study synaptic tau pathology at nanoscale resolution. Moreover, we observe a gradient of oligomeric tau from pre- to postsynaptic compartments, consistent with an anterograde trans-synaptic spread.

Furthermore, this thesis explores the correlation between tau seeding activity and synaptic oligomeric tau accumulation. This association suggests that these species may not only accumulate at synapses but may also contribute to the propagation of tau pathology.

Therefore, targeting synaptic oligomeric tau could be important not only to reduce local synaptic toxicity, but also to potentially limit tau spread and slow clinical progression.

The data presented in this thesis points at synaptic oligomeric tau as a potential culprit in the spread of tau pathology through the AD brain, highlighting the importance of developing new tools to study synaptic pathology.

RESUM

La malaltia d'Alzheimer (MA) es caracteritza neuropatològicament en l'acumulació de pèptids amiloide- β en plaques amiloides extracel·lulars, agregats intracel·lulars de tau patològica que formen cabdells neurofibril·lars, i una pèrdua progressiva de sinapsis. Cada vegada hi ha més evidència que els oligòmers de tau, espècie petites i solubles, semblen ser directament tòxics, alterant la funció sinàptica i deteriorant la memòria en models animals. Tanmateix, les dades sobre la tau sinàptica en cervell humà són escasses.

Aquesta tesi doctoral es centra en l'estudi de la tau oligomèrica a les sinapsis humanes mitjançant tècniques de microscòpia d'alta resolució. Hem demostrat la presència de formes patològiques de tau tant als compartiments pre- com postsinàptics de la sinapsis. En aquests compartiments, la tau oligomèrica ha resultat ser consistentment la forma més abundant acumulada a la sinapsi, donant suport a l'idea que aquest fet és un esdeveniment primerenc en la MA.

Mitjançant la combinació d'Array Tomography amb direct Stochastic Optical Reconstruction Microscopy, hem establert una metodologia altament sensible per resoldre sinapsis individuals i per estudiar la patologia sinàptica de tau amb resolució nanomètrica. A més, hem observat un gradient de tau oligomèrica des dels compartiments pre- cap als postsinàptics, coherent amb una propagació trans-sinàptica anterògrada.

A més, a aquesta tesi s'ha explorat la correlació entre l'activitat de tau seeding i l'acumulació d'oligòmers de tau a la sinapsi. Aquesta associació suggereix que aquestes espècies no només poden acumular-se a les sinapsis, sinó que també podrien contribuir a la propagació de la patologia tau.

Per tant, desenvolupar estratègies terapèutiques contra els oligòmers de tau sinàptics podria ser important no només per reduir la toxicitat sinàptica local,

sinó també per limitar potencialment la propagació de la tau i alentir la progressió clínica.

Les dades presentades a aquesta tesi apunten a la tau oligomèrica sinàptica com una possible medidora de la propagació de la patologia de tau a través de cervells amb MA, destacant la importància de desenvolupar noves eines per estudiar la patologia sinàptica.

CHAPTER 1 | INTRODUCTION

1.1 ALZHEIMER'S DISEASE

1.1.1 DEMENTIA AND PREVALENCE

Dementia is a clinical syndrome characterised by progressive decline in cognitive functions that is sufficient to interfere with independence in everyday life activities (1). The World Health Organization has reported that there are currently 57 million people living with dementia and, as the adult population grows, it is estimated that the number will exceed 150 million by 2050. The advanced phase of Alzheimer's disease (AD) is the most common underlying cause of dementia, accounting for 60–80% of dementia cases (2).

1.1.2 AD DEFINITION AND IMPACT

AD is a devastating progressive neurodegenerative disorder that manifests through memory loss and deterioration of cognitive functions. However, previously to the onset of symptoms, AD is believed to initialize in the brain at least two decades before (3), with a long prodromal state known as mild cognitive impairment (MCI). At first, the brain is able to overcome the pathological changes, but as the disease progresses, individuals start to lose cognitive functions. In addition, it can be manifested with behavioural changes, difficulty with speech, disorientation and impairment on the motor system, all leading to premature death (4). Typical symptoms include difficulties in remembering recent events, conversations or names. Apathy and depression are often early symptoms and may be followed by communication problems, mood swings, insomnia, confusion and impaired judgment. In the late stages, individuals may develop difficulty walking, speaking and swallowing (5).

In the European Union, the economic burden of dementia (the majority of which is AD), was more than 350 000 million euros in 2019, representing about

2.8% of the gross domestic product of the region, which reflects the enormous economic impact of the disease (6). Most of the care is performed by informal caregivers (often family members and friends), who bear a significant emotional, psychological and financial burden, increasing the average household expenditure by up to 30%. These data emphasise the urgent need for health policies that prioritise early detection, preventive intervention, and financial support for patients and caregivers to mitigate the growing global burden of disease (7).

1.1.3 NEUROPATHOLOGICAL HALLMARKS

A definitive diagnosis of AD ultimately relies on the neuropathological verification of its characteristic brain lesions. At the macroscopic level, the AD brain typically shows widespread shrinkage and cortical atrophy, reflecting the progressive neuronal degeneration and loss (Figure 1A). At the microscopic level (as assessed by light microscopy), two main hallmark lesions define the disease: extracellular accumulation of amyloid- β ($A\beta$) peptides forming the core of amyloid plaques, and intracellular aggregates of tau (often hyperphosphorylated and proteolytically processed) that form neurofibrillary tangles (NFTs) (Figure 1B) (8).

According to Braak *et al.*, tau pathology in AD first appears in the trans entorhinal and entorhinal cortex and later spreads through anatomically connected areas. Following the hippocampus and in the later stages to the neocortical areas (9). Whereas $A\beta$ deposition begins in the neocortex and subsequently extends to allocortical, subcortical, brainstem and cerebellar regions (10).

In 1991, Braak and Braak described six stages of neurofibrillary pathology. Specifically, stages I and II correspond to tau pathology predominantly affecting the trans entorhinal region; stages III and IV indicate involvement of

limbic regions such as the hippocampus; and stages V and VI reflect extensive neocortical involvement.

In addition, A β pathology is commonly classified according to the phases described by Thal *et al.* 2002, following a hierarchical sequence. In phase 1, A β deposits are found in the neocortex; in phase 2 includes allocortical regions; phase 3 involves the striatum and diencephalic nuclei; phase 4 includes several brain stem nuclei; and phase 5 is characterised by cerebellar A β deposition.

These lesions were first described by Alois Alzheimer in 1906 and remain central to pathological diagnostic criteria nowadays (4,11). Additional associated features include: dystrophic neurites (abnormal neuronal processes characterised by aberrant sprouting and dystrophic expansion that form neuritic plaques, commonly found surrounding amyloid plaques (12)), tau-positive neuropil threads (fragmented and tortuous tau-positive processes, that weave between cell bodies in the neuropil, outside of the soma, frequently dendrites with tau-positive spines (13)) and prominent neuroinflammatory response: characterised by activated microglia and reactive astrocytes (8,14).

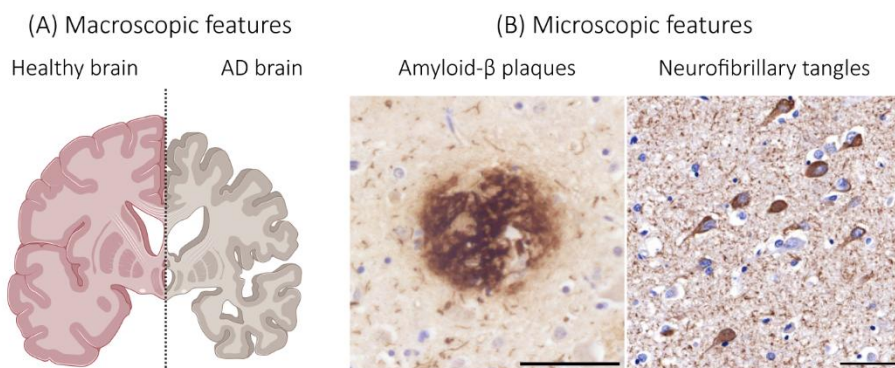


Figure 1. Neuropathological hallmarks of AD. (A) Macroscopic features, the AD brain is characterised by cortical atrophy due to the progressive loss of neurons. (B) Microscopic features: Amyloid plaques detected with anti A β x-40 and Neurofibrillary tangles detected with AT8 antibody. Scale bar is 100 μ m. Created with BioRender. Images from Sant Pau Memory Unit Lab.

Although A β plaques and NFTs are widely regarded as the two main pathological hallmarks of AD, several studies have shown that synapse density correlates more strongly with the degree of cognitive decline observed in patients than either plaque or tangle burden alone (15). This has led to the view that synapse loss may represent a third major hallmark of the disease, as it more directly reflects the functional consequences of neuronal degeneration underlying the cognitive impairment. Moreover, synapses are considered a plausible site at which toxic A β - and tau-related species may converge, ultimately leading to synaptic dysfunction and loss (16,17).

These neuropathological changes can also be detected *in vivo* through cerebrospinal fluid (CSF) biomarkers. In AD, reduced CSF A β 42 or A β 42/A β 40 ratio reflects cerebral amyloid deposition, while increased phosphorylated tau markers (such as p181 and p217) are associated with tau pathology (18). Moreover, synaptic changes that precede neurodegeneration can also be detected in CSF of patients at preclinical stages of AD (19).

1.1.4 SPORADIC AD

The majority of AD cases are sporadic (90-95%), and usually present after age 65, corresponding to late-onset AD (LOAD). In contrast, a small proportion of the cases occur before the age of 65 and are caused by autosomal dominant pathogenic variants in 3 genes: amyloid beta precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*). Although these familial forms are rare, their study has been determinant to understand the pathophysiological basis of AD.

The primary causes of sporadic AD remain incompletely understood, and the disease is considered a complex multifactorial disorder (20), that is highly heterogeneous in terms of age at onset, cognitive decline, clinical presentation and disease progression (2).

Age is the greatest risk factor for developing AD, with risk exponentially doubling after every five years from age 65 onward (21). Almost 32% of people aged 85 or older have AD (22). While age is the strongest risk factor for LOAD, sex also appears to modulate disease susceptibility and progression.

Women are more likely to develop AD, partly due to their longer life expectancy; however, other effects such as steroid hormones could have a role in this biological risk factor (23). Interestingly, for similar amyloid burden, women accumulate more tau and show more pronounced cognitive decline than men, suggesting a female-biased vulnerability in tau related pathways (21).

Although no single mutation accounts for sporadic AD, genetic factors have a substantial contribution to disease risk. Twin studies report that the hidden risk of developing AD is estimated to be 58% genetically determined at the population level (24). Almost a quarter of this heritability is explained by the allele $\epsilon 4$ of apolipoprotein E (APOE $\epsilon 4$). Individuals that carry a single $\epsilon 4$ allele have a greater risk of having AD by 3- to 4-fold, while APOE $\epsilon 4$ homozygotes have a 10- to 15-fold greater risk compared to individuals who are homozygous for the common $\epsilon 3$ allele (25). Moreover, APOE $\epsilon 4$ is also associated with younger age onset (26). Additional to APOE $\epsilon 4$, other risk factors have been identified in large genome wide association studies (27,28).

While age and genetic factors are non-modifiable, up to 45% of dementia cases could potentially be prevented or delayed by addressing 14 modifiable risk factors across the life course (29), including depression, smoking, excessive alcohol intake, obesity, diabetes, physical inactivity, untreated hearing and vision loss, among others.

1.1.5 CURRENT THERAPEUTIC APPROACHES

Over 100 years have passed since the first reported case of AD and there is still no drug capable of halting the disease. Until now, the only available option was to treat the symptoms, mainly with cholinesterase inhibitors, memantine and their combination (30), which showed modest symptomatic benefit on cognitive and behavioural measures (31).

In 2025, there were 138 drugs being tested in 182 clinical trials in the AD pipeline, with agents targeting 15 different disease processes related to the AD pathophysiology (32). Of these, 30 drugs target A β pathology and 15 target tau-related pathology, representing a 21.74% and 10.87% of the pipeline respectively.

These efforts have already led to the first disease modifying therapies for AD. In the United States, Europe and other countries, the anti-amyloid monoclonal antibodies lecanemab and donanemab have been approved for early AD. These therapies reduce amyloid burden by promoting microglia-mediated clearance. They slow cognitive decline by about 27% (lecanemab) and up to 35% (donanemab), rather than restoring the already lost cognitive function (30).

This highlights the increasing need to continue identifying additional therapeutic targets that could complement the current and future available treatments. In this context, tau pathology has re-emerged as a particularly relevant target, as amyloid clearance alone does not reverse the downstream consequences. Accordingly, several novel tau-targeted therapeutic strategies are being tested, including antisense oligonucleotides, monoclonal antibodies and gene therapies, which aim to reduce pathological tau levels or block tau propagation (33).

1.2 TAU PATHOLOGY

1.2.1 TAU GENE AND PROTEIN

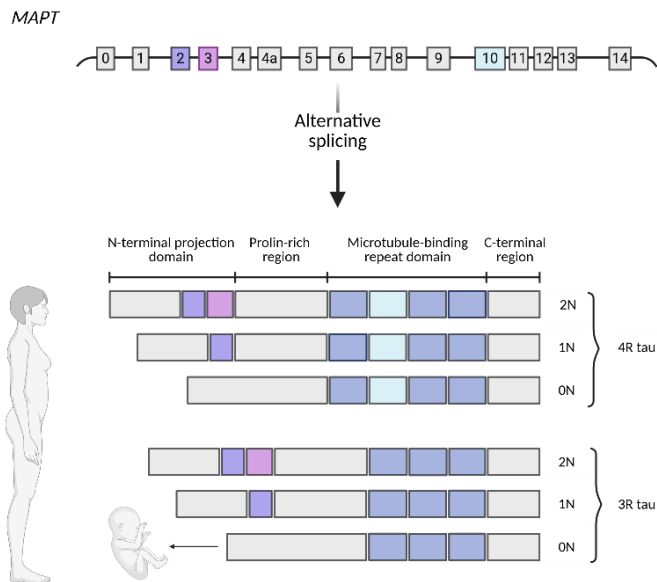
Tau protein is encoded by the microtubule associated protein tau (*MAPT*) gene. Tau was first described by Weingarten *et al.* in 1975 (34), and almost ten years later Neve *et al.* mapped the *MAPT* gene to chromosome 17 (35). Since then, extensive research has focused on understanding the role of tau. Tau is a microtubule-binding protein mainly present in neurons but is also present in glial cells in lower quantity (36). It has an intrinsically disordered nature and has little tendency for aggregation (37). The primary structure of tau can be divided into four regions: N-terminal projection domain, a proline-rich region, microtubule binding repeat domain, and a C-terminal domain (38).

The *MAPT* gene can be differentially expressed in the human body in over 30 predicted isoforms through alternative splicing of several exons, including exons 2, 3, 4a, 6, 8 and 10 (39). Although in the brain only six major isoforms, determined by exons 2, 3 and 10, are expressed (40). These isoforms vary depending on the number of N-terminal inserts (zero, one or two) and by the inclusion of exon 10, which encodes the second microtubule-binding repeat (R2), therefore generating either three-repeat (3R) or four-repeat (4R) tau isoforms (Figure 2A).

This structural heterogeneity contributes to the functional diversity of tau, since not all isoforms function in the same way. The N-terminal composition can influence interaction with other proteins and subcellular distribution (41), while the difference between 3R and 4R affects their ability to bind to microtubules (42). Furthermore, expression is regulated during development. In the human foetal brain, the form without N-terminal inserts and three-repeats (0N3R) predominates, while in the adult brain, the 3R and 4R forms are found in an equimolar ratio (Figure 2A). Alterations in this ratio are

characteristic of tau-associated diseases known as primary tauopathies, meanwhile secondary tauopathies are disorders in which tau accumulates together with other pathological proteins. These diseases may be predominantly associated with 3R tau, 4R tau or both, and they differ by the tau aggregation pattern (Figure 2B).

(A) Tau brain isoforms



(B) Classification of tauopathies

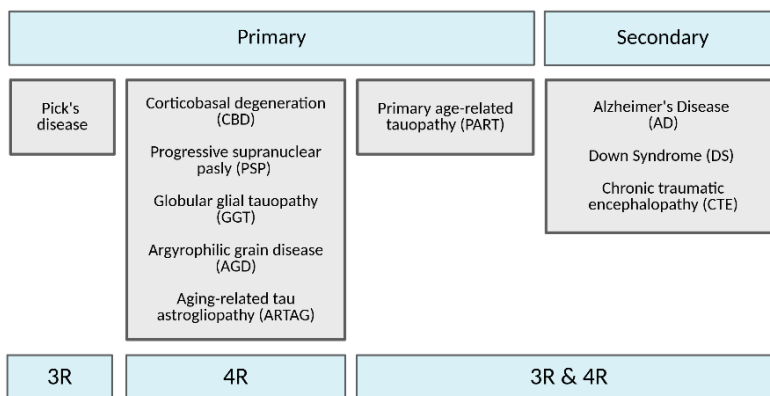


Figure 2. Alternative splicing of the human *MAPT* gene. (A) The six brain specific tau isoforms, based on the alternative splicing of exons 2 (violet), 3 (pink) and 10 (blue) (40,43). (B) Classification of tauopathies (44). Created with BioRender.

1.2.2 PHYSIOLOGICAL ROLES OF TAU

Under physiological conditions, tau is known for its role as a microtubule-associated protein involved in maintaining cytoskeletal integrity (38). Tau promotes tubulin assembly and contributes to the stabilization of microtubules, while also contributing to their dynamics, cell polarity and axonal transport (41,45). Moreover, tau is involved in the reduction of catastrophes (sudden microtubule disintegration) (46), supporting neurite outgrowth and stability of neuritic microtubules (38,41).

Beyond its cytoskeletal role, tau also participates in several other cellular processes. Tau interacts with the cell membrane during growth processes (41), contributes to synaptic organization (43) and to pre-synaptic vesicle dynamics (47). Furthermore, tau binds and protects DNA, regulates gene expression by interacting with calmodulin (48), and may contribute to neuronal DNA damage repair response (49) (Figure 3A).

1.2.3 POST-TRANSLATIONAL MODIFICATIONS OF TAU IN AD

Many of the properties and functions of tau can be explained because it is a natively unfolded protein (50). This means that tau can adopt multiple forms, which facilitates its interaction with a wide range of proteins. Moreover, proteins can undergo post-translational modifications (PTMs), resulting in different proteoforms (51). PTMs can occur either immediately after translation by ribosomes or later, once folding and localisation have taken place (38).

PTMs are responsible for fine-tuning the conformation and function of proteins, and are crucial for the physiological regulation of tau, since they are responsible for regulating the affinity of tau for microtubules.

Although PTMs are necessary for the physiological roles of tau, under pathological conditions (Figure 3B), PTMs can also lead to loss of affinity to the microtubules, increasing the concentration of soluble tau in the cytosol, facilitating its aberrant self-assembly (52,53) and altering the interaction of tau with degradation and clearance machinery (54).

Phosphorylation is the most extensively studied PTM of tau. In 1985, Brion *et al.* described that anti tau antibodies showed NFTs, where hyperphosphorylated tau was a major component (55). Subsequently and independent work reported that in the AD brain tau was abnormally phosphorylated compared with controls (56). Indeed, tau phosphorylation plays a central role in the neuropathological evaluation of AD. For instance, Braak staging is commonly evaluated using the AT8 antibody, which recognizes tau phosphorylated at Ser202 and Thr205, two sites that are associated with AD-related tau pathology (57).

Moreover, in the longest tau isoform (2N4R), there are almost 85 potential phosphorylation sites, which are mostly accessible owing to the unfolded native structure of tau (58). This means that almost 20% of tau has the potential of become phosphorylated.

A molecule of tau, under physiological conditions can contain 2 or 3 molecules of phosphate; however, in the AD brain this ratio can be duplicated or even triplicated (56). This abnormal presence of hyperphosphorylated tau provoke detrimental consequences for neurons, not only by destabilization of microtubules, but impairment of neurite outgrowth, deregulation of axonal transport (59), synaptic disorganization (60) and inducing of tau self-aggregation (61).

Proteolysis is also a major PTM of tau, where several cytosolic proteases are responsible for tau proteolysis. Although tau cleavage may initially start as a

protective mechanism of the neuron to promote the removal of abnormal tau, together with abnormal phosphorylation it can promote conformational changes that ultimately leads to the accumulation of cleaved and hyperphosphorylated tau (62,63). These species are major components of NFTs in the AD brain (38,64).

In addition to phosphorylation and proteolysis, there has been several other PTMs implicated in tau pathology in AD (53,54), such as acetylation (65), ubiquitination (63) and glycosylation (66).

Overall, tau PTMs act in an interconnected manner, shaping whether tau preserves its physiological functions or shifts towards a pathological state, promoting the formation of toxic oligomers and insoluble filaments that ultimately lead to NFTs.

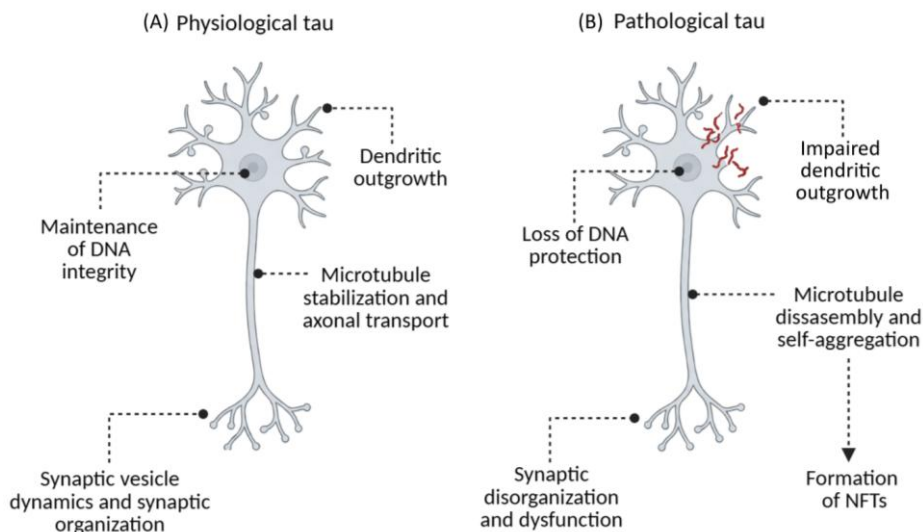


Figure 3. Physiological and pathological role of tau. (A) Tau is implicated in microtubule stabilization and dynamics, axonal transport, dendritic outgrowth, maintenance of DNA integrity, as well as involved in synaptic organization and synaptic vesicle dynamics. (B) When tau is hyperphosphorylated and truncated it dissociates from microtubules, impairs neurite outgrowth and axonal transport, provokes synaptic disorganization and induces tau self-aggregation, ultimately leading to sequestration of pathological species into NFTs. Created with BioRender.

1.2.4 OLIGOMERIC TAU

For a long time, the scientific community focused on understanding how NFTs affected neuronal cells and caused neurodegeneration. However, a series of studies in the late 1990s and early 2000s showed that, NFTs correlate with neuronal loss, but do not fully account for it, suggesting that tangles do not explain all the neurodegeneration observed in AD (67,68). In fact, neuronal loss and cognitive decline may precede NFT formation (69,70), and neurons containing NFTs are able to survive for years or even decades (71), which challenges the idea that NFTs causes imminent neuronal death.

In line with this notion, studies in AD and tauopathy models have shown that the toxicity associated with tau dysregulation can occur in the absence of NFTs. In the model studied by Santacruz *et al.*, cognitive and synaptic impairment could be reversed even though NFT accumulation continued (72). In human tau transgenic mice, neurons containing NFTs do not show markers of cell death, indicating that neuronal degeneration may occur independently of this lesion (73). These studies suggest that tau-induced toxicity may occur prior to, or independently of, NFT formation, pointing to the possibility that intermediate tau species may be responsible of neurotoxicity.

Tau oligomers are multimeric complexes formed by two or more tau molecules (74). After becoming phosphorylated and dissociating from microtubules, tau mislocalize from the axon and form bioactive oligomers that may eventually deposit as fibrils. They are highly relevant because they are the intermediate stage between physiological tau and terminal fibrillar deposits. Importantly, to determine whether oligomers were not merely transient aggregation intermediates but rather pathological species with their own biological properties, it was necessary to develop antibodies capable of recognise with high specificity these tau species (75).

In transgenic models, soluble tau species correlate with neuronal and synaptic dysfunction (76). Moreover, in 2011, Kaye's group reported that injection of tau oligomers into the hippocampus of wild-type mice caused memory impairment and promoted the misfolding and endogenous aggregation of tau (77). These findings were followed by several studies supporting the idea that oligomeric tau species, rather than NFTs, are the main drivers of neurotoxicity and neuronal death (78–80). In addition, a recent study showed that high-molecular-weight oligomers alone were sufficient to cause a severe and reversible phenotype, in the absence of fibrillar tau (81). Taken together, the literature converges on the view that oligomers are the most toxic and bioactive tau species, and those most closely linked to the causal mechanism of neuronal damage.

Tau oligomers not only act as templates to induce the misfolding of native tau, but also have the capacity to propagate from affected regions to others that were initially unaffected (82). In fact, NFTs have been proposed as a cellular sequestration or neutralization response to these pathological species, aimed at reducing their interaction with physiological tau limiting the propagation of tau aggregates from cell to cell (83,84).

1.2.5 TAU SEEDING AND PROPAGATION

Proteopathic forms of tau have the capacity to self-aggregate and to induce template misfolding in naïve tau (85), a process known as seeding. This process seems to begin in the entorhinal cortex and continue to spread throughout the brain via anatomically connected regions in a hierarchical manner (86). Tau has been shown to be physiologically secreted by neurons in an activity-dependent manner (87). However, several mechanisms have been proposed for the spread of pathological tau (88). Among them, spreading via synaptically connected neurons seems to be one of the most plausible, since

pathological tau appears to be more localised to synapses compared to non-pathological tau in animal models (89).

In order to evaluate and further quantify tau seeding activity (TSA), Diamond's laboratory designed tau biosensor cells. These cells are a stable cell line that expresses the tau repeat domain with the disease-associated P301S mutation, conjugated to either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) (90). In basal conditions, the tau reporter proteins remain in a stable soluble form within the cell. When exposed to exogenous tau seeds, the tau reporter proteins aggregate and upon excitation of CFP, they generate Förster Resonance Energy Transfer (FRET) signal that can be detected either by flow cytometry or imaging techniques (Figure 4).

This system has enabled further characterization of TSA in extracts derived from both animal models and human brains. Using this biosensor cells, TSA has emerged as a potential molecular driver of heterogeneity among patients, allowing a deeper characterization of AD cases (90–92). However, the reasons underlying the different degrees of TSA observed in AD patients and how does the seeds propagate through the brain remains unknown.

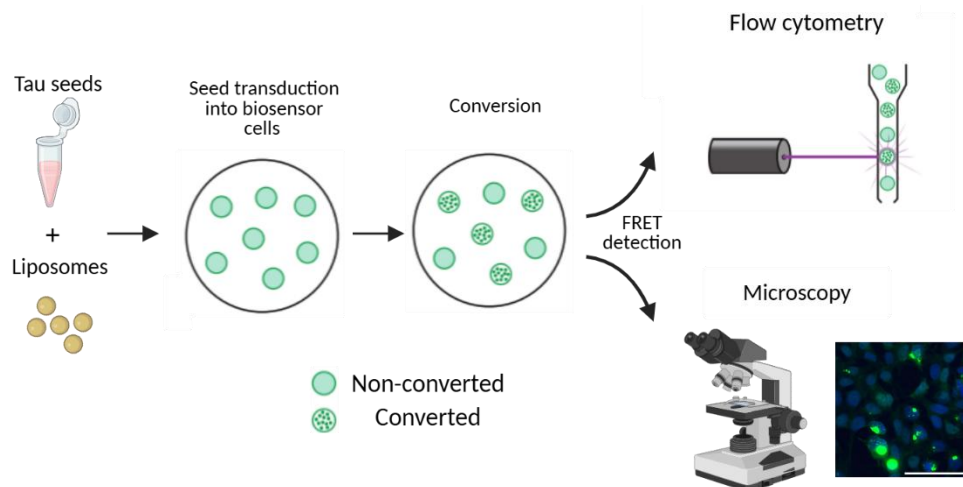


Figure 4. Schematic model of the tau biosensor cells assay workflow Using both flow cytometry and microscopy to detect FRET signal. Scale bar is 50 μm . Adapted from Holmes *et al.* (2014) (90). Created with BioRender.

1.3 SYNAPSES IN HEALTH AND PATHOLOGY

1.3.1 THE SYNAPSE

The brain is one of the most complex organs in the human body, and part of that complexity is owed to the synapse. Synapses are specialised connections between neurons where neuronal communication occurs, essential to brain function (93). It has been estimated that one neuronal cell can form up to 1,000 - 15,000 synapses, and that the human brain contains approximately 86 billion neurons (94,95), which means that average human brain contains over a trillion synaptic connections.

A single synapse is formed by pre and postsynaptic compartments that are separated by the synaptic cleft, which is approximately 20-40 nm wide (96,97). Both compartments are extremely specialised for their roles. The presynaptic compartment contains all the required machinery that is necessary for the generation and release of presynaptic vesicles containing neurotransmitters in response to action potentials. In contrast, the postsynaptic compartment contains receptors to bind neurotransmitters and induce downstream signalling cascades in response (Figure 5).

Synaptic diversity is determined, in part, by the type of neurotransmitter released and the repertory of postsynaptic receptors. For instance, excitatory synapses are typically glutamatergic and signal through α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors, whereas inhibitory synapses use mainly γ -aminobutyric acid (GABA) and signal through GABA receptors (98).

Beyond their structural diversity, synapses are highly dynamic structures. They can change their number, structure and function depending on the demands of the brain activity (99), a property known as synaptic plasticity. Through this process, neurons can strengthen or weaken synaptic contacts, which is

essential to proper wiring and maintenance of brain circuits, as well as for learning and memory (100).

Synaptic function is also modulated by astrocytes, the principal glial cells in the brain. Astrocytes respond to synaptic activity, and respond modulating synaptic transmission and plasticity, forming the tripartite synapse (101). This highly specialised and dynamic organisation makes synapses essential for normal brain function, but also particularly vulnerable to pathological processes.

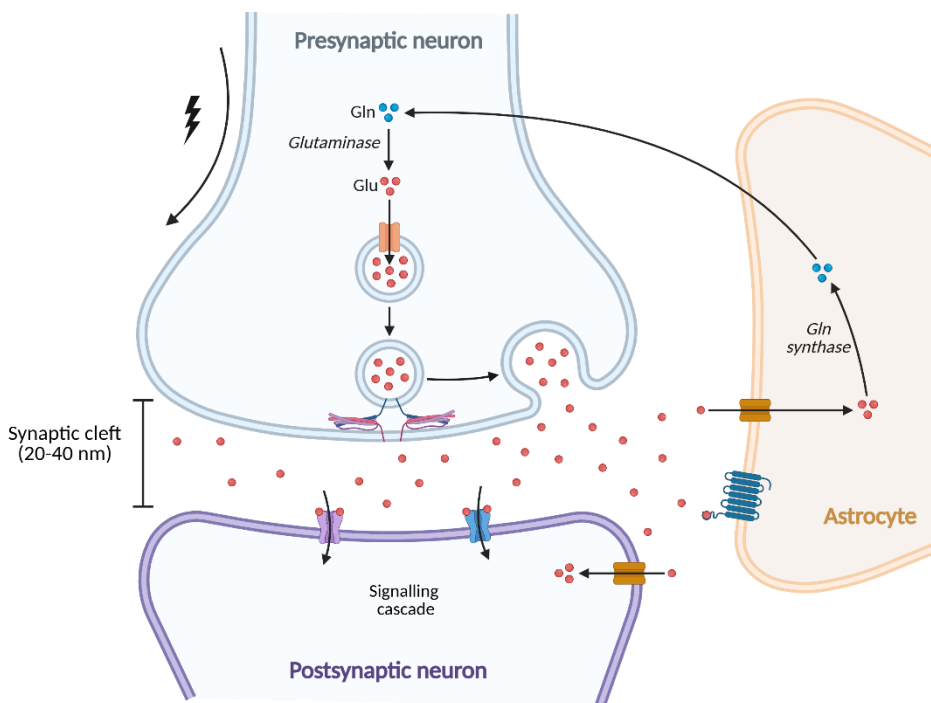


Figure 5. The architecture of an excitatory synapse. Formed by pre and postsynaptic compartments that are separated by the synaptic cleft. When the action potential arrives to the presynaptic membrane, the synaptic vesicles are released into the synaptic cleft. Postsynaptic receptors receive the neurotransmitters and start a signalling cascade. Astrocytes clear synaptic glutamate and convert it into glutamine, which can then be recycled by neurons. Created with BioRender.

1.3.2 CHALLENGES OF STUDYING HUMAN SYNAPSES

For several years, technical limitations slowed down the post-mortem characterisation of synaptic nanoarchitecture and dynamics. Because synapses are very small structures and display remarkable complexity and molecular density, conventional imaging techniques did not allow their accurate visualisation (96) .

The Abbe diffraction limit defines the size of the spot on which a light beam can be focused. This limit depends on the wavelength, the refractive index of the medium and the numerical aperture of the objective, meaning that two spots closer than 250 nm apart cannot be resolved as separate structures using conventional microscopy techniques (102,103).

Conventional light microscopy enabled the study of synaptic markers (Figure 6), such as synaptophysin, an integral protein of the synaptic vesicle (104,105). However, it lacked the spatial resolution required to define the molecular organisation of individual synapses. In order to visualise the organisational structure of synapses, the resolving power of light microscopy would need to be improved by at least a factor of 10 (106).

Another approach often used to study synapses is the biochemical isolation of synapse-enriched fractions from fresh frozen brain tissue, such as synaptosomes and synaptoneurosomes (107,108). Once synapses have been isolated, methods can be used to analyse the synaptic protein composition (19,109). While these methods provide crucial information on molecular composition they do so at the cost of losing the anatomical and tissue context, since the sample is homogenised prior to analysis.

For this reason, for many years the main technique used to study synaptic ultrastructure was electron microscopy (EM). Using a controlled beam of

electrons, instead of photons of light, the electron microscope breaks the light diffraction in the 2-dimensional planes, achieving resolutions of less than a nanometer (110). This allows neurons to be studied at the sub-compartmental level because it provides nanometre-scale resolution. However, despite being considered the gold standard for ultrastructural analysis, it has important limitations, including demanding sample preparation and fixation requirements, as well as difficulties in obtaining target-specific labelling compatible with these conditions, largely due to poor antibody penetration and high levels of non-specific staining (111).

In recent decades, novel imaging techniques have emerged to overcome the barrier imposed by light diffraction (Figure 6). These approaches are grouped under the family of Super-Resolution Microscopy (SRM), which aims to overcome the diffraction limit by reducing the spatial overlap between the fluorescence signals emitted by nearby fluorophores (112). This can be achieved either by controlling the fluorescence emission of many fluorophores simultaneously (ensemble-based deterministic approaches) or by a stochastic way (localising individual fluorophores one at a time).

Among the deterministic techniques, the two main approaches are Structured Illumination Microscopy (SIM) and Stimulated Emission Depletion (STED) microscopy. SIM, introduced in 2000, relies on a series of spatially structured, bar-code-like excitation light patterns applied in different orientations, achieving an approximate resolution of 120 nm and a two-fold improvement over the diffraction limit (113). In contrast, STED, first described in 1994, improves resolution by approximately five-fold, reaching around 50 nm (114). This technique uses two overlapping laser beams in combination with a high-resolution confocal microscope, leaving only a small central area able to emit light and thereby reducing the effective focal volume below the diffraction limit.

In 2006, a family of stochastic techniques known as Single Molecule Localisation Microscopy (SMLM) emerged, of which the three most significant are Photoactivated Localisation Microscopy (PALM) (115), Stochastic Optical Reconstruction Microscopy (STORM) (116) and DNA-based Points Accumulation for Imaging in Nanoscale Topography (DNA-PAINT) (117). The first two methods require the use of photoswitchable fluorophores to control the number of molecules emitting signal in each acquired image, thereby minimising the probability that two or more fluorescent molecules overlap spatially (118). DNA-PAINT achieves the same goal by stochastic blinking generated by the transient binding of fluorescent oligonucleotides to complementary docking strands.

Finally, in 2007, Micheva and Smith developed Array Tomography (AT), a new tool for imaging neuronal architecture and circuits in rodent brains (119). In 2013, Hyman and Spires-Jones' groups adapted this approach to study synapses in the human brain (120). AT improves the axial resolution by embedding fresh brain tissue in resin and cutting it into ribbons of ultrathin serial sections, typically 70 nm thick.

By physically separating fluorophores across consecutive sections, this approach minimises signal overlap in the z-axis and allows high-resolution three-dimensional reconstruction of tissue ultrastructure. AT is a particularly powerful tool for the study of human synapses, as it allows both the quantification of synaptic density and the characterisation of synaptic protein composition within preserved tissue architecture (121). Moreover, AT can be combined with other imaging techniques such as EM (122), STED (123) and SIM (124), providing a powerful strategy for achieving super-resolved imaging in three dimensions.

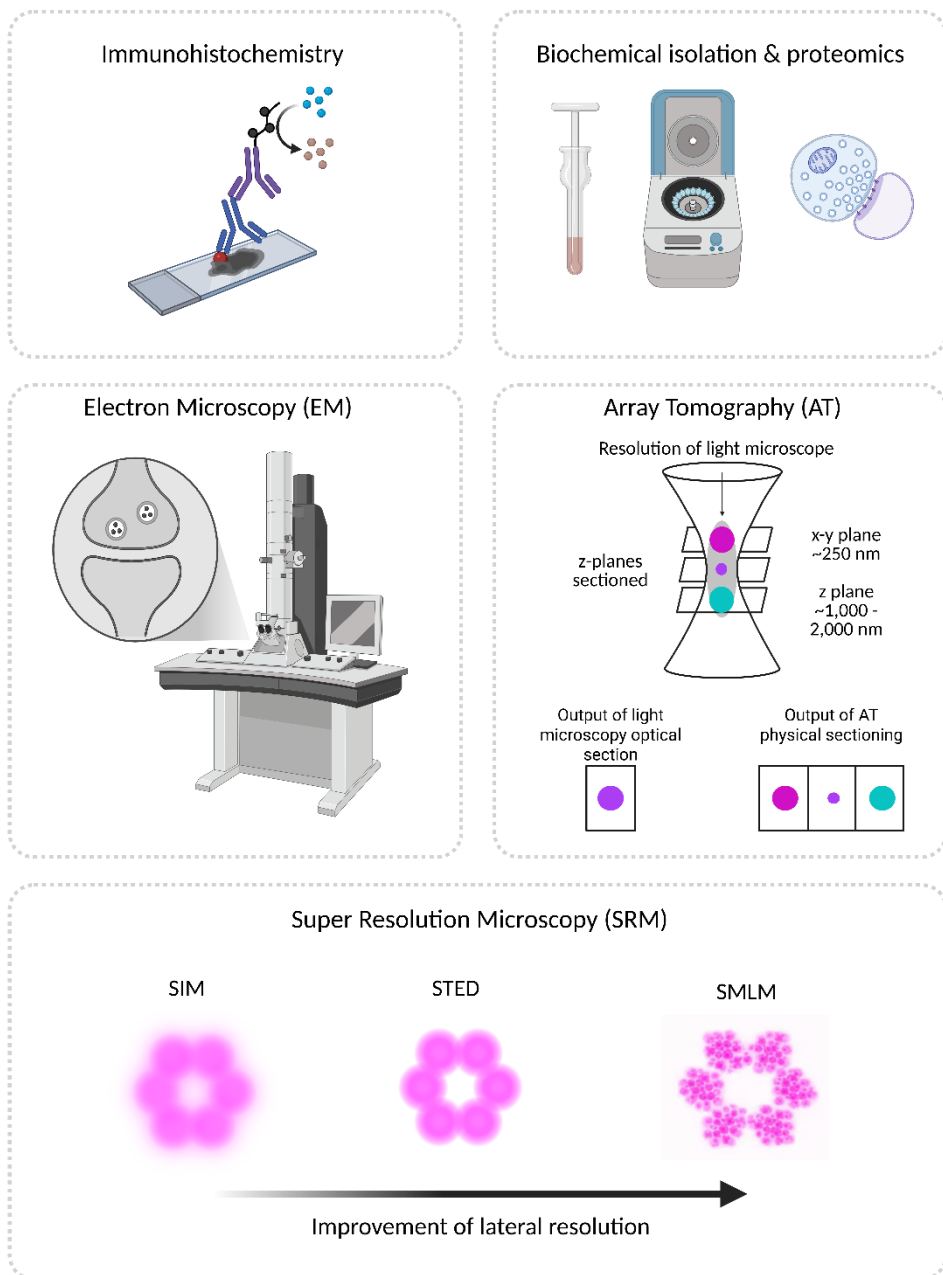


Figure 6. Main approaches to study synapses. Study of synaptic proteins using immunohistochemistry or biochemical isolation of synaptic terminals and proteomic studies. Ultrastructure study of the synapse with EM, AT and SRM. Created with BioRender. Adapted from Kay *et al.* (2013) and Nollman and Giorgeiva (2015) (120,125).

1.3.3 SYNAPTIC PATHOLOGY IN AD

AD is considered a disorder with a major synaptic component (126). One of the earliest events that occur during the progression of the disease is the development of pathological changes at the synapse, including synaptic loss, dysfunction and toxic deposition of proteins aggregates.

Synaptic loss in AD was first reported in the frontal cortex using EM (127), compared to controls. Similar findings were obtained by measuring synaptophysin immunoreactivity, which showed a 40% reduction in synaptic density in the frontal and inferior parietal cortex of AD cases (15). In both studies, synaptic loss correlated with disease progression and cognitive decline. Similar observations were subsequently made in the temporal cortex, hippocampus and inferior temporal gyrus, where synaptic loss also correlated with cognitive impairment (128–130). Across these regions, there was a 15–20% synaptic loss in the neocortex and limbic system both in MCI and early AD cases. These series of studies established synaptic loss as the major correlate of cognitive impairment, with no significant correlations observed with A β plaques or NFT burden.

A key component that drives synaptic loss in AD is mitochondrial dysfunction (131), since synaptic mitochondria are crucial for the correct functioning of synapses. In AD, mitochondria are affected at many levels. For example, genetic variants that impair mitochondrial respiration can increase vulnerability to the disease (132). Mitochondria become more likely to produce reactive oxygen species (ROS), leading to increased oxidative stress both in the organelle and the cell, which then progresses to disrupt the electron transport chain (ETC) (133). As the disease progresses, AD mitochondria fail to produce enough ATP affecting the energy and dynamics of the cell (134). Moreover, defects in both mitophagy and mitochondrial biogenesis have been reported in AD models (135,136).

APOE $\epsilon 4$ has also been linked to synaptic pathology in AD. This is not surprising as APOE is the major carrier of cholesterol in the brain, which is essential for neuronal activity (137). In animal models, APOE $\epsilon 4$ leads to synaptic deficits and impairment in memory and cognition, even in the absence of neuropathology (25). Mice expressing APOE $\epsilon 4$ showed reduced dendritic spine density and shortened dendrite length when compared to mice expressing APOE $\epsilon 3$. This result suggested that the effects of APOE $\epsilon 4$ on synaptic function may occur prior to the onset of AD (138). Moreover, studies in human living brains showed that APOE $\epsilon 4$ was associated with decreased synaptic density in patients with MCI, and that even one copy of APOE $\epsilon 4$ is sufficient to induce synaptic density loss, conferring additional risk of AD (139).

Neuroinflammation is a main factor in the early pathophysiology and progression of AD. Neuroimmune responses orchestrated by glial cells (especially astrocytes and microglia) play a crucial role in synaptic maintenance. Therefore, glial dysfunction is implicated in impairment of synaptic plasticity and synaptic loss (140). Under physiological conditions, glial cells modulate synaptic function through neurotransmitter generation and clearance, regulation of ionic concentrations, synaptic pruning and immune surveillance (141). In the early stages of AD, these cells may still provide a protective effect. However, as the disease progresses, chronic glial activation promotes the sustained release of pro-inflammatory cytokines and oxidative stress. As a result, these homeostatic mechanisms become altered, contributing to synaptic dysfunction.

Astrogliosis is a common response to AD pathology. This process is characterised by morphological, transcriptional and functional changes in astrocytes (142). They can adopt two phenotypes: neurotoxic and pro-inflammatory, typically overexpressing markers such as glial fibrillary acidic

protein (GFAP), or neuroprotective and anti-inflammatory. Post-mortem AD brains show a prevalence of pro-inflammatory astrocytes, supporting the glial contribution to synaptic and neuronal damage (143). In fact, levels of GFAP in CSF are associated with measures of synaptic dysfunction (140).

Microglia also has a key role in synaptic dysfunction, as synaptic pruning is one of the major roles of glial cells. In AD, this pruning becomes defective, generating aberrant connexions that contribute to memory loss and cognitive impairment. In early stages of the disease, microglial activation has a protective role, especially eliminating A β species through microglial receptor TREM2-mediated signal (142). However, a prolonged exposure to pathological aggregates promotes a chronic activation of the microglia, leading to the release of pro-inflammatory cytokines and ROS that ultimately damage neurons and their synaptic contacts (143). Moreover, mutations in TREM2 are linked to a higher AD risk and faster disease progression, directly linked with an impaired phagocytosis and synapse elimination (144). Soluble TREM2 is also associated with synaptic dysfunction, which seems to be mediated by phosphorylated tau (140).

Many of the mechanisms involved in synaptic pathology in AD are linked to A β and tau (Figure 7). The toxic accumulation of these proteins within the synapse contributes to synaptic dysfunction by disrupting the normal function of synaptic proteins and impairing the molecular machinery required for neurotransmission (144). Accordingly, pathogenic species of both tau and A β have been reported to alter synaptic structure and function (145,146). In terms of mitochondrial dysfunction, tau has been associated with disruption of complex I of the ETC, whereas A β preferentially disrupts Complex IV (147). Moreover, exacerbated A β and tau pathology have been reported in APOE ϵ 4 carriers, who exhibit higher levels of tau toxicity and increased A β plaque burden (148,149), both favouring neuroinflammatory responses.

Although both A β and tau proteins accumulate in the brain as insoluble fibrillar aggregates, growing evidence suggests that the most synaptotoxic species are their soluble oligomeric counterparts (150–152). These species are thought to be particularly harmful because they can interact directly with synaptic proteins during early stages of the disease, preceding the formation of larger insoluble deposits.

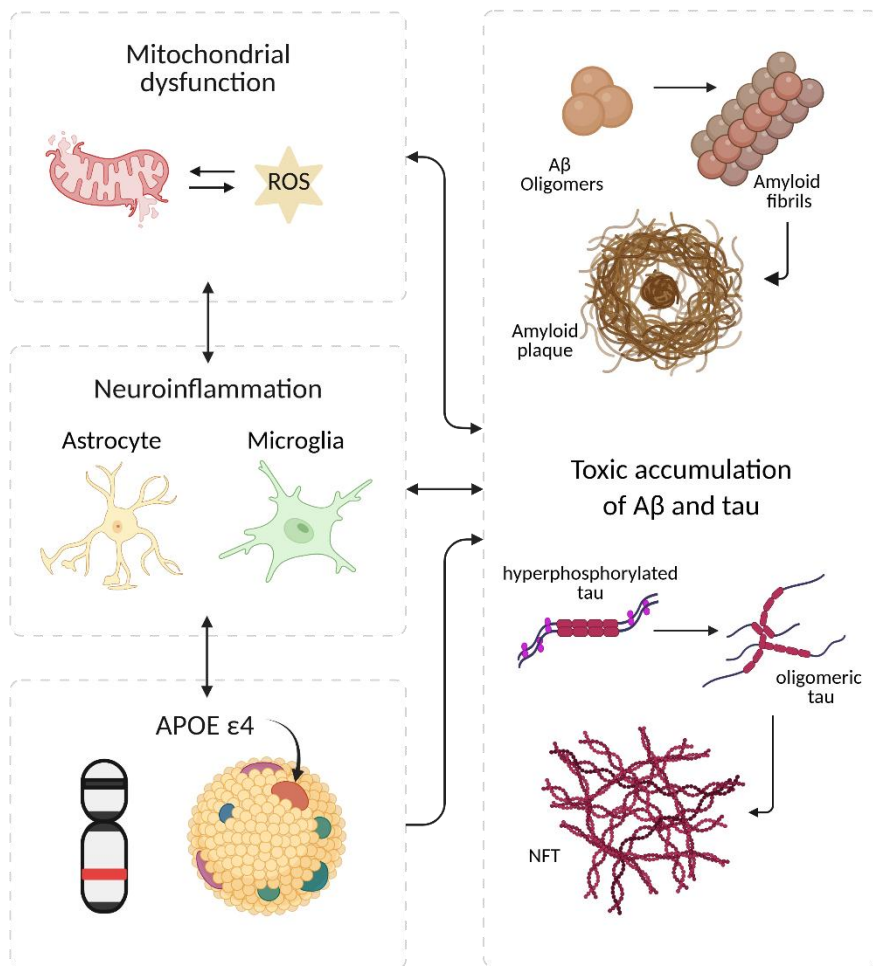


Figure 7. Schematic overview of the key mechanisms involved in synaptic dysfunction and loss in AD. Toxic accumulation of A β and tau, mitochondrial dysfunction, neuroinflammation and APOE ϵ 4 are closely interconnected processes that contribute to synaptic damage. Together, these mechanisms reinforce each other and promote progressive synaptic failure. Created with BioRender.

1.3.4 SYNAPTIC TAU PATHOLOGY IN AD

Some of the most important findings of the detrimental effect of tau oligomers at the synapses comes from studies with animal models. Early *in vivo* studies showed that intracranial administration of human recombinant tau oligomers into wild type mice was enough to impair memory and to induce synaptic dysfunction. Importantly, these effects were not reproduced by tau monomers or fibrils, suggesting that the oligomeric species are particularly relevant for toxicity rather than for aggregation (75,77).

Notably, similar results were obtained when using oligomeric tau derived either from human AD brain samples or from mice expressing human tau, indicating that these findings are not restricted to synthetic recombinant preparations alone (153,154). Moreover, the synaptotoxicity caused by tau oligomers precede neurodegeneration, supporting the idea that synapses are among the earliest compartments affected by oligomeric tau species (155).

Additional evidence of the direct pathogenic role of tau oligomers comes from immunotherapy studies. Passive immunization with tau oligomer monoclonal antibodies reversed or prevented cognitive deficits in transgenic mice, reducing oligomeric tau level, without substantially affecting NFTs or monomeric tau (156,157). These studies strengthen the view that oligomeric tau is not only an intermediate on the NFT formation, but a biologically active specie especially detrimental to the synapse.

Moreover, some studies have reported trans-synaptic tau spread in model systems (158–160), showing that trans-synaptic tau spread is biologically plausible. However, these findings should be interpreted with caution, as tau spread in such models may be influenced by artificial overexpression, and definitive evidence for this phenomenon in the human brain is still missing.

Since the first description of tau pathology by Brion, it has been known that tau accumulates along neuronally connected pathways (161). This observation has also been supported by imaging and network-based studies, suggesting that tau spreads through neuronal communication pathways (162). But tau not only spreads through anatomically connected neurons, it also accumulates within synapses. Tau oligomers have been identified in synaptic fractions from AD brains (163), and the presence of these oligomers is rather high (164), suggesting a major role for tau oligomers in synaptic dysfunction and loss.

Using tau biosensor cells, tau seeding levels were detected not only in the brain regions affected by tau pathology, but also in the subsequent region along the Braak pathway. Seed competent tau was significantly enriched in synaptic fractions (165), suggesting that tau spreading precedes the deposition of tau fibrils and that this process could be happening throughout the synapse.

Interestingly, tau oligomers have been linked to both worse cognitive outcomes and cognitive resilience. On one hand, when tau seeding activity and tau oligomers levels are high, worse cognitive outcomes are observed, which may account for some of the marked heterogeneity observed in sporadic AD patients (91). On the other hand, brain-derived tau oligomers from non-demented brains with AD pathology exhibited protease resistance and formed larger aggregates, while preserving the synaptic function, which may explain cognitive resilience (166). In line with this, functional integrity of synapses has been associated with absence of synaptic tau oligomers (167).

Taken together, the available evidence strongly supports a synaptotoxic role for tau oligomers and suggests that synaptic compartments may participate in tau propagation, although characterization of oligomeric tau in human AD synapses is missing, mainly due to limitations in imaging techniques.

CHAPTER 2 | HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

This thesis hypothesises that:

2.1.1 In AD, synapses are an early and functionally relevant site of oligomeric tau accumulation, where its localisation across pre- and postsynaptic compartments is consistent with a role in trans-synaptic spread and synaptic degeneration.

2.1.2 This synaptic pathology can be resolved in human tissue using AT combined with two-colour dSTORM.

2.1.3 Tau seeding activity derived from AD brains is linked to the synaptic accumulation of oligomeric tau.

2.2 GENERAL OBJECTIVE

To investigate the role of synaptic oligomeric tau in AD by combining high-resolution microscopy techniques in human brain tissue and functional seeding assays *in vitro*.

2.3 SPECIFIC OBJECTIVES

2.3.1 To determine the early presence of oligomeric tau and its distribution at human cortical synapses in AD.

2.3.2 To evaluate whether the combination of AT and two-colour dSTORM improves lateral resolution sufficiently to reliably resolve human synaptic terminals.

2.3.3 To determine whether tau species from sporadic AD exhibit seeding capacity *in vitro* and assess the relationship between tau seeding activity and the amount of oligomeric tau accumulated in synapses.

CHAPTER 3 | MATERIALS AND METHODS

3.1 ARRAY TOMOGRAPHY

AT consists in the ultrasectioning of brain tissue (obtaining sections of ~70 nm) in order to reconstruct the 3D ultrastructure via conventional microscopy and visualize single synapses in intact tissue. A key advantage is the ability to quantify multiple proteins of interest and precisely determine whether they localize to the pre- or postsynaptic compartment, something difficult to achieve with diffraction-limited techniques.

3.1.1 SAMPLE PREPARATION

The protocol used is detailed in Kay *et al.* (120) (Figure 8). Samples must be fresh at the time of collection, following autopsy or biopsy procedures. A 1 cm³ of tissue was collected in fixative buffer (4% paraformaldehyde and 2.5% sucrose in 0.01 M Phosphate-buffered saline (PBS)). Each sample was dissected into small blocks of 1 mm x 1 mm x 5 mm using a scalpel on a Petri dish, ensuring that blocks consist predominantly of cortical tissue. Samples were left to fix for 2-3 hours in a M24 plate.

Samples were washed using wash buffer (3.5% sucrose and 0.05 M glycine in 0.01 M PBS), which is pre-filtered through a 0.45 µm filter. The fixative buffer is removed completely with at least two washes, leaving samples for a minimum of 5 minutes.

Dehydration of fixed samples was carried out in cold ascending ethanol (EtOH) series for 5 minutes at each grade (50%, 70%, 95%, 100%, 100%) to prepare samples for infiltration with London Resin (LR) White (AGR1281, Agar Scientific) using a 1:1 LR White:EtOH mixture, followed by 100% LR White and a further change of 100% LR White. Samples were left in LR White at 4°C overnight. The following day, each block was transferred to a gelatine capsule (size 00, Electron Microscopy Sciences) and placed at 53°C until complete polymerisation of the resin.

Resin-embedded tissue was sectioned into ribbons of 70 nm-thick serial sections (~30 consecutive sections) with an ultramicrotome (Leica Microsystems) equipped with a 35° Ultra Jumbo diamond blade (Diatome). Tissue ribbons were collected on glass coverslips previously coated (0.1% fish skin gelatine and 0.01% chromium potassium sulphate) and were left to dry at room temperature overnight. All coverslips are labelled and stored at room temperature for use within 3–6 months.

3.1.2 IMMUNOHISTOCHEMISTRY

All ribbons were incubated in 50mM glycine in Tris buffered saline (TBS) for 5 minutes and washed with TBS. Once rehydrated, ribbons must not be allowed to dry out. Sections were then blocked with blocking buffer (0.1% fish skin gelatine and 0.05% Tween20 in TBS) for 30 minutes at room temperature. Primary antibodies (detailed at Chapter 4), diluted in blocking buffer, were applied to ribbons overnight at 4°C. The next day, after TBS washings, sections were incubated with the respectively secondary antibodies for 30 minutes without light. Final washes were performed with TBS and nuclei were stained with Hoechst 33258 (Life technologies) or with 4'-6-diamidino-2-phenylindole (DAPI) for 5 minutes. Finally, coverslips were mounted on slides with Immu-Mont mounting medium (9990402, EpreDia).

3.1.3 IMAGE ACQUISITION AND ANALYSIS

Regions of interest (ROIs) were acquired with references (such as nuclei or pathological aggregate) using an Olympus BX51 microscope with a 63x objective (Olympus), in a scan format of 512 x 512 pixels (0.2 µm pixel size). At least 15 consecutive sections were imaged from each ROI, with 3 ROIs for ribbon.

For AT image analysis, we employed an in-house algorithm developed using MATLAB software (The MathWorks) in combination with ImageJ, as previously described (123,168). The analysis workflow proceeds as follows: first, an image stack is generated for each channel, comprising all consecutive sections that have been acquired during imaging. These stacks are then aligned using an intensity-based registration algorithm, which is optimised through a step gradient descent approach to ensure accurate alignment across sections. Following alignment, an automated local average thresholding method is applied to segment and binarise each individual 2D slice within the stack. To eliminate noise and artefacts, objects that do not appear in at least two consecutive sections, as well as those smaller than 3 pixels in size, are classified as background and subsequently removed from the dataset. These steps allow the extraction of the main quantitative measures, including object density and colocalisation analyses.

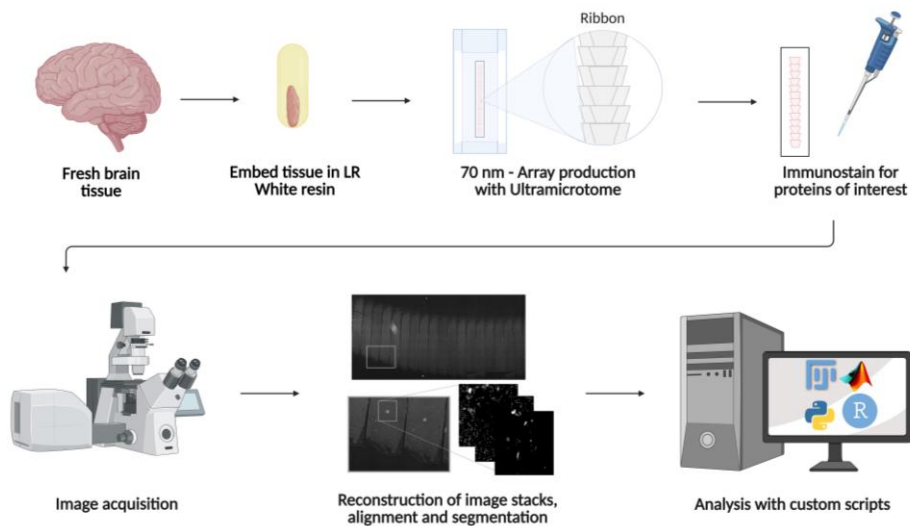


Figure 8. AT workflow. After collection, brain samples are embedded in LR White resin and ultrasectioned into ribbons of consecutive serial sections. Immunohistochemistry is then performed, followed by image acquisition and raw data processing using custom scripts for image stack reconstruction, alignment and segmentation, enabling the quantification of object density and colocalisation via custom scripts. Created with BioRender.

3.2 TWO-COLOUR DIRECT STORM

Two-colour direct STORM (dSTORM) is a method of super-resolution part of the SMLM family. It is based in the single-molecule localisation, using conventional fluorophores which are induced to blink under laser illumination and specific chemical buffer conditions. These fluorophores undergo reversible transitions between ON and OFF states through photophysical and redox-dependent processes. As a result, the majority of the molecules remain inactive and only a random subset is ON, generating stochastic blinking (Figure 9). This cyclic process is repeated multiple times, with only a scarce subset of fluorophores emitting in each round, enabling the precise identification and localisation of individual fluorophores across successive imaging cycles.

3.2.1 SAMPLE PREPARATION

Coverslips containing 2 μm (formalin-fixed paraffin-embedded, FFPE) or 70 nm (AT) thick sections were mounted with STORM buffer (PBS 8,4, MEA 100 mM, lactate 100uM, oxyrase 3%) (169), and covered with a glass slide, sample side down, immediately before image acquisition. Excess buffer was carefully wiped off and the coverslip was sealed with nail polish.

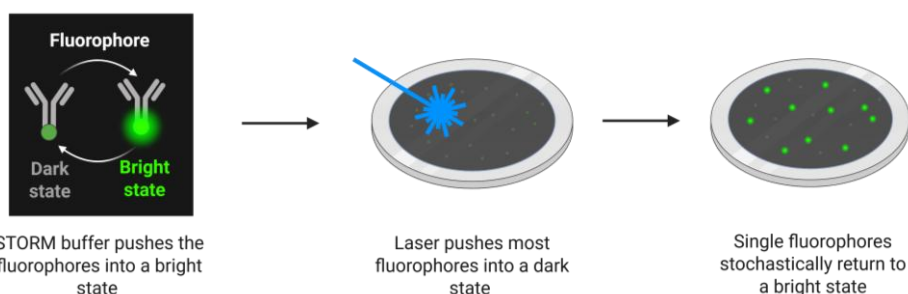


Figure 9. dSTORM principle. There is a competition between the laser and the buffer, allowing single fluorophores to stochastically blink. Extracted from Sánchez-Aced *et al.* (2025) (170). Created with BioRender.

3.2.2 IMAGE ACQUISITION AND ANALYSIS

dSTORM imaging was performed on a Nikon N-STORM system set up for total internal reflection fluorescence (TIRF) microscopy. The inclination of the laser was adjusted to maintain focus and improve the signal-to-noise ratio, typically at approximately 60°. AlexaFluor-647 was excited using the 647 nm laser line at 20% of 160 mW, while AlexaFluor-488 was excited with the 488 nm laser line at 40% of 80 mW. Fluorescence emission was collected using a Nikon 100× 1.49 NA oil immersion objective and passed through a quad-bandpass dichroic filter (97335 Nikon). For each channel, 10,000 frames were acquired with an integration time of 20 ms. Images were recorded on a 256 × 256 pixel region of a Hamamatsu ORCA-Flash 4.0 CMOS camera, with a pixel size of 0.008 μm. STORM images were processed using the STORM module in the Nikon NIS-Elements software (171).

Similar to the array tomography analysis, using a semiautomated mean local threshold images from serial sections were stacked, aligned and segmented. Nonspecific staining, defined as signal not present in at least two consecutive sections, was considered background and removed. Three-dimensional reconstructions of representative synapses were generated in ImageJ using the Volume Viewer plug-in with tricubic smooth interpolation. For centroid distance analysis, stacks of protein images were loaded and processed frame by frame. Object shape was then parametrised obtaining values such as roundness, circularity and eccentricity. To estimate synaptic cleft width, pixels from both protein signals were listed, all pairwise distances were calculated, and the minimum distance was retained.

The scripts used for these analyses have been deposited at https://github.com/MemoryUnitSantPau/Synseg_py with the names: density, distance and shape, and segmentator.

3.3 HOMOGENIZATION AND SYNAPTONEUROSOME ISOLATION

Using frozen brain tissue, both total homogenates and synaptically enriched fractions (synaptoneurosomes) can be obtained (Figure 10). Homogenates provide a general representation of the molecular composition of the tissue, whereas synaptoneurosomes consist in the presynaptic, intact postsynaptic density (PSD), glial processes, and some components of the extracellular matrix, allowing a more specific study of synaptic proteins and pathological species located at the synaptic environment. Both fractions can be further used for downstream biochemical analyses, proteomic studies or functional *in vitro* assays.

3.3.1 HOMOGENIZATION

To obtain the crude homogenate fraction, 200 mg of frozen tissue from temporal cortex was homogenised on ice using a Dounce homogenizer in 1ml of buffer A (25 mM HEPES pH 7.5, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, protease inhibitors (Roche complete mini), phosphatase inhibitors (Millipore, 524 629)). The homogenate was filtered through an 80 µm nylon filter (Millipore, NY8002500) and 300 µl was saved as crude homogenate with buffer B (100mM Tris-HCl pH 7.6, 4% SDS, protease inhibitor cocktail EDTA-free 100x Thermo Fisher Scientific) at -80°C until their use.

3.3.2 SYNAPTONEUROSOME ISOLATION

The remaining crude homogenate was further filtered through a 5 µm filter (Millipore, SLSV025NB) and centrifuged at 1000 g for 5 min. The supernatant was discarded and the pellet was washed with buffer A and centrifuged again, yielding the synaptoneurosomes pellet. For storage it was mixed with buffer B. Protein concentrations from both fractions were determined using a protein assay (Micro BCA TM Protein Assay Kit, Thermo Fisher Scientific, 23235).

3.3.3 VALIDATION

Both preparations (2.5-10 $\mu\text{g}/\mu\text{l}$) were denatured at 95° C for 5 min for validation: 15 μg of protein were resolved using 15% Tris–Glycine gel and transferred onto nitrocellulose membranes (Biorad, 1620112) in Trans-Blot turbo buffer (Biorad, 1704270) supplemented with 20% ethanol at 25 mV for 7 min in the Trans-Blot® Turbo™ Transfer System (Biorad, 1704150). After blocking 1h with Intercept® Blocking Buffer (Li-COR; 927-60001), the membrane was incubated with the indicated primary antibodies: α -VAMP2 antibody (Cell Signalling, 13508, 1:250) and α - β -actin antibody (Cell Signaling, 3700,1:1000) overnight at 4°C. The protein bands were visualised using corresponding fluorescent secondary antibodies (Odyssey, 1:5,000). Infrared fluorescence was measured with the Odyssey CLx imager (LI-COR) and quantified using Image Studio software (Li-COR Biosciences).

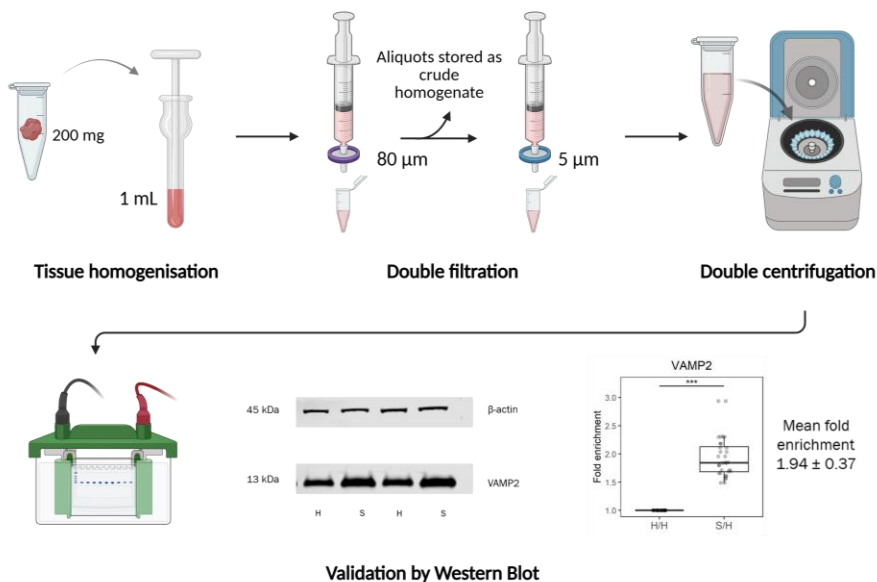


Figure 10. Synaptoneurosome isolation from brain homogenates. Frozen tissue undergoes homogenization, a first filtration with nylon filter to obtain crude homogenate and then another filtration, followed by double centrifugation to obtain the synaptoneurosome fraction. Both fractions are resolved to validate synaptic enrichment. Created with BioRender.

3.3.4 WESTERN BLOTTING FOR OLIGOMERIC TAU

For oligomeric tau detection, 75 µg of protein were resolved using a 4–12% Bis-Tris gel (MOPS running). The remaining steps were performed using the same protocol described in section 3.3.3, except that transfer time was 13 min to improve the transference of high molecular weight species and the primary antibodies were α-tau5 antibody (BioLegend, 806401, 1:250) and α-GAPDH antibody (Cell Signaling, 2118, 1:1000).

3.4 TAU SEEDING ASSAY

For tau seeding assays we used Tau RD P301S FRET Biosensor Tau cells (line CRL-3275™), these cells show stable expression of the PS19-mutant tau repeat domain conjugated to either CFP or YFP (90). In basal conditions, reporter proteins remain mostly soluble, producing low or no FRET signal. However, when cells are exposed to tau seeds, the pathological tau species induce aggregation of the CFP- and YFP-tagged tau repeat domains, bringing both fluorophores close enough to generate FRET signal when CFP is excited (Figure 11).

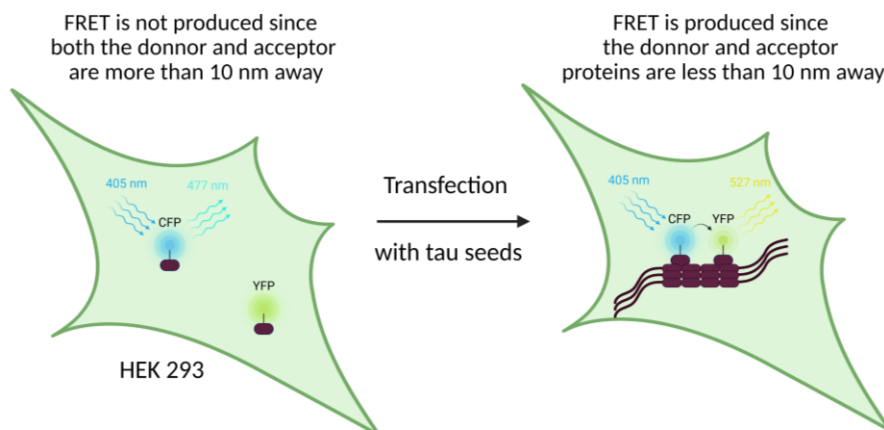


Figure 11. Biosensor Tau cells. When cells are exposed to tau seeds, CFP- and YFP-tagged tau repeat domains come closer producing FRET signal upon excitation of CFP. Created with BioRender.

3.4.1 TAU SEEDS ISOLATION

Based on an initial working estimate of the tau content in the brain homogenate, an amount of homogenate corresponding to approximately 800 ng of tau was ultracentrifuged at $150,000 \times g$ for 30 min at 4°C. The resulting pellet, enriched in tau seeds, was resuspended in 50 μ L of 0.01 M PBS. Total tau concentration was quantified by ELISA (Invitrogen, KHB0041). Aliquots were stored at -80°C until use.

3.4.2 BIOSENSOR CELLS AND SEEDING ASSAY

The stable Tau RD P301S FRET Biosensor cell line (CRL-3275™) was maintained in 100 mm plates with complete medium, consisting of Dulbecco's Modified Eagle Medium (DMEM, Gibco™ 11965092) supplemented with 10% of foetal bovine serum (FBS) and 1% of penicillin/streptomycin. When cells reached 90% confluence, they were seeded in 24-well plates containing coverslips previously coated with Matrigel (Merck CLS354234, diluted 1:50 in cold DMEM) and incubated overnight at 37°C to enhance cell adhesion.

After 24 h, cells were transfected with Lipofectamine 2000 (Invitrogen™ 11668019; 1% v/v) and tau seeds (16 ng), both diluted in Optimized Minimal Essential Medium (Opti-MEM, Gibco™ 31985070). Lipofectamine and tau seeds were incubated together for 20 min before transfection to allow complex formation. Cells were transfected for 5 h, after which the medium was replaced with complete medium.

At 72 h post-transfection, cells were fixed with 4% paraformaldehyde for 20 min, followed by quenching with 50 mM NH_4Cl and 20 mM glycine. Cells were then stained with Hoechst 33258 (Life technologies) and mounted with Imm-Mont mounting medium (9990402, Eprelia) for imaging.

3.4.3 IMAGE ACQUISITION AND ANALYSIS

FRET signal resulting from tau aggregation was acquired using a 3DHISTECH fluorescence scanner and quantified with ImageJ. From each coverslip image, four 2 mm² sections were selected using SlideViewer and SlideMaster, and ten smaller random ROIs of 1000 × 1000 pixels were extracted from each section using MATLAB. All ROIs were analysed with ImageJ, and results were expressed as percentage of FRET-positive signal.

3.5 STATISTICAL ANALYSIS

Statistical analyses were performed in R version 4.3.1. Data distributions were examined using histograms and Shapiro–Wilk tests. Age and post-mortem interval (PMI) differences between groups were assessed using Kruskal–Wallis tests and one-way ANOVA, respectively. Data points exceeding three times the interquartile range were excluded from the analysis. For most tissue-based analyses, mixed-effects models were fitted using the *lmerTest* package to evaluate the effect of disease status, APOE genotype and sex on the variable of interest, while controlling for potential confounding variables and including random effects to account for repeated measures. Statistical assumptions were checked using model residuals, and data were transformed when needed. Main and interaction effects were tested using Type III ANOVA. Significant effects were followed by post hoc comparisons with Tukey-adjusted tests.

For comparisons between microscopy techniques, data distribution was evaluated before applying Mann–Whitney tests to assess differences in object dimensions and distances between synaptic proteins. The contribution of PMI to synaptic cleft width was assessed using linear regression with PMI as a covariate. Differences in area, length and other morphological measurements of synaptic proteins were evaluated using Wilcoxon tests.

For FRET-based seeding analyses, data were summarised per case to avoid pseudoreplication. Group differences in mean FRET positivity were assessed using the Wilcoxon rank-sum test. Associations between FRET positivity and clinical, neuropathological, biochemical or tissue-based variables were evaluated using Spearman's correlation. Pearson's correlation was used only when data met the assumptions for parametric testing.

All tests were two-sided, and statistical significance was set at 5% ($\alpha = 0.05$). Further statistical details, including the test used, exact n values and measures of dispersion, are provided in the corresponding figure legends and results text (Chapter 4).

CHAPTER 4 | STUDIES

SYNAPTIC OLIGOMERIC TAU IN ALZHEIMER'S DISEASE – A POTENTIAL CULPRIT IN THE SPREAD OF TAU PATHOLOGY THROUGH THE BRAIN.

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ABSTRACT

In Alzheimer's disease, fibrillar tau pathology accumulates and spreads through the brain and synapses are lost. Evidence from mouse models indicates that tau spreads trans-synaptically from pre- to postsynapses and that oligomeric tau is synaptotoxic, but data on synaptic tau in human brain are scarce. Here we used sub-diffraction-limit microscopy to study synaptic tau accumulation in post-mortem temporal and occipital cortices of human Alzheimer's and control donors. Oligomeric tau is present in pre- and postsynaptic terminals, even in areas without abundant fibrillar tau deposition. Furthermore, there is a higher proportion of oligomeric tau compared with phosphorylated or misfolded tau found at synaptic terminals. These data suggest that accumulation of oligomeric tau in synapses is an early event in pathogenesis and that tau pathology may progress through the brain via trans-synaptic spread in human disease. Thus, specifically reducing oligomeric tau at synapses may be a promising therapeutic strategy for Alzheimer's disease.

INTRODUCTION

Of the neuropathological hallmarks of Alzheimer's Disease (AD), synapse loss is the strongest correlate of cognitive decline (1-3), and progression of tau pathology through the AD brain closely correlates with synaptic loss and cognitive symptoms (4,5). Tau protein binds synaptic vesicles and is thought to have physiological roles in synapses (6). In animal and neuronal culture model systems, accumulation of hyperphosphorylated and misfolded tau within synapses causes synaptic dysfunction and synapse loss (7-13).

While neurofibrillary tangles (NFT) were historically considered neurotoxic lesions, data over the past decades indicate that the tau fibrils in tangles are relatively inert and that soluble oligomeric forms of tau are more toxic to synapses and neurons in mouse models of tauopathy (14,15). Much less is known about synaptic tau and in particular synaptic oligomers in human brain, which is an important knowledge gap to address in order to develop tau-directed therapeutics to protect synapses from degeneration. Phosphorylated and misfolded tau have previously been observed in human AD synapses using biochemical and imaging methods (12,16,17). There is a small amount of evidence from human cases suggesting that diffuse forms of tau precede NFT formation (18,19), that those diffuse forms are mainly oligomeric species (20,21), and that oligomeric tau is important for seeding activity (22). Both high seeding activity and high concentrations of oligomers have been linked to worse cognitive outcomes (22,23).

In addition to synaptotoxic effects, synaptic localisation of tau is potentially important for propagation of tau pathology through brain networks. Seminal observations of the patterns of tau pathology post-mortem by Braak and Braak (24) and more recent observations using tau ligands for positron emission tomography (PET) (25,26) demonstrate that NFTs accumulate in a

stereotypical spatiotemporal sequence beginning in the brainstem and entorhinal cortex and progressing through synaptically connected neural circuits. Because tau is secreted by presynaptic terminals in an activity-dependent manner (27) and misfolded tau can act as a seed to initiate pathological changes in naive tau *in vitro* and *in vivo* (28,29), a likely method of pathological propagation is via synaptic connections. It is also possible that anatomically connected brain regions are sequentially vulnerable to tau. A recent analysis of tau PET imaging and post-mortem data indicates local replication rather than spreading between brain regions may be more closely related to regional tau pathology (30). These two ideas are not mutually exclusive, and indeed the local accumulation of tau may at least partially reflect *trans*-synaptic spread in local circuits, which one might predict would occur faster due to the shorter axons of local connections.

We and others have previously demonstrated *trans*-synaptic tau spread in model systems including transgenic mice expressing human tau in the entorhinal cortex and viral mediated tau expression (31-35). These studies prove that *trans*-synaptic tau spread is biologically possible. However, one limitation is that it might have been artificially induced by overexpression in models, and this phenomenon has not been conclusively demonstrated in human brain. Here, we use sub-diffraction-limit optical imaging of synapses with multiple techniques and multiple tau antibodies (detecting oligomeric, misfolded, and phosphorylated tau) to explore in human post-mortem brain whether there is evidence of tau present in both the pre- and postsynaptic sides of the same synapses, which could be due to *trans*-synaptic spread. Furthermore, we characterize tau pathology in detail to determine whether synaptic tau accumulates in areas without substantial NFTs.

MATERIALS AND METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human brain tissue

Brain samples were obtained with ethical approval via the University of Edinburgh Sudden Death Brain Bank and the Alzheimer Scotland Brain and Tissue Bank. This study was reviewed and approved by the Edinburgh Brain Bank ethics committee and the Academic and Clinical Central Office for Research and Development, a joint office of the University of Edinburgh and NHS Lothian (approval number 15-HV-016). The Edinburgh Brain Bank is a Medical Research Council funded facility with research ethics committee (REC) approval (16/ES/0084).

Inclusion criteria for Alzheimer's disease cases were a clinical dementia diagnosis, Braak stage V-VI, and neuropathological diagnosis of AD. Control subjects were donors without neurological or psychiatric diagnoses and were included based on age, sex, and APOE genotype matching the AD cases as closely as possible. All cases were genotyped in-house to determine APOE genotype, as described previously (67). Exclusion criteria for both groups were neuropathological findings indicative of substantial secondary pathology (e.g., haemorrhage or Lewy bodies within brain region of interest). A total of 23 AD and 19 control cases were examined in this study as outlined in Table 1 showing demographic data and the experiments they were used in. Sex was included as a fixed effect in statistical models to examine sex effects in all measures.

A statistical power analysis was performed for sample size estimation, based on data from study 1 (Table 1, Supplementary Table 1), comparing presynaptic terminals with tau to diagnosis (control or AD) by linear mixed effect model (effect size $\beta=0.521$, see statistical analysis section). With an alpha = 0.05 and

power = 0.80, the projected sample size needed with this effect size in approximately $N=10$. The analysis was performed as described in (68).

Brain samples from inferior temporal cortex (Brodmann Areas 20 and 21, BA20/21) and primary visual cortex (BA17) were collected at autopsy and preserved by fixation and paraffin embedding, and fixation for array tomography as detailed previously (42). These regions were selected to compare areas with high pathological burden (BA20/21) to those with low pathology (BA17).

Mouse brain tissue

Mice expressing human P301L mutant tau, cytoplasmic tdTomato, and green fluorescent protein tagged synaptophysin in entorhinal cortex neurons were used to determine whether oligomeric tau can spread anterogradely from pre to post synapses. Tissue was used from mice generated in a previous study (34,69). Briefly, FVB-Tg(tetO-TauP301L)⁴⁵¹⁰ mice (70,71) were crossed with the Tg(tetO-tdTomato-Syp/mut4EGFP)^{1.1Luo/J} line from Jackson laboratories. Offspring expressing both Tg(tetO-TauP301L)⁴⁵¹⁰ and Tg(tetO-tdTomato-Syp/mut4EGFP) transgenes were crossed with a line expressing the tetracycline sensitive transcriptional activator controlled by the Klk8 neuropsin promotor (EC-tTA), which expresses the tTA heavily in the EC and not at all in the dentate gyrus (72). These crosses generated mice expressing both rTgTau^{EC} + EC-tdTomato/Syp-GFP. Blocks containing entorhinal cortex and hippocampus were sectioned for array tomography from both male and female animals at 3 months ($n=3$), 9 months ($n=4$) and 18 months ($n=4$). A mouse without P301L Tau expression which did express tdTomato and GFP was included as a negative control for T22 staining and there was a no primary negative control condition. Animals were maintained on a 12h light/dark cycle with ad libitum access to food and water. All animal experiments were

approved by the Harvard Medical School Institutional Animal Care and Use Committee and the UK Home Office.

METHOD DETAILS

Array tomography

Tissue samples were fixed in 4% paraformaldehyde for 3 hours, dehydrated, and embedded in LR White resin. 70nm serial sections were cut with a diamond knife (Diatome) using an ultracut microtome (Leica). Ribbons of 15-30 serial sections were collected onto gelatin coated coverslips and immunostained with antibody combinations shown in Table S1. Images were obtained with a 63x 1.4 NA objective on an AxioImager (Zeiss) or Leica TCS confocal (Leica). In study 1, images were taken in regions containing NFTs, dystrophic neurites surrounding plaques, or no pathological lesions. Pathological lesions were identified by 2 experienced investigators (CD and MCC) based on international guidelines as we have used previously (Figure S1) (73–75). Only one microscope was used for imaging each experimental condition. Image from the same location in each serial section along a ribbon were aligned, thresholded, and parameters quantified using in house scripts in Image J, MatLab (Mathworks), and Python. All software is freely available on GitHub (<https://github.com/Spires-Jones-Lab>). Saturation was minimized during image acquisition and only applied for figure visualization. 3D reconstructions were performed with Imaris software (Bitplane). For more details, please see our methods video demonstrating this technique at <https://doi.org/10.7488/ds/297>.

Immunogold-electron microscopy

Tissue samples were collected at autopsy and fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB) for 48 hours at 4°C. After fixation, the tissue was washed with 0.1M PB twice and stored at 4°C

prior to processing and embedding. Tissue processing was carried out using an Electron Microscope Tissue Processor (Leica). In the processor, the tissue was first placed in 0.1% osmium tetroxide (O014, TAAB Laboratories) in boiled distilled water for 1 hour. Thereafter, samples were dehydrated in a series of solutions in preparation for embedding: 3 cycles of 5 minutes each in boiled distilled water, 50% ethanol, 70% ethanol and 90% ethanol. This is followed by 2 cycles of 15 minutes in 100% ethanol, and 2 cycles of 2 minutes in propylene oxide. The samples were then placed in a mixture of half propylene oxide (AGR1080, Agar Scientific) and half LR white resin (AGR1281, Agar Scientific) for 30 minutes, before being transferred to a full solution of LR white resin for 2 cycles of 30 minutes. These tissues were then taken out of the processor, placed in a gelatin capsule (C087/1, TAAB Laboratories), and filled up with LR white resin to the brim. Polymerisation of the LR white resin was allowed to occur by thermal curing at 52°C for 24 hours. These resin-embedded tissue blocks were then stored at room temperature until ready for immuno-EM staining. The resin-embedded tissue blocks were cut into 50nm ultrathin sections using an ultramicrotome (UC6, Leica) fitted with a Histo Jumbo Diamond Knife (Diatome Knives) and placed onto formvar-coated nickel grids (S162N1, Agar Scientific). Prior to immuno-EM staining, residual osmium tetroxide in the tissue were removed by incubating the tissue-covered nickel grids in saturated sodium metaperiodate in boiled distilled water for 1 minute. The nickel grids were then incubated in 1% sodium borohydride in 0.1M PB for 5 minutes to reduce free aldehydes, and thereafter incubated in 50mM glycine in 0.1M PB for 10 minutes to inactivate residual aldehyde groups. The nickel grids were then blocked in blocking buffer (0.1% BSA, 0.1% fish skin gelatin, 0.05% Tween in 0.2M PB, pH7.4) for 1 hour at room temperature. Rabbit T22 primary antibody (1:50, courtesy Dr Rakez Kayed) was diluted in antibody dilution buffer (0.1% BSA, 150mM sodium chloride in 0.2M PB, pH7.4) and incubated with the nickel grids overnight at 4°C. The next

day, the nickel grids were washed in 6 rinses of 0.2M PB. Goat anti-rabbit 10nm gold-conjugated secondary antibody (1:50, ab39601, Abcam) was diluted in antibody dilution buffer and incubated with the nickel grids for 90 minutes at room temperature. The nickel grids were then washed in 6 rinses of 0.2M PB, fixed in 2.5% glutaraldehyde in 0.2M PB for 15 minutes, and washed in 6 rinses of 0.2M PB again. Negative staining of the nickel grids was carried out to improve imaging contrast. This was done in a carbon dioxide free environment: incubated the nickel grids with 3% uranyl acetate in 50% ethanol for 15 minutes and 3% lead citrate in boiled distilled water for 150 seconds, rinsed thrice in boiled distilled water in between the steps. Once the nickel grids were dry, images were captured using a Transmission Electron Microscope (JEM-1400 Plus, JOEL) and viewed using ImageJ (Version 1.44).

Direct stochastic optical reconstruction microscopy (dSTORM) acquisition and image reconstruction

For the reconstruction of the images using dSTORM, sections 70 nm thick were stained as previously described (76), using the primary antibodies T22 (Millipore) or AT8 (Innogenetics) and Synaptophysin (R&D Systems), and the secondary antibodies Alexa 488 (ThermoFisher) and Alexa 647 (ThermoFisher), finally stained with Hoechst 33258 (Life Technologies) to visualise the nuclei, then the coverslips were mounted with OXEA buffer (77). STORM images were acquired using a Nikon N-STORM system configured for total internal reflection fluorescence (TIRF) imaging. Laser inclination was tuned to adjust focus and to maximize the signal-to-noise ratio. AlexaFluor-647 was excited illuminating the sample with the 647 nm (20% of 160 mW) and AlexaFluor-488 with the 488 (40% of 80 mW) lasers line built into the microscope. Fluorescence was collected by means of a Nikon 100x, 1.49 NA oil immersion objective and passed through a quad-band pass dichroic filter (97335 Nikon). 10,000 frames at 20 ms integration time were acquired for

each channel. Images were recorded onto a 256×256 pixel region of a Hamamatsu ORCA Flash 4.0 CMOS camera ($0.16 \mu\text{m}$ pixel size). STORM images were analysed with the STORM module of the NIS element Nikon software (78).

DNA points accumulation for imaging in nanoscale topography (DNA-PAINT)

DNA Conjugation to the AT8 and T22 antibodies and donkey anti-rabbit IgG (H+L) cross-absorbed secondary antibody (Invitrogen, 31238) was performed using the Siteclick Antibody Azido modification Kit (Thermo Fisher Scientific, S20026) according to the manufacturer's instructions. Briefly, after buffer exchange, $200 \mu\text{g}$ of the AT8, T22 antibodies and secondary antibody were incubated for 6 h at 37°C with β -galactosidase. To conjugate an azido group to the antibodies, they were incubated over-night at 30°C in a mixture of UDP-GalNAz and GalT enzyme. After purification, the modified antibodies were incubated with DBCO-DNA (2-ACCACCACCACCACCACCA, $2 = 5'$ DBCO TEG, ATDBio Ltd.) in a 1:5 ratio over-night at 25°C . The remaining unconjugated DNA was removed with 100 kDa Amicon columns (Merck, UFC510024).

Frame seal slide chambers ($9 \times 9 \text{ mm}$, Biorad, Hercules, USA) were affixed to the AT sections onto the coverslip, which were stained as previously described (Kay K. R. *et al.* 42) 1) using AF488-labelled synaptophysin (1/100 dilution) and either DNA-conjugated AT8 (100 nM) or DNA-conjugated T22 (100 nM) antibodies or 2) using PSD95 (Cell Signaling Technology, D27E11 XP rabbit mAb) (1/50 dilution) and DNA-conjugated donkey anti-rabbit IgG (H+L) cross-absorbed secondary antibody with either DNA-conjugated AT8 (100M) or DNA-conjugated T22 (100nM) antibodies. ATTO655-tagged imager strand (GGTGGT-ATTO655, ATDBio Ltd.) was diluted in GLOX buffer ($40 \mu\text{g/mL}$ Catalase, 0.5mg/mL glucose oxidase, 10% w/v glucose and 200 mM β -mercaptoethanol) and used at a final imaging concentration of 2nM or 4 nM. Cy3B-tagged imager strand (AGAGAGAX-Cy3B, ATDBio Ltd.) was diluted in

imaging buffer (5 mM Tris-HCl pH 8.0, 1mM EDTA, 75 mM MgCl₂, 0.05% Tween-20) and at a final imaging concentration of 2 nM.

DNA-PAINT for synaptophysin and AT8 or T22 combined with dSTORM was performed on an Oxford Nanolmager (ONI) super-resolution microscope equipped with four laser lines (405, 488, 561, and 638 nm) and a 100x oil-immersion objective (Olympus 1.49 NA). Fluorescence images were acquired by exciting multiple 50 µm x 80 µm areas with 488 nm laser for AF488-labelled synaptophysin (dSTORM, laser power 44–47 mW, interval excitation with 405 nm laser per 200 frames, 5,000 frames with an exposure time of 50 ms) or 638 nm laser for ATTO655-tagged imaging strands targeting the conjugated AT8 or T22 antibodies (DNA-PAINT, laser power 60–66mW, 5,000 frames with an exposure time of 50 ms). The laser was set at 53° incidence angle. Images were recorded by NimOS software associated with the ONI instrument.

DNA-PAINT for PSD95 and AT8 or T22 was performed using a custom-built TIRF microscope, restricting excitation of fluorophores within the sample to 200 nm from the sample-coverslip interface. The fluorophores were excited at either 561 nm (Cy3B) or 638 nm (ATTO655). Collimated laser light at wavelengths of 561 nm (Cobolt DPL561-100 DPSS Laser System, Cobalt, Sweden) and 638 nm (Cobolt DPL638-100 DPSS Laser System, Cobalt, Sweden) were aligned and directed parallel to the optical axis at the edge of a 1.49 NA TIRF Objective (CFI Apochromat TIRF 60XC Oil, Nikon, Japan), mounted on an inverted Nikon TI2 microscope (Nikon, Japan). A perfect-focus system corrected the imaging process for any stage-drift. Fluorescence was collected by the same objective and separated from the TIR beam by a dichroic mirror Di01-R405/488/561/635 (Semrock, Rochester, NY, USA). Collected light was then passed through appropriate filters (561 nm: LP02-568-RS, FF01-587/35 (Semrock, NY, USA, 638 nm: FF01-432/515/595/730-25, LP02-647RU-25 (Semrock, NY, USA)). The emission beam was passed through a 2.5x beam

expander and focussed onto an EMCCD camera for image collection (Delta Evolve 512, Photometrics, Tucson, AZ, USA) operating in frame transfer mode (EMGain = 11.5 e⁻/ADU and 250 ADU/photon). Pixel size was 103 nm. Images were recorded with an exposure time of 50 ms with 638 nm illumination, followed by 561 nm excitation. The microscope was automated using the open-source microscopy platform Micromanager (NIH, Bethesda).

DNA-PAINT analysis

The positions of the transiently immobilized DNA imager strands within each frame were determined using the PeakFit plugin (an imageJ/Fiji plugin of the GDSC Single Molecule Light Microscopy package (http://www.sussex.ac.uk/gdsc/intranet/microscopy/imagej/gdsc_plugins) for imageJ using a 'signal strength' threshold of 50 and a precision threshold of 20 nm.

Immunohistochemistry (IHC)

Paraffin sections were stained with AT8 using the Novolink polymer detection system and visualized using 3,3'-diaminobenzidine chromogen then counterstained with haematoxylin to stain nuclei. ROIs containing cortical grey matter were delineated using QuPath 78 and colour deconvolved in ImageJ. Using a custom Matlab script, neurofibrillary tangles were identified minimizing the bias from manual counting.

Synaptoneurosome isolation and Western blot analysis

Brain homogenates (H) and synaptoneurosomes (S) were prepared as described in (48). In brief, ~ 200 mg of tissue from BA20/21 was homogenized on ice in 1ml homogenization buffer (25 mM HEPES pH 7.5, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, protease inhibitors (Roche complete mini), phosphatase inhibitors (Millipore, 524,629). The homogenate was filtered through an 80 µm nylon filter (Millipore, NY8002500) and 300 µl was saved as crude homogenate with buffer (100mM/L Tris-HCl pH 7.6, 4% SDS,

protease inhibitor cocktail EDTA-free 100x Thermo Fisher Scientific, Loughborough, UK). The remaining crude homogenate was further filtered through a 5 µm filter (Millipore, SLSV025NB) and centrifuged at 1000 g for 5 min. The supernatant was discarded and the pellet was washed with buffer and centrifuged again, yielding the synaptoneurosome pellet. For storage it was mixed with buffer (100mM/L Tris-HCl pH 7.6, 4% SDS, protease inhibitor cocktail EDTA-free 100x Thermo Fisher Scientific, Loughborough, UK). Protein concentrations were determined using a protein assay (Micro BCA TM Protein Assay Kit, Thermo Fisher Scientific, 23235).

Synaptoneurosomes preparations (2.5-10 µg/µl) were denatured at 95° C for 5 min. To detect oligomeric tau, 75 µg of protein were resolved using 4%–12% Bis–Tris gels (MOPS running) and transferred onto nitrocellulose membranes (Biorad, 1620112) in Trans-Blot turbo buffer (Biorad, 1704270) supplemented with 20% ethanol at 25 mV for 13 min in the Trans-Blot® Turbo™ Transfer System (Biorad, 1704150). After blocking 1h with Intercept® Blocking Buffer (LI-COR; 927-60001), the membrane was incubated with the indicated primary antibodies (α-tau5 antibody (Biolegend, 806401, 1:250) and α-GAPDH antibody (Cell Signaling, 2118,1:1000) overnight at 4°C. The protein bands were visualized using corresponding fluorescent secondary antibodies (Odyssey, 1:5,000). Infrared fluorescence was measured with the Odyssey CLx imager (LI-COR) and quantified using Image Studio software (LI-COR Biosciences).

Study design

The immunostaining, image acquisition, image processing and analyses were performed blinded to the clinicopathological diagnosis. Bias was also minimized by setting the same parameters for image acquisition and image analysis for all cases.

Quantification and statistical analysis

Differences in age and PMI were assessed using Kruskal-Wallis and one-way ANOVA, respectively. Distributions of data were examined using histograms and Shapiro-Wilks tests. The majority of statistical analyses used mixed effect models (lmerTest package in R) to investigate the effect of disease status, APOE and sex on the variable of interest, while controlling for potentially confounding variables and specifying the random effect to account for repeated measures. Models were generated and residuals assessed. If data failed to meet model assumptions data was transformed using Tukey or Box-Cox transformations.

After running the mixed effects model, an ANOVA (stats package - Type III Analysis of Variance Table with Satterthwaite's method) was performed on the model output to determine the main and interaction effects. If the interaction was significant or if the main effect was significant and had more than 2 levels, the emmeans package was used to compute multiple comparisons, where the Kenward-Rogers method for degrees of freedom and the Tukey method for P value adjustment were employed.

Correlations used Pearson's or Spearman's methods, dependent on whether data met assumptions for parametric testing or not. Statistical significance was set at 5% ($\alpha = 0.05$). Statistical details can be found in the figure legends and results text, including the statistical tests used, exact value of n, what n represents, and dispersion and precision measures. All the analyses were performed with R (80) and the scripts and full statistical results can be found at <https://doi.org/10.7488/ds/3837>.

RESULTS

Excitatory synapses are lost in Alzheimer's disease

Using human post-mortem brain samples prepared for array tomography (Table 1), we first replicated the observations from previous studies where synapse loss has been observed in AD (1-3,36,37). Using array tomography, we imaged 1,315,583 individual excitatory synaptic pairs in inferior temporal cortex (BA20/21). Synaptic pairs were defined as a synaptophysin-positive presynaptic puncta with a PSD95 puncta within $0.5\ \mu\text{m}$ (distance between centroids). This distance was chosen based on electron microscopy data and analysis of synaptic pairs in this array tomography dataset with differing cut-off distances (Figures S1A and S1B).

We observed a 1.25-fold decrease in excitatory synapse density in AD when compared to controls (Control median= $4.29 \times 10^8 \pm 7.49 \times 10^7$ (n=12); AD median= $3.45 \times 10^8 \pm 9.99 \times 10^7$ (n=20); $\beta = -5314.01$; $p = 0.023$; Figures 1A and B). Next, we investigated the relationship between pathological aggregates and synaptic loss in the immediate environment. To do this, regions of interest were chosen at 63x magnification with either a neurofibrillary tangle, neuritic plaque, or no aggregate in the centre of the field of view ($140.6 \times 106.6\ \mu\text{m}$ area; Figure S1C). A decrease in synapse density was observed around clumps of dystrophic neurites (DNs) containing tau which surround amyloid aggregates in neuritic plaques (Control $\beta = 3862$; $p = 0.005$; AD $\beta = 3862$; $p = 0.005$; 35,36). In contrast to DNs, the presence of NFTs was not associated with a decrease of excitatory synapse in the immediate environment (Figures 1A and C).

Table 1. Demographic, neuropathological & genetic characteristics of human subjects AD,Alzheimer’s disease; APOE,Apolipoprotein E; BBN,Medical Research Council Brain Bank Number; SD,Sudden Death Brain Bank number; NA,not available; PMI,post-mortem interval. Study number as included in Table S1.

BBN	Clinical Diagnosis	APOE genotype	Age (yrs)	Sex	PMI (hrs)	Brain weight (g)	Brain pH	Braak stage	Thal phase	Study
BBN_19686	Control	3/3	77	F	75	1320	6.5	I	I	1,9
001.26495	Control	3/3	78	M	39	1290	6.17	I	0	1,2,5,7,8,9
001.28402	Control	3/3	79	M	49	1503	6.33	I	2	1
001.28406	Control	3/3	79	M	72	1437	6.13	II	2	1,2,5,6,7,8,9
001.28793	Control	3/3	79	F	72	1219	5.95	II	1	1,2,5,6,7,8,9
001.28797	Control	3/3	79	M	57	1301	6.11	0	0	1,2,5,6,7,8
001.32577	Control	3/3	81	M	74	1313	6.07	II	3	1
001.35215	Control	3/3	82	M	40	1246	6.01	I	NA	1
001.35549	Control	3/3	82	M	56	1326	6.01	I	1	1
001.29082	Control	3/4	79	F	80	1339	5.96	III	5	1,2,5,6,7,8
001.34131	Control	3/4	82	M	95	1472	5.97	IV	3	1,2,5,6,7,8,9
001.35181	Control	3/4	82	M	49	1496	6.12	II	2	1,2,5,6,7,8,9
001.28794	Control	2/3	79	F	61	1289	5.76	I	0	2,5,7,8,9
001.29086	Control	3/3	79	F	68	1468	6.2	0	1	2,5,6,7,8,9
001.35866	Control	NA	83	F	78	1158	5.96	IV	4	2,5,6,7,8,9
001.36435	Control	3/3	84	M	95	1222	6.00	II	1	3
001.35511	Control	2/3	86	M	123	1553	6.17	II	1	3
001.36135	Control	3/3	84	F	30	1214	5.93	II	1	3
001.36809	Control	3/3	85	M	3	1400	6.10	II	1	3

BBN_24527	AD	3/3	81	M	74	1160	6.1	V	4	1,2,5,6,7,8,9
001.28410	AD	3/3	62	F	109	1029	6.04	VI	5	1
001.28771	AD	3/3	85	M	91	1183	5.95	VI	5	1,2,5,6,7,8,9
001.29081	AD	2/3	90	F	110	943	6.06	VI	5	1
001.32929	AD	3/3	85	F	80	1354	6.03	VI	5	1,2,5,6,7,8,9
001.35182	AD	3/3	66	M	49	1225	6.12	VI	5	1,9
BBN_24322	AD	3/4	80	M	101	1410	6.10	VI	5	1,2,5,7,8
BBN_24526	AD	3/4	79	M	65	1300	6.05	VI	NA	1
BBN_24668	AD	3/4	96	F	61	1082	6.11	VI	NA	1
BBN_25739	AD	3/4	85	F	45	1375	5.77	VI	5	1,2,5,6,7,8,9
001.26718	AD	3/4	78	M	74	1367	6.13	VI	5	1,2,5,6,7,8,9
001.29135	AD	3/4	90	M	73	1275	6.44	VI	3	1
001.29521	AD	3/4	95	M	96	1221	6.08	VI	5	1
001.29695	AD	3/4	86	M	72	1300	6.1	VI	5	1,2,5,7,8,9
001.30142	AD	3/4	88	F	112	1054	5.96	VI	5	1,2,5,6,7,8,9
001.30883	AD	3/4	61	F	69	1060	6.15	VI	5	1
001.30973	AD	3/4	89	F	96	1210	6.03	VI	5	1,2,5,6,7,8,9
001.33636	AD	3/4	93	M	43	1117	5.92	VI	5	1
001.35096	AD	3/4	72	M	103	1209	6.18	VI	5	1,2,5,6,7,8
001.35535	AD	3/4	83	F	95	1400	6.55	VI	4	1
001.35564	AD	3/4	90	F	52	1075	5.99	VI	5	3
001.36297	AD	3/4	84	M	87	1163	5.82	VI	5	3
001.36346	AD	NA	90	F	60	932	6.1	VI	5	3
	AD	NA	90	F	18	1110	NA	V	5	4

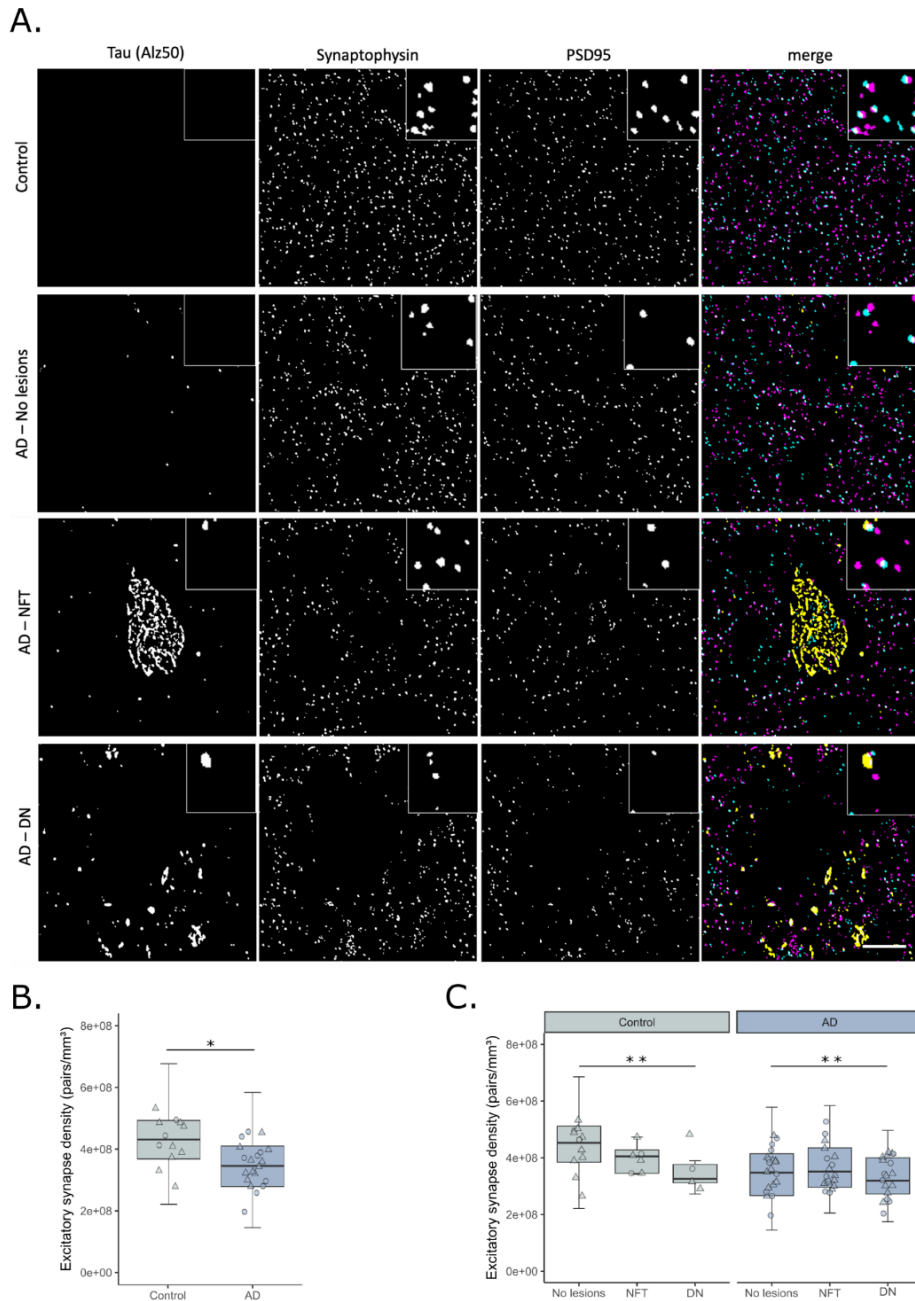


Figure 1. Excitatory synapse loss in BA20/21 of AD cases and its relationship to hallmark tau aggregates. (A) Representative single 70-nm-thick segmented images of a control case and AD cases with no hallmark tau aggregates (AD-no lesions), a neurofibrillary tangle (AD-NFT), or dystrophic neurite clumps around neuritic plaques (AD-DN). In merged composite images, synaptophysin presynaptic terminals are shown in magenta, PSD95 postsynaptic terminals in cyan, and misfolded tau (Alz50) in yellow. (B) Excitatory synapse density was decreased in AD cases when compared to controls. (C) The quantification of synaptic terminals split by the presence of tau aggregates. Boxplots show quartiles and medians calculated from each image stack. Data points refer to case means (females, circles; males, triangles). Analysis was with linear mixed effects models, including diagnostic group, sex, and pathology in the image (no effect of sex). Scale bar represents 10 μ m. Insets, 5 $\times$ 5 μ m. NFT, neurofibrillary tangle; DN, dystrophic neurite. * $p < 0.05$, ** $p < 0.01$. See also Figure S1 and Table S1.

Oligomeric, misfolded, and phosphorylated tau species accumulate in postsynaptic terminals of AD cases

We next investigated the presence of tau at postsynaptic terminals. Three species of tau were studied: oligomeric (T22 antibody, characterized by Lasagna-Reeves (21)), misfolded (Alz50 antibody) and Ser202, Thr205 phosphorylated (AT8 antibody) (Figures 2A-E and S2A-S2I). All three tau species were found in postsynaptic terminals of AD cases, with a higher presence of oligomeric tau stained with T22 than the other markers (Figure 2F). Control cases also had a small amount of postsynaptic tau pathology. Interestingly, T22 and AT8 were often found in the same postsynapses while Alz50 was largely found in different synapses of AD cases (Figure 2G). There was a stronger correlation between postsynaptic tau species in AD than control cases (Figure 2G).

PSD95 puncta size did not differ between control and AD cases (Figure S2J). However, there were differences in PSD95 volume when tau species were present (ANOVA after linear mixed effects model $F[3,102.3]=6.85$, $p<0.001$). All tau species had a tendency to colocalise with bigger postsynaptic terminals, with T22 being the highest observed increase, especially in control cases. We observed similar results when looking at PSD95 signal intensity as a proxy for protein levels. When looking at all postsynaptic terminals, signal intensity did not differ between control and AD cases (Figure S2K). Again, there were differences when looking at the presence of different tau species and signal intensity (ANOVA after linear mixed effects model $F[3,98.3]=8.97$, $p<0.001$). There was an overall increase of postsynaptic intensity when each tau species were present, with T22 being the highest observed increase in control cases. Taken together, both the PSD95 size and intensity seem to be related to the presence of tau species, with oligomeric tau (T22) associated with the highest increases.

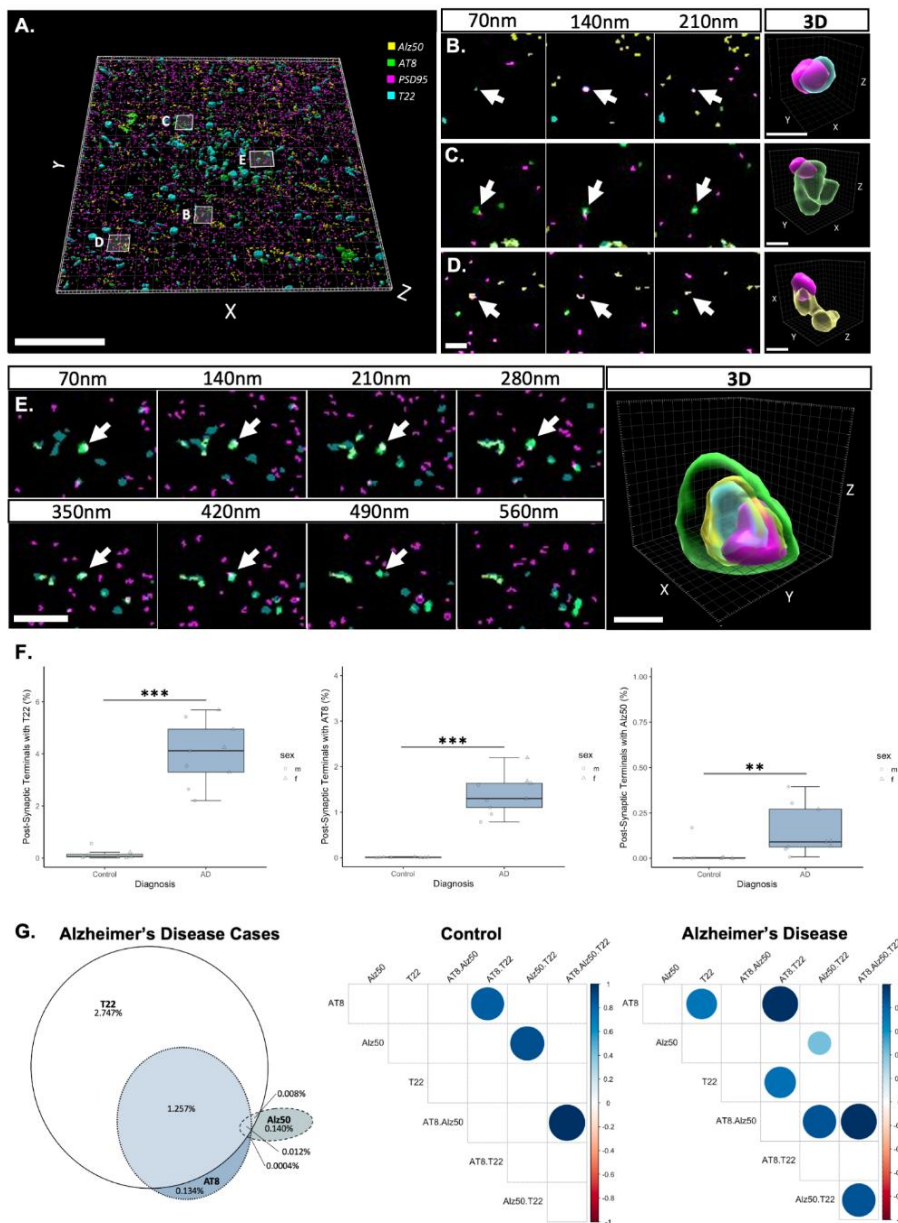


Figure 2. Different tau species accumulate in postsynaptic terminals. (A) An aligned and segmented three-dimensional image stack from an AD case stained for oligomeric tau (T22, cyan), misfolded tau (Alz50, yellow), Ser202, Thr205 phosphorylated tau (AT8, green), and postsynaptic densities (PSD95, magenta). (B–E) All three types of tau can be observed in postsynapses, as shown in serial sections and three-dimensional reconstructions of individual synapses. (F) Quantification (F) reveals increases in synaptic terminals containing tau in AD compared to control. Boxplots show quartiles and medians calculated from each image stack. Data points refer to case means (females, circles; males, triangles). *** $p < 0.001$, ** $p < 0.01$. (G) A Venn diagram of the percentage of synapses that contain tau staining in AD cases (G, left) reveals that T22 is found in more synapses than the other species and that it colocalizes often with AT8. Alz50 colocalizes less with the other species. Correlation plots (G, right) show differences ($p < 0.01$) between markers in control and AD cases. For example, synaptic T22 correlates with synaptic AT8 in AD but not in control cases. Scale bars represent 20 μm in (A), 2 μm in left panels of (B)–(E), and 0.5 μm in 3D reconstructions in (B)–(E). See also Figure S2.

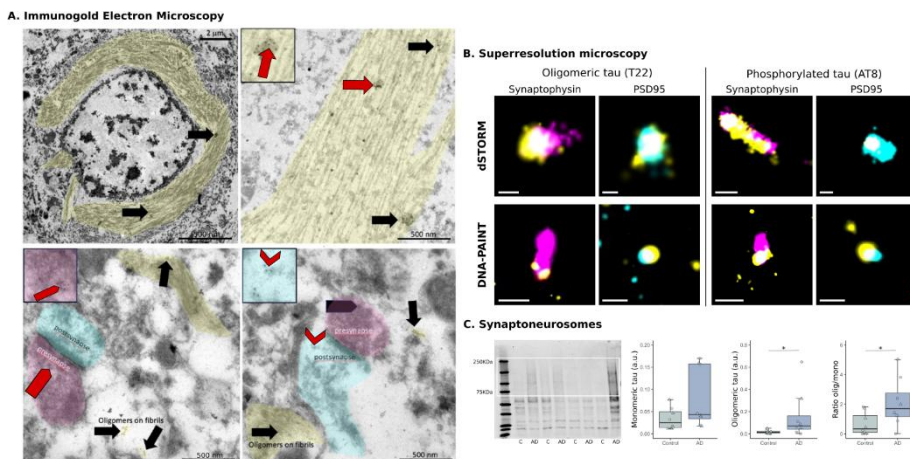


Figure 3. Confirmation of AD synaptic tau aggregates. (A) Immunoelectron microscopy with gold-conjugated secondary antibodies (black dots) shows T22 labels globular-appearing oligomers associated with fibrils (arrows) in neurofibrillary tangles and on fibrils in neurites (shaded yellow). T22 immunogold staining is also observed on presynaptic vesicles (pentagons, presynapses shaded magenta) and in postsynaptic terminals near the postsynaptic density (chevrons, postsynapses shaded cyan). (B) dSTORM and DNA-PAINT were used on array tomography sections to confirm tau (yellow) and synaptophysin (magenta) or PSD95 (cyan) colocalization. (C) Full western blot from synaptoneurosomes isolated from control or AD cases is shown at left and the quantification of oligomeric and/or monomeric tau bands at right. Boxplots show quartiles and medians (females, triangles; males, circles). * $p < 0.05$. C, control; SAD, Alzheimer's disease. Scale bars EM: 500 nm (insets 250 $\times$ 250 nm), dSTORM: 100 nm, DNA-PAINT: 200 nm. See also Figure S3.

Synaptic oligomeric tau precedes the presence of neurofibrillary tangles

To determine whether oligomeric tau accumulation in synapses is an early pathological event, we next investigated the regional relationship between the presence of NFTs and the synaptic accumulation of oligomeric tau.

Temporal cortex (BA20/21) and primary visual cortex (BA17) samples from 10 AD cases and 10 controls were investigated for the presence of oligomeric tau at synaptic terminals by array tomography microscopy and for the presence of NFTs in larger fields of view by IHC on paraffin sections from the same cases and brain regions (Figure 4A). We found that in areas with no or low NFT counts, there was already oligomeric tau accumulating at presynaptic terminals both in control and AD cases (Figures 4A and B). There was a positive correlation between NFTs and the total amount of oligomeric tau, especially in brain areas highly affected by tau pathology (AD cases at BA20/21; $R=0.81$, $p=0.022$; Figure 4C). However, the proportion of oligomeric tau at synaptic terminals was reduced as more oligomeric tau was present, especially in BA17 where there is less overt pathology (AD cases at BA17; $R=-0.87$, $p=0.0027$; Figure 4D).

To confirm whether synaptic oligomeric tau is an early phenotype, we compared staining of AT180 which labels tau phosphorylated at residue 231 (reported to be an early marker of tau pathology (39)), with oligomeric tau in synapses. While both types of tau are more prevalent in AD than control synapses, we observe 3.3-fold more T22 than AT180 labelled presynaptic terminals in BA20/21 of AD cases (presynaptic terminals with T22: Control median= $0.297 \pm 2.038\%$ ($n=9$); AD median= $5.061 \pm 3.389\%$ ($n=9$); $\beta=4.15$; $p=0.004$; Figure S4B. Presynaptic terminals with AT180: Control median= $0.0219 \pm 0.697\%$ ($n=9$); AD median= $1.534 \pm 1.441\%$ ($n=9$); $\beta= 1.537$; $p=0.01$; Figure S4C).

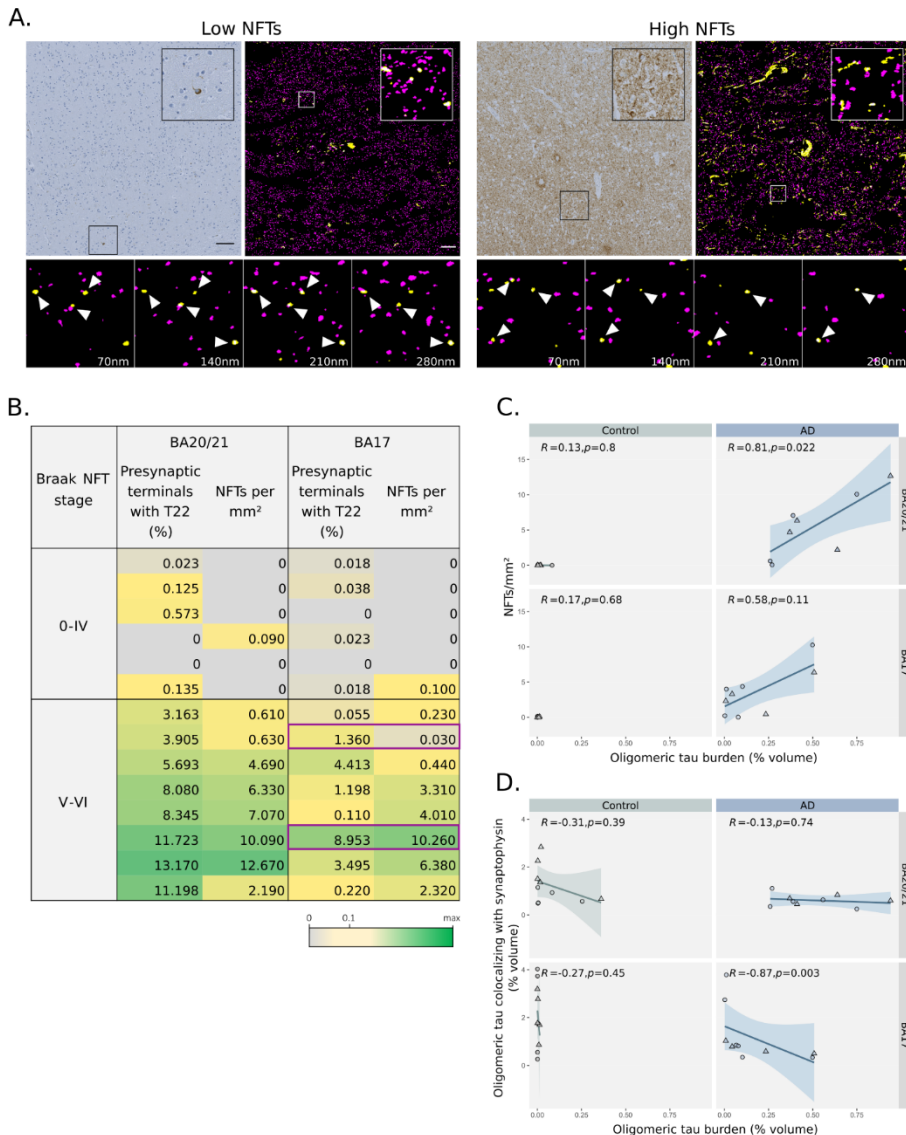
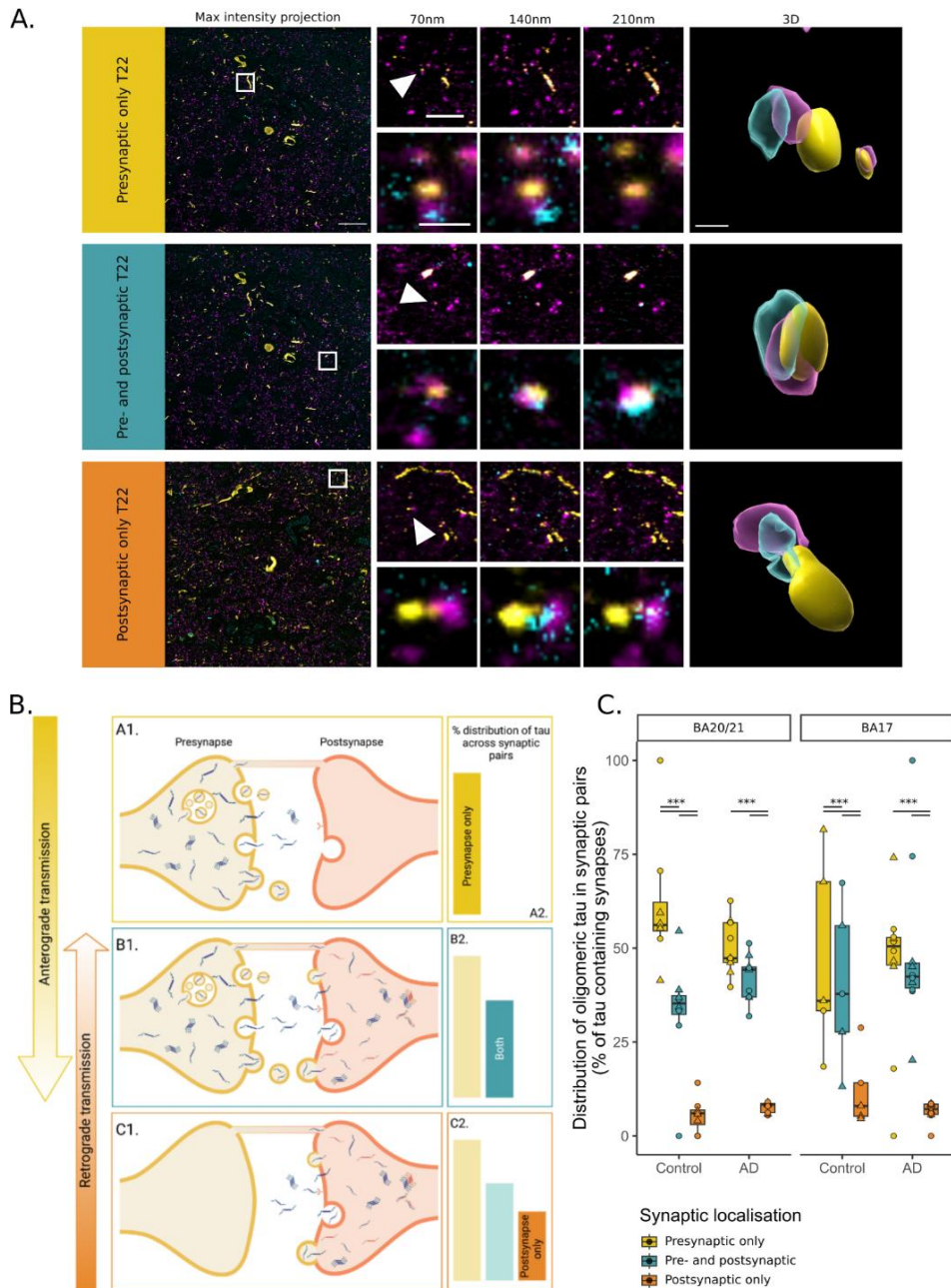


Figure 4. Synaptic oligomeric tau precedes the presence of neurofibrillary tangles. (A) Representative image of a case with spare (left panel) and high (right panel) NFT density. Top left images show tau AT8 immunoreactivity by immunohistochemistry and the inset highlights the presence of NFTs. At top right is a maximum-intensity projection of 12 consecutive 70-nm-thick sections by array tomography microscopy. Presynaptic synaptophysin-positive terminals (magenta) and oligomeric tau (T22, yellow) are shown. The inset highlights the presence of presynaptic terminals with oligomeric tau that are pointed by arrowheads in the horizontal panel of four consecutive 70-nm-thick sections. (B) Table displaying the density of synaptic terminals that contained oligomeric tau and the NFT density per case, area, and Braak NFT stage. Highlighted in purple are the cases shown in (A). (C and D) (C) Correlation plots between the density of NFTs and the percent volume of the image occupied by T22 and (D) correlation plots between the percent of T22 that colocalizes with presynaptic terminals and the total amount of T22. Spearman correlation results are shown in each condition. Data points refer to case means (females, circles; males, triangles). Scale bars: left, 100 μ m; right, 10 μ m. Inset sizes: left, 160 $\times$ 160 μ m; right, 10 $\times$ 10 μ m. See also Figure S4.

Indirect evidence of transsynaptic transmission of tau aggregates

The finding of tau accumulating at synapses of AD cases even in areas with little tau pathology is in line with the hypothesis that tau may spread *trans-synaptically*. To further explore this possibility, the subset of tau-containing excitatory synaptic pairs were explored for pre- and postsynaptic location of tau (Figure 5A). Oligomeric tau was differentially located in the synaptic compartments (ANOVA after linear mixed effects model $F[2,88]=73.61$, $p<0.0001$); it was more commonly found in presynaptic terminals only, with a gradient towards postsynaptic localization (Figure 5B). In inferior temporal cortex, BA20/21, we confirmed that misfolded tau antibody (Alz50) was also found in both sides of the synapse (Figures S5A-S5E).

Finally, to overcome the descriptive nature of post-mortem studies, we investigated transsynaptic spreading of oligomeric tau in an animal model. We previously demonstrated that human tau can spread from pre- to postsynaptic terminals in a mouse model with overexpression of human P301L mutant tau and synaptophysin tagged with GFP in entorhinal cortex (34). Here, tissue from this same mouse model was stained with T22 to show that oligomeric tau can spread from pre- to postsynaptic terminals in mammalian brain (Figure S5F).



DISCUSSION

In the present work, we show that oligomeric, phosphorylated and misfolded tau can be detected within synapses of human AD cases. As far as we know, this study represents the first comprehensive visualization of oligomeric tau at synaptic terminals in human tissue. The presence of oligomeric tau at synaptic terminals was observed even in cases with low NFT pathology and represented a higher proportion of the tau burden in regions with little NFT pathology, suggesting an early synaptic localisation of oligomeric tau. The distribution of oligomeric tau at synapses was asymmetric with the highest proportion in presynapses and a gradient towards the postsynapse. This is indirect evidence for transsynaptic anterograde transmission of tau.

Synaptic loss and synaptic tau

Synaptic loss and alterations in synaptic markers represent one of the earliest findings in AD-affected brain regions and are the best pathological correlate of cognitive decline (2,40,41). Here, we used array tomography microscopy (42,43), immunogold-electron microscopy, dSTORM, and DNA paint, which allowed us to resolve different tau species in single synaptic terminals.

With this approach we first replicated the synaptic loss associated with AD, as we and others have shown with several techniques (36,37,41). Previous studies using array tomography in human post-mortem brain identified synapse loss in areas surrounding amyloid- β (A β) plaques, with synapse loss correlating with distance from the plaque (36,37). In this study, paired presynaptic and postsynaptic terminals were reduced in AD and reduced further near neuritic plaques identified by surrounding dystrophic neurites. In contrast, we did not observe synapse loss in the direct vicinity of neurofibrillary tangles. While amyloid plaques are extracellular lesions surrounded by oligomeric A β that is known to be toxic to synapses,

neurofibrillary tangles are intracellular lesions which are thought to be harmful to the neuron containing them or to reflect a sequestering of toxic tau. This toxicity could induce loss of synapses formed by the tangle-bearing neuron, which are typically distant from the cell body, sometimes even in the opposite hemisphere. Some of the postsynaptic dendritic spines of tangle-bearing neurons are likely within the same image stack as the soma, but many of these will also be out of frame. We thus postulate that while plaques cause local synaptic toxicity, tau-induced synaptotoxicity is more diffuse throughout the brain. Second, we found that oligomeric, phosphorylated and misfolded tau all accumulate in synaptic terminals. We observed an increase in signal intensity and volume in tau-containing postsynaptic terminals, which may reflect compensation for the injured environment, something that has been suggested previously by others (40,44). However, we need to be cautious interpreting these data, as light microscopy techniques are sensitive and subtle changes may affect both size and intensity. We have previously observed synaptic tau with antibodies raised to total tau, misfolded tau (Alz50) and tau phosphorylated at serine 202 and threonine 205 (AT8) (11-13). Here, we combined them and found that oligomeric tau is the most common tau species in postsynapses. This is an important finding as animal studies indicate oligomeric tau is particularly synaptotoxic (15). In previous post-mortem studies, analysis of synaptosomes (45-49) and synaptic fractions of homogenised tissue (50) have identified tau to be localised to both synaptic compartments in control and AD tissue. However, methods involving biochemical isolation of synapses fail to consider the overall architecture of the intact tissue and may unwittingly select for certain populations of synapses. Tissue homogenisation can also be damaging to synapses, allowing soluble proteins to leak in or out of the preparations. Further, this method does not generally allow for study of multiple proteins within the same synapse. Array tomography overcomes some of these limitations, offering a

means for high-resolution characterisation of a large number of synapses *in situ* in human post-mortem tissue, and enables the assessment of synaptic density and protein composition (42).

The oligomeric tau we detected with immuno-electron microscopy is remarkably similar in morphology to oligomers biochemically isolated from human AD extracellular vesicles observed by atomic force microscopy (51). Here we demonstrate that globular structures containing oligomeric tau are associated with fibrils and within some synapses. We also observe tau labelling of synaptic vesicles consistent with observations in *Drosophila* that tau binds synaptic vesicle proteins and our previous work showing with array tomography that tau is present in presynaptic terminals (12,13). This finding was further confirmed with dSTORM and DNA-PAINT, which both demonstrated colocalization between pathological tau and presynaptic vesicle proteins with nanoscale resolution.

The finding of tau, especially oligomeric tau, accumulating at synapses may have implications for the understanding of AD pathogenesis. While many studies have focused on A β as the trigger for AD-related synaptic damage, tau can mediate synapse loss, both alone and in concert with A β (52). Most tauopathy models show synapse dysfunction and loss (7,53-55). And mounting evidence suggests that oligomeric, misfolded forms of tau mediate synaptotoxicity. Tau oligomers may induce morphological changes, impair plasticity and dysregulate synaptic transmission (56-59). Oligomers also induce synapse loss and impairment in memory function, which is rescued upon reduction of oligomer levels (60-62). Thus, at the synapse, tau oligomers may reduce synaptic proteins, alter neuronal signalling, promote synapse loss and impair memory function.

In this study, we also found oligomeric tau in cases with no or low NFT presence. These findings are in line with evidence from human cases

suggesting that diffuse and neuropil forms of tau precede NFT formation (18,39) and may involve oligomeric species (20,21). This early (according to NFT burden and the well established Braak staging) oligomeric tau was found to accumulate at synapses. The seeding activity of tau in synaptoneurosomes preparations has also been found to precede the amount of hallmark pathology as reflected by Braak stages (63), and seeding closely correlated with oligomeric tau (22). Of note, both high tau seeding and high levels of tau oligomers have been linked to worse cognitive outcomes (22,23). We also found that the proportion of oligomeric tau in presynaptic terminals was inversely correlated with the total burden of oligomeric tau. Paralleling Braak staging, these findings may imply that i.e. while primary visual cortex does not have enough pathology to be classified as Braak stage VI, we already find oligomeric tau at synapses that may be affecting neuronal circuits.

Synaptic localization of tau may be also important for propagation of tau through the AD brain. Oligomeric tau was distributed asymmetrically across synaptic pairs. This possibly suggests a model where tau accumulates presynaptically, before release and uptake at the postsynapse, leading to tau localization in both compartments. It is unlikely that transference of pathological tau to the postsynapse from the presynaptic terminal is the only propagation mechanism. Synaptic pairs in which tau is localized to the postsynapse only could arise from retrograde transport to the postsynapse (i.e., along dendrites and spines) (64). Further, tau could undergo retrograde transport across the synapse to the presynaptic site (65,66). However, considering the stepwise decrease in oligomeric tau localization observed at synaptic pairs (presynapse only > both terminals > postsynapse only), it is likely that tau predominantly traverses synapses in the anterograde direction. Oligomeric tau followed this pattern of distribution across synaptic pairs in both control and AD brain. However, the proportion of synapses with tau was significantly increased in AD brain, indicating that although the mode of

spread may be the same, propagation likely occurs at greater frequency in AD. While these findings gave us insights into the pathogenesis of AD and can have implications in therapeutical approaches, we need to be cautious because of the inherent descriptive cross-sectional nature of post-mortem studies.

Conclusions

In the present work we found that oligomeric, misfolded, and phosphorylated tau species are found at synaptic terminals of AD cases even when NFT burden is low. Given the potential early nature of these events in AD pathogenesis, we believe that these findings support the notion of multiple target and synaptic based approaches for therapeutic interventions.

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SUPPLEMENTARY MATERIAL

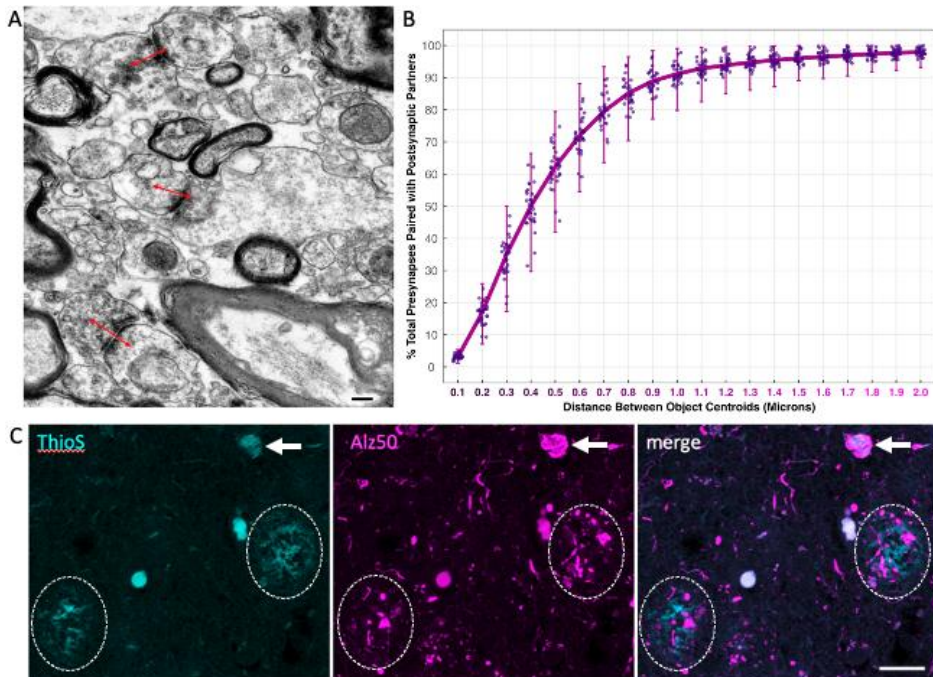


Figure S1. Synaptic pairs and identifying pathological lesions. Related to Figure 1. Synaptic pairs in array tomography were defined as pre and post-synaptic puncta with centroids within 500 nm ($0.5\ \mu\text{m}$). This is based on electron microscopy data (example in panel A) showing that the centre of presynaptic terminal staining is within 500nm of the PSD of the majority of synapses (red arrows 500nm in length). Further this cut-off in our array tomography data results in 60% of presynaptic terminals having a postsynaptic partner (B). This is lower than the expected 80-90% excitatory synapses but reduces the chances of counting false positives which is important for inferring trans-synaptic tau spread in this study. In the array tomography study, synapse density was assessed in proximity to Alz50 positive neurofibrillary tangle pathology or neuritic plaques defined by Alz50 positive dystrophic neurite patterns. To confirm these patterns of dystrophic neurites are indeed neuritic plaques, we used confocal imaging of paraffin sections stained with thioflavin S to label plaque and tangle fibrils and Alz50 immunohistochemistry and confirmed that Alz50 labels tangles (C, arrows) and that Alz50 positive dystrophic neurite surround ThioS positive plaques (C, circles). Scale bars represent 500nm in A, $20\ \mu\text{m}$ in C.

Different tau species accumulate in postsynaptic terminals

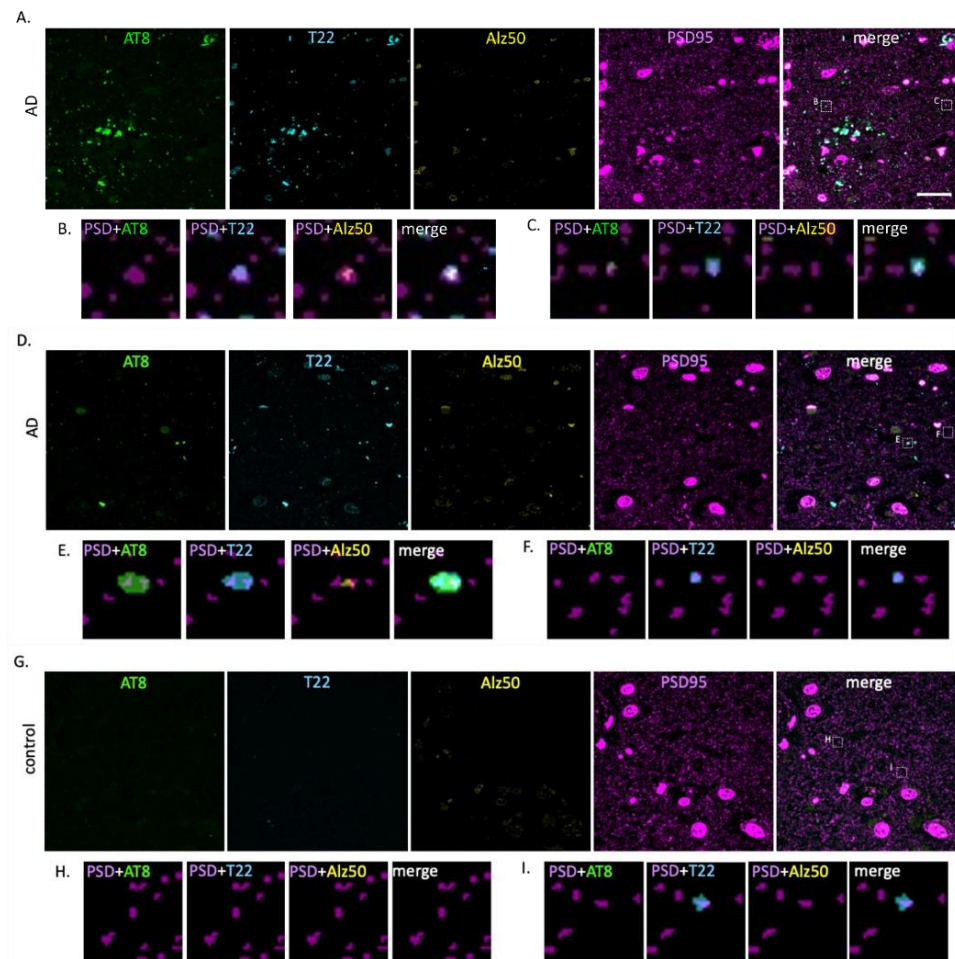
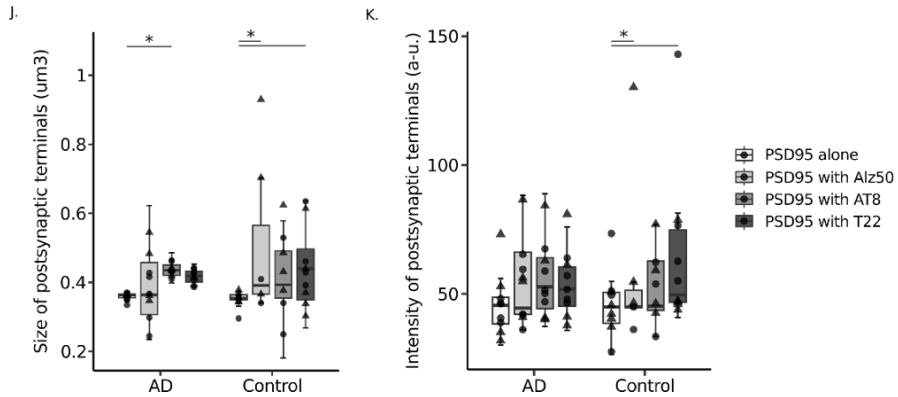


Figure S2. Different tau species accumulate in postsynaptic terminals. Related to Figure 2. Top panel shows three more examples of postsynaptic terminals containing tau. A., D and G shows a single 70nm-thick section from two AD and a control case stained with oligomeric tau (T22, cyan), misfolded tau (Alz50, yellow) and Ser202, Thr205 phosphorylated tau (AT8, green) and postsynaptic (PSD95, magenta). All three types of tau can be observed in postsynapses as shown in 5 μ m $\times$ 5 μ m insets below (B, C, E, F, H, I).

Postsynaptic terminals volume and intensity

Bottom panel of Figure S2. Shows quantification of postsynaptic terminals volume and intensity in relation to tau species present. In A are plotted the size of PSD95 puncta in AD and control cases depending on the presence of tau species. In B are plotted the mean signal intensity of postsynaptic terminals in AD and control cases in relation to the presence of tau species. Boxplots show quartiles and medians calculated from each image stack. Data points refer to case means (females, triangles; males, circles). Analysis was with linear mixed effects models including diagnostic group, sex and tau specie presence. * $p < 0.05$ in post-hoc emmeans test after linear mixed effects model. Abbreviations: a.u., arbitrary units. Scale bars represent 20 μm .

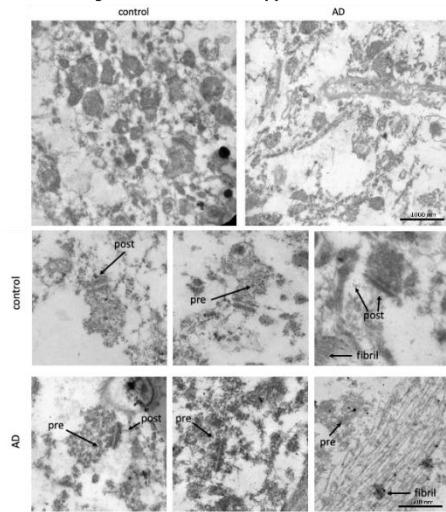
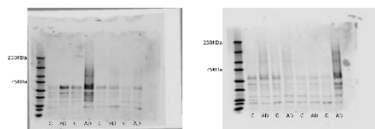
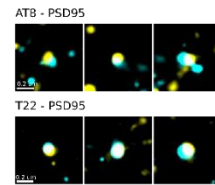
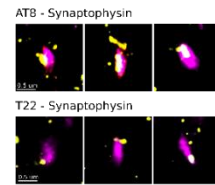
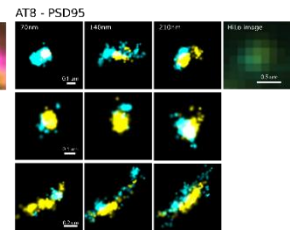
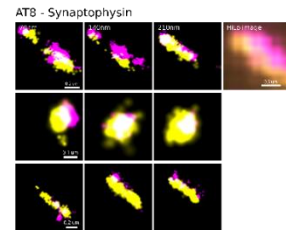
A. Immunogold Electron Microscopy**B. Synaptoneurosomes****C. DNA-PAINT****D. dSTORM**

Figure S3. Confirmation of AD synaptic tau aggregates. Related to Figure 3. A Immuno-electron microscopy with gold-conjugated secondary antibodies (black dots) shows T22 at pre- and postsynaptic terminals. Scale bar top = 1000nm bottom = 500nm. B Full western blots from synaptoneurosomes isolated from 8 control and 8 AD cases. C Examples of DNA-PAINT array tomography 70nm-thick sections confirm tau (yellow) and synaptophysin (magenta) or PSD95 (cyan) colocalization. D Examples of dSTORM array tomography 70nm-thick consecutive sections confirm tau (yellow) and synaptophysin (magenta) or PSD95 (cyan) colocalization after reconstruction of HiLo images.

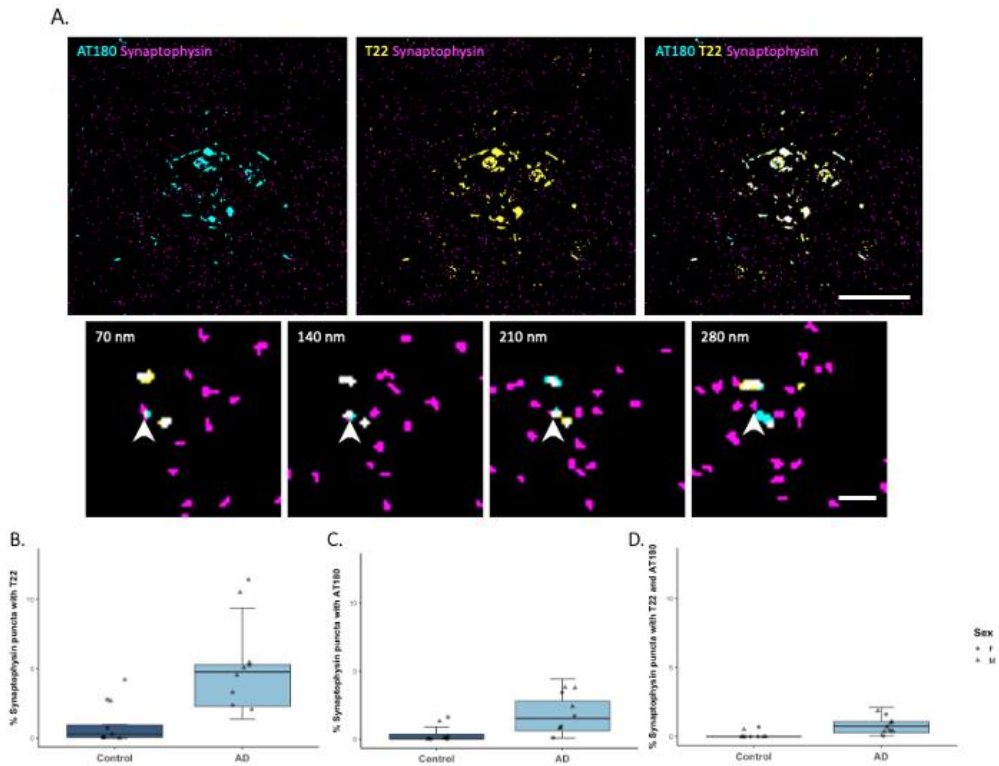


Figure S4. Oligomeric tau is more prevalent than an early phosphoepitope in presynaptic terminals in AD cases. Related to Figure 4. A At top, maximum intensity projection of a representative segmented image from array tomography showing presynaptic terminals (magenta), ptau231 (AT180, cyan) and/or oligomeric tau (T22, yellow). The inset below shows four consecutive 70nm thick sections where a presynaptic terminal containing both tau species is pointed by an arrowhead. B Percent of presynaptic terminals containing oligomeric tau (T22). C Percent of presynaptic terminals containing ptau231 (AT180 clone). D Percent of presynaptic terminals containing both oligomeric and ptau231. Boxplots show quartiles and medians calculated from each image stack. Data points refer to case means (females, circles; males, triangles). Analysis was with linear mixed effects models including diagnostic group and sex. Scale bars represent 20 μm and 2 μm .

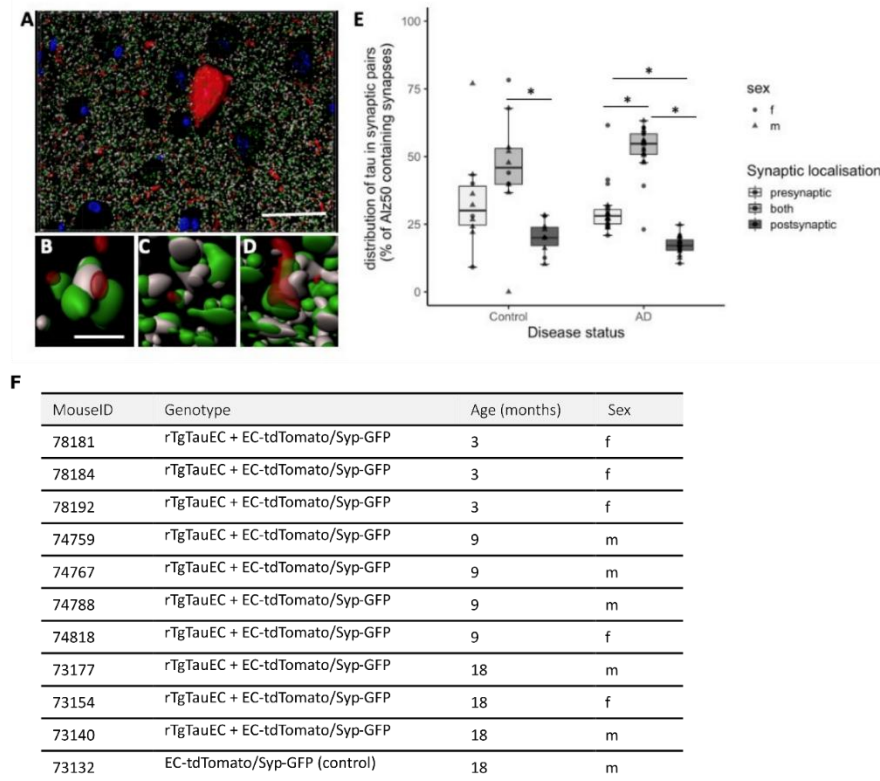
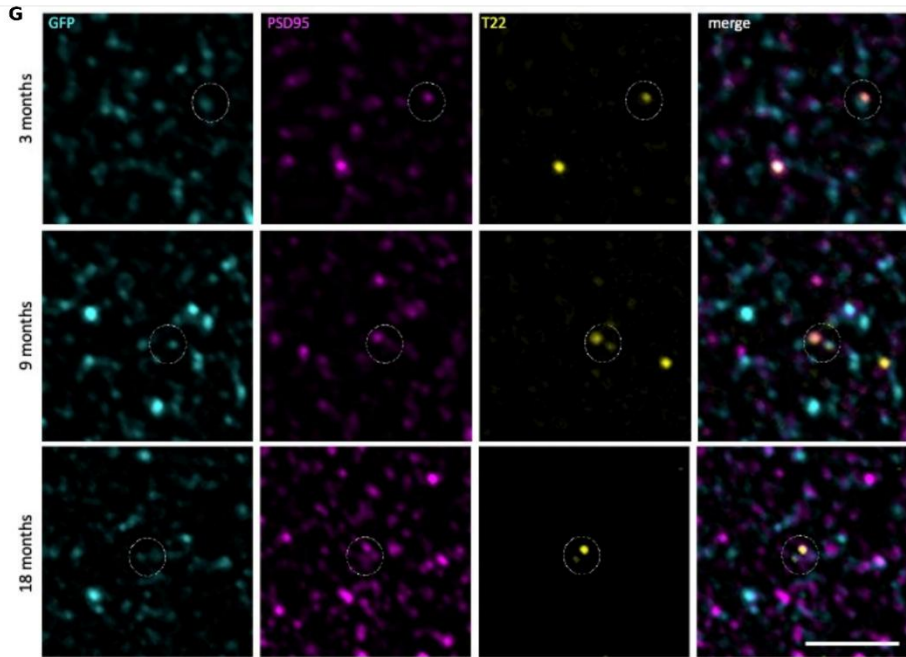


Figure S5. Tau distribution at synaptic terminals. Related to Figure 5. Panel A Shows a 3D reconstruction made in Imaris of array tomography images of misfolded tau (Alz50, red), presynaptic terminals (grey), post synaptic terminals (green) and DAPI (blue). We observe tau accumulation in presynaptic terminals (B), postsynaptic terminals (C) and both sides of synaptic pairs (D). Quantification of synaptic pairs containing tau (E) reveals most tau accumulation differs between presynaptic only, both sides of the pair, and postsynaptic only (ANOVA after linear model $F=62.93$, $p<0.0001$), asterisks represent post-hoc Tukey corrected tests $p<0.05$. Array tomography imaging of mice expressing P301L mutant human tau and GFP tagged synaptophysin (F) in entorhinal cortex stained for GFP, PSD95, and T22.



Bottom panel of Figure S5. (G) show that oligomeric tau can spread from pre to post synapses at 3 ages studied. Scale bars represent 20 μm in A, 1 μm in B-D, 5 μm in F.

Table S1 – Antibody combinations by experiment. Related to Table 1 and STAR methods. AT: Array tomography microscopy. EM: Immunogold electron microscopy. dSTORM: Direct Stochastic Optical Reconstruction Microscopy. WB: Western blot. IHC: Immunohistochemistry.

Study	Technique	Antibodies	Host species	Reactivity species	Supplier	Catalogue #	Isotype	Dilution
1. Synaptic density and Alz50 localisation in BA20/21	AT	Primary						
		Synaptophysin (SY38)	Mouse	Human	Abcam	ab8049	IgG1	1:50
		PSD95 (D27E11)	Rabbit	Human	Cell Signaling Technology	3450	IgG	1:50
		Alz50	Mouse	Human	Courtesy of P. Davies		IgM	1:50
		DAPI			Sigma Aldrich	D9542		1:10000
		Secondary						
		Alexa Fluor 488 [®]	Donkey	Rabbit	Invitrogen	A-21206	IgG	1:50
		Alexa Fluor 594 [®]	Goat	Mouse	Invitrogen	A-21044	IgM	1:50
2. Synaptic Tau species comparison in BA20/21	AT	Primary						
		PSD95	Guinea Pig	Human	Synaptic Systems	124 014	IgG	1:50
		Tau (AT8)	Mouse	Human	ThermoFisher	MN1020	IgG1	1:50
		T22 (serum)	Rabbit	Human	Courtesy of Rakez Kayed		IgG	1:50
		Alz50	Mouse	Human	Courtesy of P. Davies		IgM	1:50
		Secondary						
		Alexa Fluor 594 [®]	Goat	Mouse	Invitrogen	A-21044	IgM	1:50
		DyLight [®] 405	Goat	Mouse	BioLegend	409109	IgG1	1:50
3,4,5,6 Synaptic tau confirmation in BA20/21	3. EM	Primary						
		T22 (serum)	Rabbit	Human	Courtesy of Rakez Kayed		IgG	1:50
		Secondary						
		10nm Gold	Goat	Rabbit	Abcam	ab39601	IgG	1:50
	4. dSTORM	Primary						
		Synaptophysin	Goat	Human	R&D Systems	AF5555	IgG	1:50
		Tau (AT8)	Mouse	Human	Innogenetics	90206	IgG	1:50
		T22 (purified)	Rabbit	Human	Millipore	ABN454-I	IgG	1:50
		PSD95 (D27E11)	Rabbit	Human	Cell Signaling	3450	IgG	1:50
		PSD95 (K28/43)	Mouse	Human	Neuromab	MABN68	IgG	1:10
		Secondary						
		Alexa Fluor 488 [®]	Donkey	Rabbit	ThermoFisher	A21206	IgG	1:50
		Alexa Fluor 488 [®]	Donkey	Mouse	ThermoFisher	A21202	IgG	1:50
		Alexa Fluor 647 [®]	Donkey	Rabbit	ThermoFisher	A21447	IgG	1:50
		Alexa Fluor 647 [®]	Donkey	Goat	ThermoFisher	A31573	IgG	1:50
	5. DNA-PAINT	Primary						
		Synaptophysin (YE269) - AF488	Rabbit	Human	Abcam	ab196379	IgG	1:100
		Tau (AT8)	Mouse	Human	ThermoFisher	MN1020	IgG1	1:50
		T22 (serum)	Rabbit	Human	Courtesy of Rakez Kayed		IgG	1:50
		PSD95 (D27E11)	Rabbit	Human	Cell Signaling Technology	3450	IgG	1:50
		Secondary						
3,4,5,6 Synaptic tau confirmation in BA20/21	5. DNA-PAINT	Unconjugated	Donkey	Rabbit	Invitrogen	31238	IgG	1:50

6. WB		Primary					
		Tau5	Mouse	Human	BioLegend	806401	IgG 1:250
		GAPDH	Rabbit	Human	Cell Signaling	2118	IgG 1:1000
		B-actin	Mouse	Human	Cell Signaling	3700	IgG 1:1000
		VAMP2	Rabbit	Human	Cell Signaling	13508	IgG 1:1000
		Secondary					
		Goat anti-Rabbit IR Dye 800	Goat	Rabbit	LI-COR BioSciences	926-32211	IgG 1:5000
		Goat anti-Mouse IR Dye 700	Goat	Mouse	LI-COR BioSciences	926-32220	IgG 1:5000
7. Tau pathology burdens in BA20/21 and BA17	IHC	Primary					
		Tau (AT8)	Mouse	Human	ThermoFisher	MN1020	IgG1 1:850
		Secondary Antibodies					
		Novolink Polymer Detection System		Mouse	Leica Biosystems	RE7200	
8. Synaptic oligomeric tau in BA20/21 and BA17	AT	Primary					
		Synaptophysin (YE269) - AF488	Rabbit	Human	Abcam	ab196379	IgG 1:100
		PSD95 (K28/43)	Mouse	Human	DSHB	K28/43	IgG2A 1:50
		T22 (serum)	Rabbit	Human	Courtesy of Rakez Kayed		IgG 1:50
		DAPI			Sigma Aldrich	D9542	1:10000
		Secondary					
		Cy3*	Donkey	Mouse	Jackson Immuno	715-165-150	IgG 1:50
		Cy5*	Donkey	Rabbit	Jackson Immuno	711-175-152	IgG 1:50
9. Synaptic pTau231 and oligomeric tau in BA20/21	AT	Primary					
		AT180	Mouse	Human	ThermoFisher	MN1040	IgG1 1:50
		T22 (serum)	Rabbit	Human	Courtesy of Rakez Kayed		IgG 1:50
		Synaptophysin (YE269) - AF488	Rabbit	Human	Abcam	ab196379	IgG 1:50
		Secondary					
		DyLight* 405	Donkey	Mouse	Abcam	Ab175658	IgG1 1:50
10. Mouse array tomography study	AT	Primary					
		PSD95	Guinea Pig	Mouse, human	Synaptic Systems	124014	IgG 1:50
		T22 (serum)	Rabbit	Human	Courtesy of Rakez Kayed		IgG 1:25
		GFP	Chicken	Jellyfish	Aves labs	GFP-1020	IgY 1:50
		Secondary					
		Alexa Fluor 647*	Goat	Rabbit	ThermoFisher	A21244	IgG 1:50
		Alexa Fluor 488*	Goat	Chicken	ThermoFisher	A11039	IgY 1:50
		Alexa Fluor 594*	Goat	Guinea Pig	ThermoFisher	A11076	IgG 1:50

ENHANCING LATERAL RESOLUTION USING TWO-COLOUR DIRECT STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY TO UNRAVEL SYNAPTIC TAU PATHOLOGY IN ALZHEIMER'S DISEASE.

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ABSTRACT

In Alzheimer's disease (AD), the pathological accumulation of tau in synapses contributes to synapse dysfunction and loss. However, the small and complex structure of synapses limits the investigation when using conventional techniques. In this work, we describe the combination of Array Tomography (AT) with two-colour direct stochastic optical reconstruction microscopy (dSTORM) to enhance lateral resolution for resolving synaptic terminals in human post-mortem brain. We applied this combination to study synapses in brain samples (from biopsy and post mortem) from healthy subjects and pathological synaptic tau (aggregates and oligomers) in samples from AD patients. AT combined with dSTORM allowed the characterisation of the orientation and shape of the synaptic terminals and the synaptic cleft. In addition, this combination confirmed the presence of oligomeric tau in synaptic terminals in AD. Overall, we found that the combination of AT and two-colour dSTORM provides optimal resolution to detect pathological synaptic tau and its spatial relationship with pre- and postsynaptic terminals.

INTRODUCTION

Alzheimer's disease (AD) is characterised by the presence in the brain of extracellular aggregation of Amyloid- β (A β) peptides, which form amyloid plaques, and the intracellular deposition of hyperphosphorylated tau, which forms neurofibrillary tangles (NFTs). While the burden of NFTs correlates with neuronal loss and clinical severity, it does not account for total neuronal loss (1-3), suggesting the potential involvement of soluble tau species or other factors in the pathophysiology (4). Synapse loss post mortem is known to correlate with cognitive decline in AD (5,6). However, the small and complex structure of synapses poses a significant challenge to elucidating their role using conventional techniques. Traditional biochemical, genetic, structural, and imaging methods have yielded valuable insights into biological processes; nevertheless, they fall short in offering a detailed molecular perspective on the nanometre scale organisation (7). Advances in microscopy techniques in the last decades have opened a window to investigate synaptic pathology in neurodegenerative diseases. We and others have used Array Tomography (AT), a novel microscopy technique especially suited for visualising synapses in human brain and for investigating synaptic aggregates in patients with AD and dementia with Lewy bodies (DLB) (8-11). AT offers an increased spatial resolution by combining ultrathin sectioning of the tissue with immunofluorescence, allowing high-resolution quantitative analysis of different ultrastructures, such as synapses or pathological aggregates in DLB and AD (9,12,13). The small size of synaptic aggregates has led other groups to look for microscopy techniques with higher resolution.

In a previous study, we combined AT with two-colour direct stochastic optical reconstruction microscopy (dSTORM), a Single Molecule Localisation Microscopy (SMLM) technique, capable of detecting interactions within nanometre scale (14). Using this combination, we showed that oligomeric,

misfolded, and phosphorylated tau species are found at synaptic terminals of AD brains even in areas with low NFT burden (11). In the present work, we describe the full capabilities of the combination of AT with dSTORM to achieve enhanced lateral resolution for resolving synaptic terminals in human post-mortem brain samples from healthy subjects and synaptic tau oligomers in samples from AD patients. The enhancement of the lateral resolution enables a more precise study of the spatial relationship between pathological proteins and the synaptic terminals, which could provide crucial insights into synaptic pathology in neurodegenerative diseases.

MATERIALS AND METHODS

Protocol approval, patient consent and post-mortem human brain samples

The study was approved by the local ethics committee of Hospital de Sant Pau, Barcelona. All research was performed in accordance with relevant guidelines and regulations. All brain tissue samples were obtained from patients after they or their legal representatives gave written informed consent for the use of their brain tissue and medical records for research purposes, as approved by the Ethics Committee of the Hospital Clínic-FRCB-IDIBAPS, in accordance with the Declaration of Helsinki. Neuropathological examination was performed according to standardised protocols at the Neurological Tissue Bank of the Hospital Clínic-FRCB-IDIBAPS, a brain bank with expertise in neurodegenerative diseases. Cortical biopsies were obtained from neurosurgical procedures directed at removing deep brain tumours from patients at the Hospital de la Santa Creu i de Sant Pau (Barcelona, Spain). All samples were processed using previously described methods (15) and generally recommended (8). The demographic and clinical data of the cohort are outlined in Table 1.

Table 1. Demographic and clinical data of the cohort. F: Female; M: Male; PMI: Post-mortem interval. HC: Healthy control, SAD: Sporadic Alzheimer’s Disease, ^aCortical brain biopsy. Studies: (1) AT+conventional epifluorescence microscope, (2) AT+dSTORM, (3) Formalin-fixed paraffin-embedded+Confocal, (4) Formalin-fixed paraffin-embedded + dSTORM.

Cases No.	Clinical diagnosis	Gender	Age at biopsy or age of death	PMI (h)	Region	Braak NFT stage	Study
1	HC (biopsy) ^a	F	62	NA	Temporal	NA	1, 2
2	HC (biopsy) ^a	M	46	NA	Temporal	NA	1, 2
3	HC	F	83	7.5	Frontal	II	1, 2
4	HC	F	89	8.8	Frontal	I	1, 2
5	SAD	F	90	18.0	Frontal	V	1, 2
6	SAD	M	64	17.9	Frontal	VI	1, 2, 3, 4
7	SAD	F	82	13.8	Frontal	IV	1, 2, 3, 4

Immunofluorescence Staining

Formalin-fixed paraffin-embedded (FFPE) sections from the frontal cortex were deparaffinised and rehydrated following standard procedure (16). Then, the antigen retrieval step was performed. Next, sections were blocked with 0.2% bovine serum albumin for 1 hour, followed by incubation of the samples overnight with primary antibodies diluted 1:500 at 4°C (anti-Synaptophysin, AF5555, R&D Systems; anti-PSD95, 3450, Cell Signaling; anti-AT8, 90206, Innogenetics). The next day, AlexaFluor secondary antibodies diluted 1:1000 were incubated for 1 hour at room temperature (Donkey anti-Rabbit, Alexa Fluor 488, A21206, ThermoFisher; Donkey anti-Mouse Alexa Fluor 488, A21202, ThermoFisher; Donkey anti-Goat Alexa Fluor 647, A21447,

ThermoFisher). After washing with PBS, the sections were incubated with Sudan Black B (199664, Sigma-Aldrich) for 5 minutes and washed with PBS again to remove any precipitates that may have been generated. For visualisation of the nuclei, the samples were incubated with Hoechst 1:5000 (33258, Life Technologies). Finally, the sections were mounted on slides with Immu-Mount mounting medium (Thermo Scientific™). Images were recorded by Leica inverted fluorescence confocal microscope (Leica TCS SP5-AOBS, Wetzlar, Germany), and cover slides were viewed with HCX PL APO 63X/1.4 NA IMM Corr objective. Fluorescent images were acquired in a scan format of 1024 x 1024 pixels, and a 3x scanner zoom factor was used (0.4 µm pixel size). A pulsed 405-nm laser was used for Hoechst visualisation, collecting images in a spectral range of 450 to 460 nm.

Array Tomography and image acquisition

The array tomography (AT) protocol was applied using previously described methods (10,16). Briefly, London Resin white embedded brain tissue blocks were sectioned using a diamond knife (Diatome, UK) generating ribbons of 70 nm thick consecutive sections. Then, ribbons were incubated with Tris-Glycine solution at room temperature for 5 min and blocked for 30 min with cold water fish blocking buffer (G7041, Sigma-Aldrich). Sections were incubated overnight with primary antibodies diluted 1:50 at 4°C (anti-Synaptophysin, AF5555, R&D Systems; anti-PSD95, 3450, Cell Signaling; anti-T22, ABN454-I, Millipore). After TBS washes, secondary antibodies diluted 1:50 were applied for 30 min at room temperature (Donkey anti-Rabbit, Alexa Fluor 488, A21206, ThermoFisher; Donkey anti-Mouse Alexa Fluor 488, A21202, ThermoFisher; Donkey anti-Goat Alexa Fluor 647, A21447, ThermoFisher). Sections were washed with TBS and stained with Hoechst diluted 1:100 (33258, Life Technologies) for 5 min for nuclei visualisation. Finally, coverslips were mounted on microscope slides with Immu-Mount mounting medium

(9990402, Epredia). Images of three regions of interest were obtained on at least 12 consecutive 70 nm thick serial sections using the nuclei as a guide with a fully automated epifluorescence upright microscope (BX51, Olympus) with a 63x1.2 NA Plan Apochromat objective.

Direct STORM Acquisition (dSTORM) and Image Reconstruction

For image reconstruction using dSTORM, sections 70 nm (AT) and 2 μ m (FFPE) thick were stained as aforementioned using the same primary and secondary antibodies, Hoechst dye diluted 1:100 (33258, Life Technologies) was used to visualise the nuclei (Fig. 1A), then the coverslips were mounted with OXEA buffer (STORM buffer) (17). To pinpoint individual fluorophores within the sample, fluorescent dyes go through a switching cycle, transitioning between an "on" and "off" state known as "blinking". During this phenomenon, most dyes remain inactive while a few of them randomly activate, allowing for their individual identification. This cyclic process repeats multiple times, enabling the identification of the emitting fluorophore in each successive cycle (Fig. 1B).

STORM images were acquired using a Nikon N-STORM system configured for total internal reflection fluorescence (TIRF) imaging. Laser inclination was tuned to adjust focus and maximize the signal-to-noise ratio. AlexaFluor-647 was excited illuminating the sample with the 647 nm (20% of 160 mW) and AlexaFluor-488 with the 488 (40% of 80 mW) laser line built into the microscope. Fluorescence was collected using a Nikon 100x, 1.49 NA oil immersion objective and passed through a quad-band pass dichroic filter (97335 Nikon). 10,000 frames at 20 ms integration time were acquired for each channel. Images were recorded onto a 256×256-pixel region of a Hamamatsu ORCA Flash 4.0 CMOS camera (0.16 μ m pixel size). Also, using the 405 nm laser, a low-resolution image of the nucleus was taken and

subsequently utilized for 3D image alignment. STORM images were analysed with the STORM module of the NIS element Nikon software (Fig. 1C) (171)

Image analysis

Image analysis for both AT and AT+dSTORM images was performed using Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA), Matlab software (Mathworks Inc., MA, USA) and Python (Centrum voor Wiskunde en Informatica Amsterdam, The Netherlands). The scripts have been deposited at https://github.com/MemoryUnitSantPau/Synseg_py with the names Density, Distance and Shape, and Segmentator. Images from serial sections were stacked, aligned and segmented using a semi-automated mean local threshold. The non-specific staining (not present in at least 2 consecutive sections) was considered background and removed. 3D reconstructions of representative synapses were generated using the ImageJ Volume Viewer plug-in with tricubic smooth interpolation.

To assess the distance between centroids: first, stacks of protein images are loaded and processed frame by frame. Each frame undergoes thresholding to eliminate intensity values below 20% of the maximum intensity of that frame. Subsequently, the frames are labelled to extract their properties, utilizing the "spicy" and "scikit image" libraries. The detected object's shape is parametrised using "diplib" and "cv2" measurement tools obtaining measurements about the roundness, circularity, and eccentricity, among other characteristic values. The distance between centroids is then computed using the Pythagorean theorem. Finally, to calculate the synaptic cleft width, the pixels of both proteins are listed, and for each pair of points, the distance is calculated, and the minimum is recognised.

Statistical analyses

Statistical analyses were performed in R version 4.3.1. We excluded data points from the analysis that exceeded three times the interquartile range. We evaluated the distribution of our data (Shapiro-Wilk, $p < 0.05$) before performing a Mann-Whitney test to assess the differences between techniques when measuring the object dimension and distance of the synaptic proteins. To evaluate the contribution of PMI in the synaptic cleft width, we used a linear regression with the PMI as a covariate. Differences in area, length and other measurements of synaptic proteins were evaluated with Wilcoxon's test.

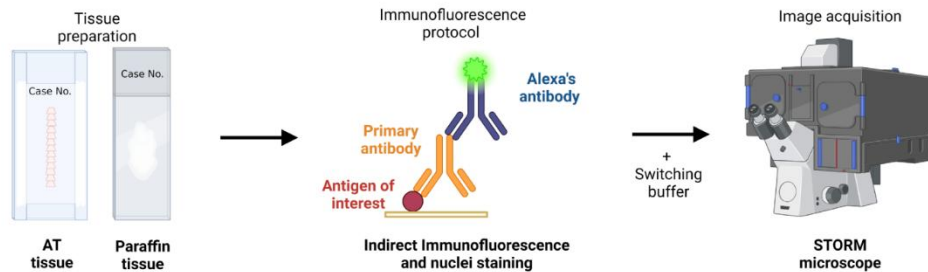
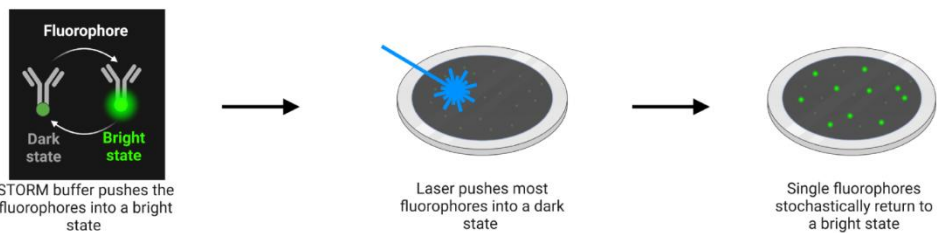
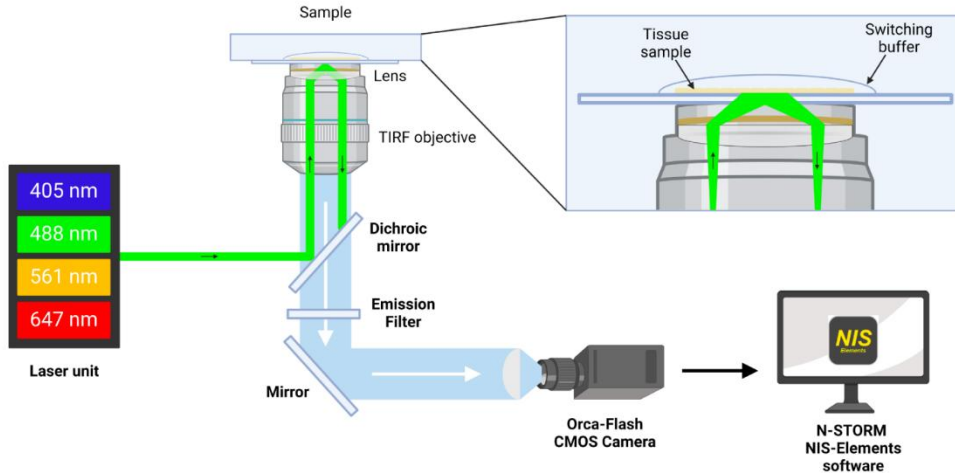
A. TECHNIQUE OVERVIEW**B. STORM BUFFER PRINCIPLE****C. STORM SET-UP**

Figure 1. Visual summary of dSTORM imaging. (A) Technique overview showing the 3 main steps: tissue preparation, immunofluorescence of the samples and image acquisition with a two-colour STORM microscope. (B) Schematic representation of the STORM buffer principle and the stochastic activation of fluorophores. (C) The STORM microscope has a laser unit with 4 channels, allowing the acquisition of two-colour images as well as recognition of the nuclei of the field of interest for reconstruction using the STORM module of the NIS element Nikon software for analysis.

RESULTS

Enhancement of lateral resolution with dSTORM to detect tau aggregates in formalin-fixed paraffin-embedded brain samples

To compare the resolution achieved using confocal microscopy and dSTORM, we immunostained FFPE samples from patients with SAD with a phosphorylated tau marker (p-tau, AT8) to detect neurofibrillary tangles and tau immunoreactive neurites. The samples were imaged with confocal microscopy (Figure S1A) and subsequently with dSTORM, resulting in a significant improvement in lateral resolution (Figure S1B). This enhanced resolution revealed the discontinuous nature of tau+ neurites in contrast to the classical continuous appearance observed with conventional microscopy (CM) due to light diffraction. As the thickness of the FFPE sample is greater than the diffraction limit of the microscope, resolution along the z-axis is poor in FFPE tissue and degrades faster than on the XY plane (12). As a result, it was not possible to visualise the interaction of tau aggregates with synaptic terminals using either imaging technique.

Enhancement of lateral resolution with dSTORM to visualize single synapses in AT samples

We next investigated the potential of the combination of dSTORM with our classical AT protocol (8,10,13) to visualise synaptic pairs in human brain samples. As previously described, we selected 70 nm thick AT samples from an HC (frontal cortex) and immunostained them using an anti-synaptophysin (SYPH) antibody, as a presynaptic marker, and anti-PSD95 (PSD) antibody, as a postsynaptic marker (Figure 2A). Using fluorescence microscopy, synaptic pairs appear as puncta with a distance between the pre- and postsynaptic centroids within a 0.5 μm range (11). The resolution obtained with AT allows the 3D reconstruction of single synapses, which enables the localisation of pre- and postsynaptic structures separately (Figure 2B) and the presence of

synaptic aggregates (8,10). We next performed the same experimental design combining AT with two-colour dSTORM. The dSTORM image is the final reconstruction of 10,000 frames per channel. The improved resolution achieved with AT+dSTORM allowed detailed visualisation of the pre- and postsynaptic terminals and the synaptic cleft (Figure 2C,D). In addition, the combination allows a better characterisation of the orientation and shape of the synapse. This result indicates that the combination of AT and dSTORM offers increased properties for the characterisation of the synapses in the healthy human brain.

Assessing Protein-Protein Distances, Synaptic Cleft Size, and Object Dimensions with AT+dSTORM

The improved lateral resolution of AT+dSTORM allowed a precise characterisation of the synaptic compartments. This allowed the quantification of the distances between the pre- and postsynaptic structures. We performed image binarisation with a pre-established threshold (Figure 2E), resulting in increased accuracy for distance calculations. We analysed over 400 single synapses (200 for each technique) using frontal cortex AT samples from HC (n= 2). We observed a significant difference in the median distance between centroids when comparing conventional epifluorescence microscopy with respect to dSTORM (409 ± 80 vs 190 ± 70 nm, $p < 0.01$) (Figure 2F). The use of AT+dSTORM revealed that the area of PSD95 labelling is almost twice as large as that of SYPH labelling (Fig. 2H). Furthermore, using the combination AT+dSTORM, we quantified the width of the synaptic cleft, revealing a median value of 64 ± 59 nm. This allowed us to discriminate objects without overlap at a distance as small as 8 nm (Figure 2G). To confirm that the values obtained with AT-STORM were not influenced by the PMI or the AT protocol, we also measured the synaptic cleft in samples obtained from surgical cortical biopsies (n=2). We did not observe significant differences in

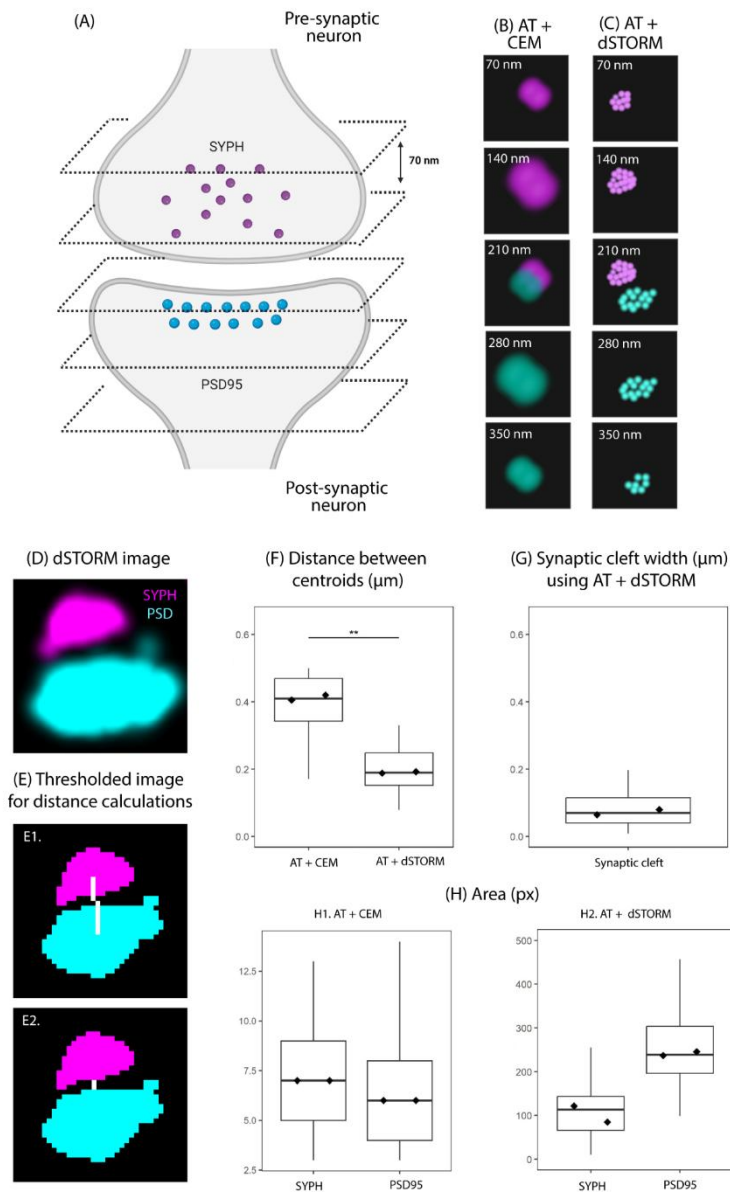


Figure 2. Imaging single synapses from human brain. (A) Graphical representation of 70 nm consecutive sections, immunolabelled with antibodies raised against SYPH and PSD95. The production of ultrathin ribbons enables the subsequent reconstruction of the ultrastructure of the brain tissue. (B-C) Graphical synaptogram comparing the resolution of AT + conventional epifluorescence microscopy (CEM) (B) and AT+dSTORM (C). (D) dSTORM image of a single synapse as a result of the rendering process. (E) Thresholded image used for measuring the distance between centroids (E1, white diagonal represents 101.84 nm) and the width of the synaptic cleft (E2, white line represents 21.5 nm). (F) The centroid's distance obtained with AT samples and conventional epifluorescence microscope and in combination with dSTORM. (G) The median value of synaptic cleft width using the AT+dSTORM images and the dispersion of the data represented in the boxplot. Each point corresponds to a single synapse. Error bars indicate medians with standard deviations. ** p<0.01. (H) Area quantifications (pixels) of objects using (H1) AT samples with CEM and (H2) AT with dSTORM. The boxplot represents the distribution of all data points, while the statistical analysis was performed on the mean values for each case, which are displayed as diamonds. Error bars indicate medians with standard deviations.

synaptic cleft distance between post-mortem AT controls and cortical biopsy samples (Supplementary Fig. 2). In addition, PMI was added as a covariate in the linear regression model, showing no significant contribution (adj. r^2 : -1.032, p-value: 0.82). This result supports that the PMI does not affect the measurements of the synapses using AT.

AT+dSTORM detects oligomeric tau in synapses in AD brains

We next confirmed our previous result using AT+dSTORM, showing the presence of tau oligomers at the synapse using the T22 antibody (Figure 3A) (11). We expanded the number of frontal cortex samples of SAD patients to characterise synaptic tau aggregates ($n=3$). We observed that the centroid of the presynaptic oligomeric tau immunoreactivity was closer to the centroid of the presynaptic terminal (SYPH) when compared to the immunoreactivity observed in the postsynaptic density (PSD95, Figure 3B). Furthermore, we characterised the morphology of the aggregates, their area, eccentricity and length (Figure 3C-E). Oligomeric tau immunoreactivity was larger than the synaptic markers (SYPH and PSD95), but there were no significant differences between them. When evaluating the eccentricity of the three proteins, both SYPH and T22 were significantly more circular than PSD95 ($p<0.05$). Furthermore, we measured the length of the major axis of oligomeric tau immunoreactivity present in the synapse, and we did not detect significant differences (median length of 315.27 ± 117.69 nm in the presynaptic compartment and of 332.43 ± 163.59 nm in the postsynaptic compartment). Finally, of a total of 200 tau+ synapses analysed in three SAD cases, tau oligomers were found more frequently in presynaptic terminals than in postsynaptic terminals.

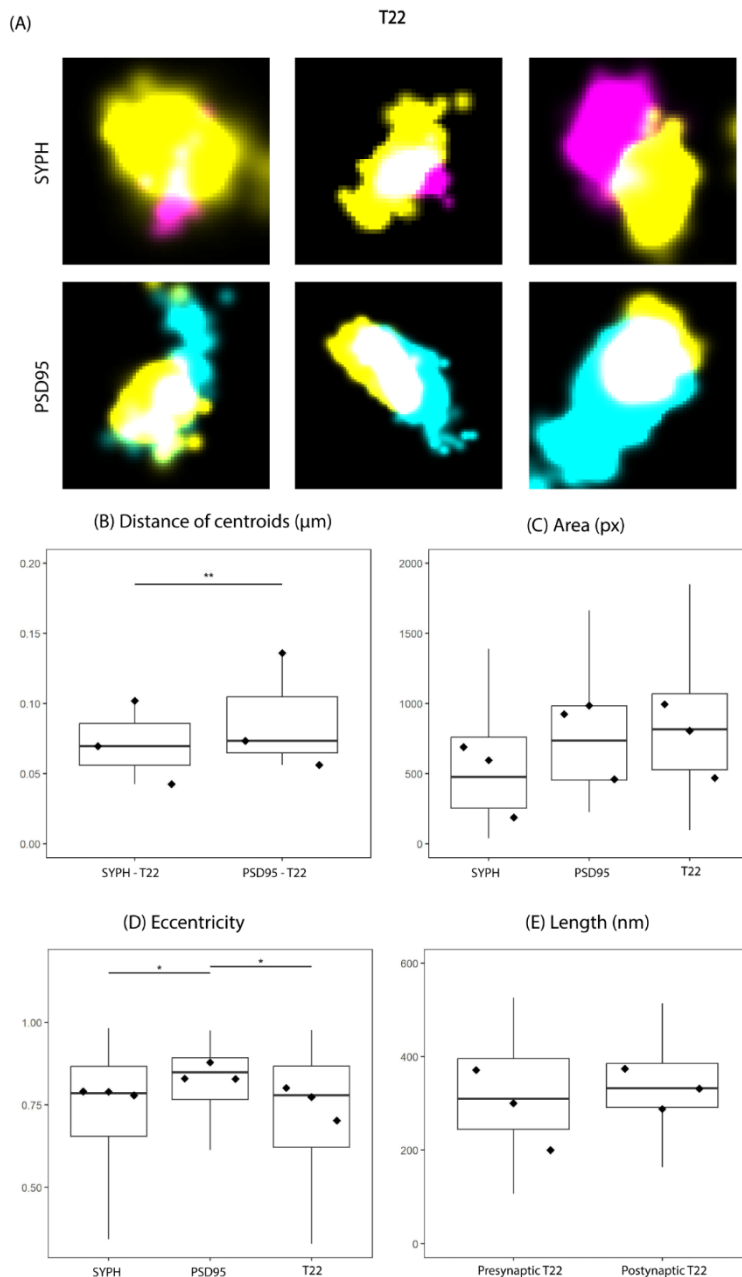


Figure 3. Assessment of the presence of oligomeric tau in pre and postsynaptic compartments. (A) Representative images of oligomeric tau (T22, yellow) in the presynaptic compartment (SYPH, magenta) and the postsynaptic compartment (PSD95, cyan). (B) Distance calculation between centroids of tau aggregates and synaptic compartments. Evaluation of (C) area in pixels and (D) eccentricity of the three markers. (E) Measurement of the length of the major axis in nanometres of T22 oligomers in the two synaptic compartments. The boxplot represents the distribution of all data points, while the statistical analysis was performed on the mean values for each case, which are displayed as diamonds. Error bars indicate medians with standard deviations. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

In this work, we describe the potential of the combination of AT and two-colour dSTORM to study synaptic tau pathology in AD brains. Combining these two microscopy techniques, we achieved nanoscale resolution that allows an optimal visualisation of single synapses and confirms the presence of oligomeric forms of tau at synaptic compartments in human brain.

dSTORM is an SMLM that consists of photo-switchable fluorescent molecules that stochastically turn on and off within a diffraction-limited area at different times, preventing their signals from overlapping significantly. When isolated in space, the position of each fluorophore can be determined by localising the centre positions of their signal. dSTORM can currently achieve a lateral resolution of 10-30 nm (20). dSTORM offers some advantages over other traditional techniques, such as transmission electron microscopy, especially in terms of sample preparation and multiple targets (13).

Several studies have reported the presence of pathological tau at the synapse in the AD brain (21-26). Pathological tau at the synapse is believed to contribute to synaptic dysfunction and loss in AD (27). In this study, we assessed the presence of synaptic p-tau aggregates (AT8) in SAD frontal cortex FFPE samples using dSTORM. The increased lateral resolution revealed a discontinuous nature of tau+ neurites.

To investigate the relationship between synapses and the oligomeric forms of tau in AD, we next applied the combination of AT and two-colour dSTORM. Using both techniques, we were able to visualise and identify with high precision the pre- and postsynaptic terminals. In addition, we were able to visualise the synaptic cleft with a median width of approximately 64 nm, which closely approximates the range reported by electron microscopy of 20-50 nm

(28-31). This combination also allowed us to observe the orientation in space and the morphology of synaptic terminals. Our results were also validated in cortical biopsy samples, indicating that PMI or the AT sample preparation protocol does not affect synaptic ultrastructure.

There is increasing evidence that oligomeric forms of tau are one of the early events in AD pathology and the most synaptotoxic tau species, contributing to synaptic dysfunction and loss (24,25,32-34). Here, using AT with two-colour dSTORM, we were able to detect tau oligomers (using T22 antibody) colocalising with pre- and postsynaptic markers (SYPH and PSD95, respectively) confirming the presence of this pathological form of tau at the synapse. T22 immunoreactivity was found more frequently in presynaptic terminals in the frontal cortex, consistent with our previous results in the temporal and primary visual cortex (11).

Taking advantage of the improved resolution of AT+dSTORM, we defined the characteristics of eccentricity and length, with results similar to those seen in primary cell cultures (35). Taken together, this study establishes the combination of AT+dSTORM as an optimal technique to study synaptic tau aggregates, paving the way for the study of other proteins that are toxic to the synaptic compartment.

This study has limitations. First, dSTORM requires long acquisition times and extensive post-processing. Second, the intrinsic composition of the sample tends to distort and blur the single-molecule emission patterns (36). To overcome this distortion, TIRF microscopy is used, wherein the laser is tilted to an angle at which the sample is illuminated in an exceptionally thin axial region (37). This allowed us to obtain an enhanced spatial resolution. Finally, the study is limited in sample size due to the scarcity of the AT samples and

the findings need to be replicated in additional cases including different subtypes of AD.

CONCLUSIONS

In this study, we demonstrate that the combination of AT with two-colour dSTORM offers an improvement of the lateral resolution suitable to investigate synaptic pathology in the human AD brain. These novel techniques offer valuable information for the study of intact human synapses and provide insight into the relevance of synaptic pathology in AD.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval was obtained from the ethics committee at Hospital de la Santa Creu i Sant Pau and Hospital Clínic- Fundació Recerca Clínic Barcelona - Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

AVAILABILITY OF DATA AND MATERIALS

The Image analysis scripts are freely available on GitHub (https://github.com/MemoryUnitSantPau/Synseg_py). The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

COMPETING INTERESTS

The authors declare no competing interests.

FUNDING

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AUTHOR CONTRIBUTIONS

É.S-A., S.P., T.S-J., S.S. and AL designed the research; S.T., C.Q-S, À.B., L.M-P., I.A. collected and prepared the samples; É.S-A., B.M-L. and S.T. performed the experiments; É.S-A., J.A.E., J.P. and M.C-C. designed the scripts and analysed the data; J.F., O.B., S.P., S.S. and A.L. supervised the study. All authors read and approved the final manuscript.

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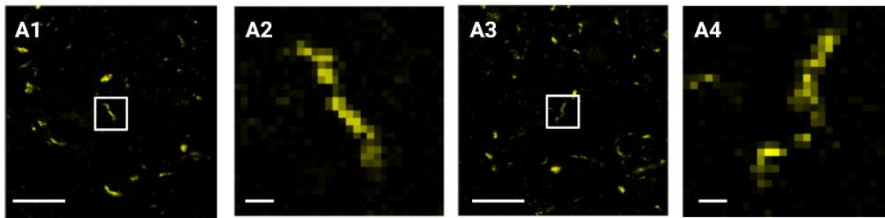
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SUPPLEMENTARY MATERIAL

A. FFPE + CONFOCAL MICROSCOPY



B. FFPE + dSTORM

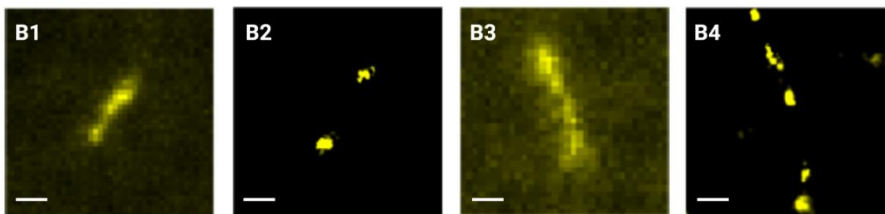


Figure S1. Comparison of lateral resolution to detect AT8 immunoreactive neurites. (A) Visualisation of AT8 immunoreactive neurites and aggregates in SAD FFPE samples imaged with confocal microscopy. A2 and A4 show a zoom-in view of A1 and A3. Scale bar: 0.50 μm . (B) Visualisation of the neurites with enhanced lateral resolution using SAD FFPE samples with dSTORM. B2 and B4 show a super-resolved representation of the diffracted limited version shown in B1 and B3. Scale bar: 0.5 μm .

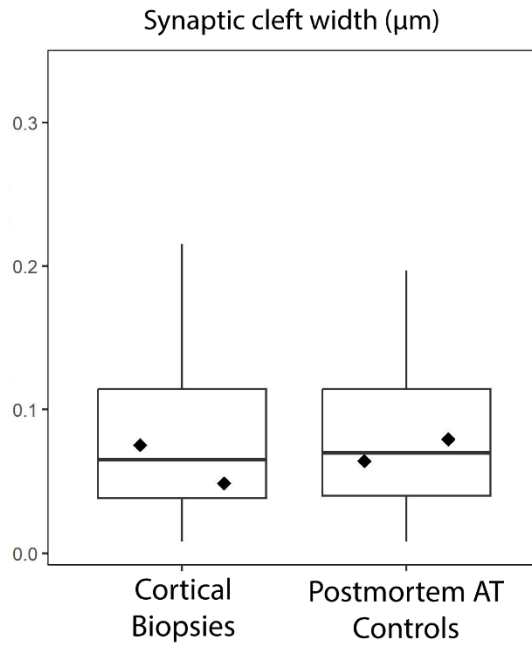


Figure S2. Synaptic cleft width is not significantly different between cortical biopsy samples and postmortem AT controls. The median of synaptic cleft distance in biopsies was 65 ± 50 nm and in controls was 64 ± 59 nm, showing not significant differences between the two types of samples. The boxplot represents the distribution of all data points, while the statistical analysis was performed on the mean values for each case, which are displayed as diamonds.

TAU SEEDING ACTIVITY CORRELATES WITH SYNAPTIC OLIGOMERIC TAU IN ALZHEIMER'S DISEASE.

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**(Manuscript in preparation
for a Brief Communication)**

ABSTRACT

The underlying mechanisms of tau propagation along brain circuits in Alzheimer's disease (AD) remain largely unknown. Here we investigated the association between tau seeding activity (TSA) and synaptic tau pathology in human AD samples. Using a deeply characterized cohort, we performed tau seeding assays and integrated these data with previously reported synaptic measures. TSA showed a strong correlation with synaptic oligomeric tau (T22) accumulation in both presynaptic ($R = 0.96$, $p = 0.0028$) and postsynaptic compartments ($R = 0.82$, $p = 0.034$) of the temporal cortex. Our findings demonstrate that synaptic oligomeric tau, rather than total tau burden or established pathological lesions, is a strong correlate of seeding activity. These results reinforce the hypothesis that targeting synaptic tau oligomers is particularly relevant for understanding disease mechanisms and limiting tau propagation and clinical progression.

INTRODUCTION

Alzheimer's disease (AD) is characterized by the extracellular deposition of amyloid- β (A β) into A β plaques and the intracellular accumulation of hyperphosphorylated tau forming neurofibrillary tangles (NFTs) (1). While NFTs and A β plaques follows a remarkably consistent pattern, AD is a highly heterogeneous disorder in terms of age at onset, clinical presentation, rate of cognitive decline, and overall survival (2). Current evidence indicates that tau pathology in AD distributes through anatomically connected areas, however, the underlying mechanisms of tau propagation remain largely unknown. Although NFTs correlate with cognitive impairment and neuronal loss, they do not fully explain all the neurodegeneration observed in AD patients (3).

Recent studies have shown that soluble tau oligomeric species exhibit higher seeding competence and neurotoxicity than mature fibrillar aggregates or end-stage neuropathological lesions (4). In addition, in the absence of fibrils, oligomers alone are sufficient to cause a severe and reversible phenotype (5), highlighting the importance of study this tau species in AD.

Recent evidence suggest that distinct molecular forms of tau contribute differently to disease progression and clinical aggressiveness (6,7). In this context, differences in tau seeding activity (TSA) among patients has emerged as a potential molecular driver of disease heterogeneity. TSA reflects the ability of pathological tau species to template the misfolding and aggregation of native tau (8), a process that may underlie the progressive spread of tau pathology across connected neuronal networks. This could explain, at least in part, how tau pathology seeds appear in new neurons (9-11).

Importantly, evidence by us and others indicates that tau propagation may occur through trans-synaptic mechanisms, where pathological tau species are transferred between synaptically connected neurons (12-15). In fact, the

synapse is a critical localization of disease progression, being both synaptic dysfunction and synaptic loss the earliest and strongest correlates of cognitive decline in AD (16-17). In line with this, we previously reported the early presence of tau oligomers at both pre- and postsynaptic terminals of the same synapse in AD brains, supporting the hypothesis of trans-synaptic spread of tau oligomers (12). Given that soluble and oligomeric tau species are considered more neurotoxic than end-stage neuropathological lesions (4), we investigated the association between TSA and the accumulation of synaptic oligomeric tau in a uniquely well-characterized AD cohort (12).

METHODS

Brain samples were obtained via the University of Edinburgh Sudden Death Brain Bank and the Alzheimer Scotland Brain and Tissue Bank, as published previously (see Table 1 and Table S1) (12). Written informed consent for brain donation and research was obtained from all donors or next of kin. Characteristics of participants and study allocation are detailed in Supplementary Table S1. Use of human tissue for post-mortem studies has been reviewed and approved by the Edinburgh Brain Bank ethics committee and the ACCORD medical research ethics committee, AMREC (ACCORD is the Academic and Clinical Central Office for Research and Development, a joint office of the University of Edinburgh and NHS Lothian, approval number 10/S1103/10). The Edinburgh Brain Bank is a Medical Research Council funded facility with research ethics committee (REC) approval (HV-016).

Table. Characteristics of the study cohort. The table summarizes diagnostic group, sex, age at death, APOEε4 genotype and post-mortem interval (PMI).

	Controls (n=8)	AD (n=8)
Age, mean ± SD	80.25 ± 1.75	82.88 ± 5.64
Sex (% Female)	4 (50%)	4 (50%)
APOEε4 carriers (%)	3/7 (42.9%)	5/8 (62.5%)
PMI, mean ± SD (hours)	71.38 ± 14.12	84.38 ± 20.96

Tau seed isolation

Human temporal cortex homogenates were obtained from frozen tissue (200 mg). Brain homogenates fractions used in this study were previously prepared for (12) from the same donor cohort, following the protocol described in (12). Aliquots were stored at -80 °C and were not subjected to additional freeze-thaw cycles prior to use. Total protein concentration was determined by Micro BCA Protein Assay Kit (Thermo Scientific, No. 23235). From this data, the proportion of tau protein (0.1-0.5% of total brain protein) was estimated and aliquots were adjusted to a standardized concentration of 800 ng tau (Tau seed). Subsequently, samples were subjected to ultracentrifugation at 150,000 g for 30 min at 4 °C, the pellet was resuspended in 50 µL of 1X PBS. Final aliquots were stored at -80 °C for further experimental use. A total tau ELISA (KHB0041) was performed to quantify total tau concentrations in the samples, in accordance with the manufacturer's protocol.

Cell culture and seeding assay

TSA was assessed using Tau RD P301S FRET-Biosensor cells (CRL-3275™)(3). Cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and plated 60,000 cells/well on Matrigel-coated 12mm coverslips in 24-well plates. Tau seed preparations were adjusted to 16

ng total tau per well and complexed with Lipofectamine 2000 (Invitrogen; 1% v/v) in Opti-MEM for 10 min at room temperature before addition to cells. After 72 h, cells were fixed in 4% paraformaldehyde for 20 min, followed by quenching with 50 mM NH₄Cl and 20 mM glycine. Cells were then stained with Hoechst and mounted for imaging. TSA signal was acquired using a 3DHISTECH fluorescence scanner. For each coverslip, four 2 mm² regions were selected, and a MATLAB script was used to randomly extract ten ROIs from each region, that were analysed with ImageJ.

Western blotting of oligomeric tau

Isolated tau seeds were denatured at 95 °C for 5 min, and 12 µg protein per sample was resolved on 4–12% Bis–Tris gels (MOPS running) and transferred to nitrocellulose membranes (Bio-Rad, 1620112) using Trans-Blot Turbo buffer (Bio-Rad, 10026938) supplemented with 20% ethanol. Transfers were performed in the Trans-Blot Turbo Transfer System (Bio-Rad, 1704150) at 25 mV for 13 min. Membranes were blocked and incubated overnight at 4 °C with antibodies against Tau5 (BioLegend, 806401; 1:250) and GAPDH (Cell Signaling, 2118; 1:1000), followed by incubation with fluorescent secondary antibodies (Odyssey; 1:20,000). Bands were detected using the Odyssey M imaging system and quantified with Image Studio software (LI-COR Biosciences).

Previously acquired measurements

For correlation analyses, immunohistochemistry and synaptic measures were obtained from our prior study in this cohort (12), which analysed the same donor cohort. These previously reported metrics were not re-measured here and were integrated with the newly generated seeding and biochemical data.

Statistical Analysis

All statistical analyses were performed in R (version 4.3.1). The normality of the distribution was checked prior to analysis, and the corresponding parametric or non-parametric tests were used. Outcomes were summarised per case and these case means were used for analysis to avoid pseudo replication. For the FRET-based seeding assay, group differences were evaluated on per-case mean FRET positivity using the Wilcoxon rank-sum test. Associations between seeding activity and neuropathological and clinical covariates, biochemical or tissue-based measures were assessed using Spearman's correlations. All tests were two-sided, and statistical significance was set at $p < 0.05$.

RESULTS

FRET-based TSA distinguishes AD from controls cases

To assess TSA, we employed a cell-based FRET biosensor assay using tau seeds isolated from temporal cortex brain homogenates (Fig. 1A). We observed a 6.2-fold increase in the percentage of FRET-positive cells in AD compared to control cases ($W = 8$, means: 5.71 vs 0.92, $p = 0.01$) (Fig. 1B). These findings are consistent with previous reports showing that FRET-based biosensor assays can discriminate SAD from control samples, supporting its use as a sensitive readout of tau seeding activity.

TSA is not associated with demographic or genetic factors

Next, we examined whether TSA was associated with key clinical and neuropathological measures. No significant correlation was observed between TSA and age at death ($R = 0.38$, $p = 0.14$) (Fig. 1C). A similar degree of TSA was observed in tau seeds derived from APOE $\epsilon 4$ carriers and non-carriers ($W = 18$, $p = 0.16$, Fig. 1D). No significant differences were detected by sex ($W = 19$, $p = 0.20$, Fig. 1E).

Associations of TSA with tau pathological burden

We next examined the relationship between TSA and multiple measures of tau pathology burden in AD cases. TSA was not significantly associated with total tau levels quantified by ELISA ($R=-0.38$, $P=0.36$) or oligomeric tau measured by western blot from isolated tau seeds from the brain homogenates ($R=0.6$, $p=0.35$) (Fig. S1A,B). However, this analysis was limited to five cases due to restricted sample availability and should be interpreted as exploratory. TSA showed no significant relationship with tau in dystrophic neurites ($R=0.64$, $p=0.14$) (Fig. S1C) or neurofibrillary tangle burden ($R=0.57$, $p=0.2$) (Fig. S1D) quantified with AT8 antibody on immunohistochemical sections (9). These findings suggest that TSA is relatively independent from the extent of conventional tau pathological burden markers. No correlations were found in the control group (data not shown).

TSA correlates with synaptic T22 accumulation in AD

Finally, we tested whether TSA was associated with synaptic tau pathology in AD cases. We did not find correlation between TSA and excitatory synapse density in the temporal cortex (BA20/21) (Fig. S2A). When specifically analysing synaptic tau pathology assessed by AT in our previously published study (12), TSA showed a strong correlation with oligomeric tau (T22) accumulation in both postsynaptic ($R = 0.82$, $p = 0.034$) (Fig. 1F) and presynaptic compartments ($R = 0.96$, $p = 0.0028$) (Fig. 1G). No significant association was found between TSA and accumulation of misfolded (Alz50, $R=0.68$, $P=0.11$) or phosphorylated tau (AT8, $R=0.64$, $P=0.14$) in the postsynaptic compartment of AD cases (Fig. 1F). No correlations were found in the control group with any of the synaptic markers. There was no correlation between TSA and excitatory synapse density or the accumulation of T22 in the synapses in primary visual cortex (BA17) (Fig. S2B and 3).

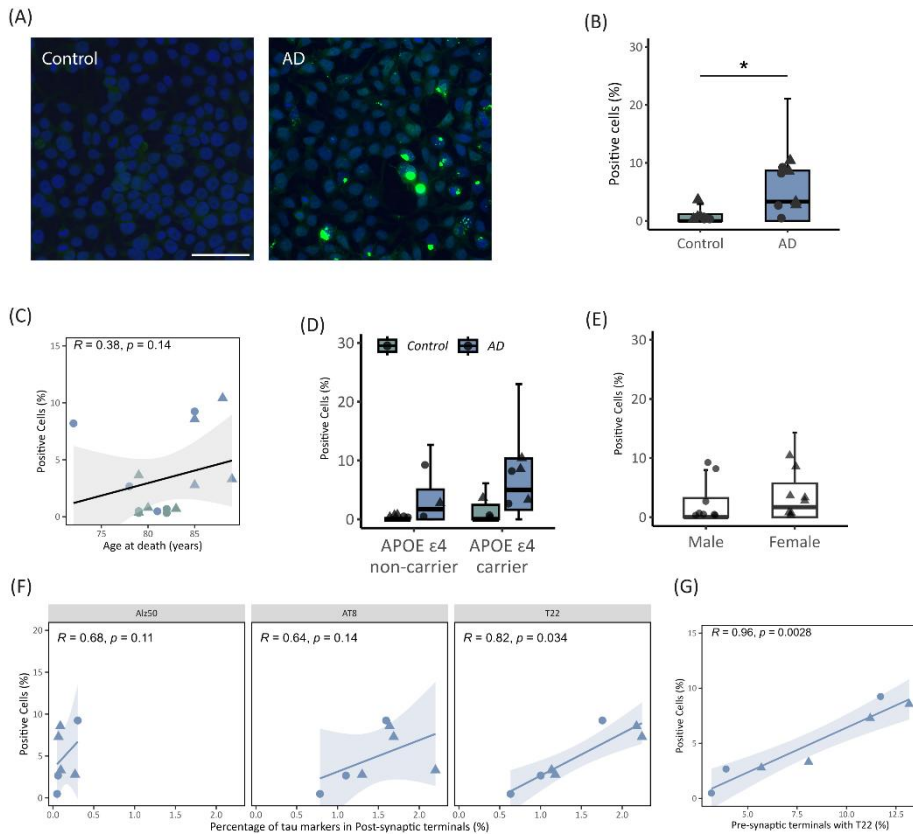


Figure 1. Increased TSA in AD is associated with synaptic oligomeric tau accumulation. (A) Representative images from each group. Scale bar is 50 μm . (B) Quantification of FRET positive cells. Age at death, APOE status and sex did not influence seeding capacity (C, D, E). Correlation plots of AD cases between the percentage of FRET positive cells and tau markers accumulated in the post-synaptic terminals (F) and T22 in presynaptic terminals (G). Boxplots show quartiles and medians calculated from each image. Spearman correlation results are shown for each condition. Data points refer to case means (females, circles; males, triangles).

DISCUSSION

In the present study, we demonstrate that TSA is preferentially associated with the synaptic accumulation of oligomeric tau in AD, rather than with overall tau pathology burden. Using a FRET-based biosensor assay, we confirmed that tau seeds isolated from temporal cortex homogenates of AD cases exhibit higher TSA than those from controls, consistent with previous reports (3,4,10). Recent studies have shown that TSA in AD is highly heterogeneous across patients (1,11). Up to a ten-fold difference in TSA has been reported between cases, which strongly correlates with the levels of oligomeric and phosphorylated tau species (1,10,12). In a cohort of PSP patients, individuals classified as “high-seeders” were associated with high molecular weight tau species and shorter disease duration (13). Collectively, these findings have encouraged the search for factors underlying variability in seeding capacity, with particular emphasis on the contribution of distinct pathological tau species.

In our cohort, TSA was not associated with age, sex, or APOE ϵ 4 status. These findings suggest that TSA is not primarily driven by demographic or APOE genotype, but rather by specific molecular features of AD pathology.

A key observation of this study is that TSA did not correlate with conventional biochemical or histopathological measures of tau burden. Total tau levels in the isolated seeds, as measured by ELISA, were not associated with TSA. Likewise, no significant associations were observed with AT8-positive NFTs or dystrophic neurites. These findings support the notion that TSA does not simply reflect the overall extent of tau pathology (8), but rather the presence of specific, highly bioactive tau species (14-16). However, the analysis of oligomeric tau levels in the isolated seeds was limited by sample availability and should therefore be interpreted with caution.

We and others have demonstrated the presence of oligomeric tau at the synapse in AD (9,17,18), with a 3.3-fold higher abundance of T22-reactive tau species compared to phosphorylated tau. This early accumulation precedes the appearance of NFTs, suggesting a role in the propagation of the pathology across neural circuits (9). Consistent with these observations, we identified a strong correlation between TSA and the accumulation of oligomeric tau (T22) at synaptic terminals, particularly within presynaptic compartments. This finding is in line with prior reports indicating that oligomeric tau predominates in presynaptic rather than in postsynaptic sites (19,20). By contrast, although TSA showed positive correlations with Alz50- and AT8-reactive tau in the postsynaptic compartment, these associations did not reach statistical significance. Taken together, these results extend previous observations and suggest that the synaptic accumulation of oligomeric tau may be mechanistically linked to tau seeding and propagation. Finally, the lack of significant correlations in the primary visual cortex (BA17) suggests that this relationship may be region-dependent and more pronounced in areas with greater pathological vulnerability, such as the temporal cortex. The limited sample size reflects the scarcity of AD cases with tissue suitable for array tomography.

Overall, our results support the notion that synaptic oligomeric tau, rather than total tau burden or mature lesions, is more closely associated with TSA in AD. These findings reinforce the hypothesis that targeting synaptic tau oligomers may be particularly relevant for improving our understanding of disease mechanisms and, ultimately, for limiting tau propagation and clinical progression.

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AUTHOR CONTRIBUTIONS

É.S-A., T.S-J., S.S. and AL designed the research; É.S-A, A.B-A, B.M.L., L.Y. and J.T. collected and prepared the samples; É.S-A. and A.B.A performed the experiments; É.S-A., K.H. and M.C-C. designed the scripts and analysed the data; S.S. and A.L. supervised the study. All authors read and approved the final manuscript.

POTENTIAL CONFLICTS OF INTEREST

The authors declare that they have no financial conflicts of interest related to this paper.

DATA AVAILABILITY

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

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SUPPLEMENTARY MATERIAL

Table S1. Demographic and neuropathological characteristics of the cohort of the present study (n = 16). The table shows UK brain bank number (BBN), diagnosis group, sex, age at death, post-mortem interval (PMI), and neuropathological staging (Braak stage and Thal phase), APOE genotype, and indicates the experimental allocation. 1 ELISA and FRET, 2 Western Blot of oligomeric tau, 3 AT8 Immunohistochemistry in formalin fixed paraffin embedded samples, 4 Array Tomography.

BBN	Clinical Diagnosis	Sex	Age (years)	PMI (h)	Braak stage	Thal phase	APOE genotype	Study
001.28797	Control	M	79	57	0	0	3_3	1,2,4
001.29086	Control	F	79	68	0	1	3_3	1,3,4
001.28406	Control	M	79	72	II	2	3_3	1,2,3,4
001.28793	Control	F	79	72	II	1	3_3	1,2,3,4
001.29082	Control	F	79	80	III	5	3_4	1,2,3,4
001.35181	Control	M	82	49	II	2	3_4	1,2,4
001.34131	Control	M	82	95	IV	3	3_4	1,4
001.35866	Control	F	83	78	IV	4	NA	1,2,4
001.35096	AD	M	72	103	VI	5	3_4	1,2,3,4
001.26718	AD	M	78	74	VI	5	3_4	1,3,4
BBN_24527	AD	M	81	74	V	4	3_3	1,3,4
001.28771	AD	M	85	91	VI	5	3_3	1,2,3,4
001.32929	AD	F	85	80	VI	5	3_3	1,2,4
BBN_25739	AD	F	85	45	VI	5	3_4	1,2,3,4
001.30142	AD	F	88	112	VI	5	3_4	1,3,4
001.30973	AD	F	89	96	VI	5	3_4	1,2,3,4

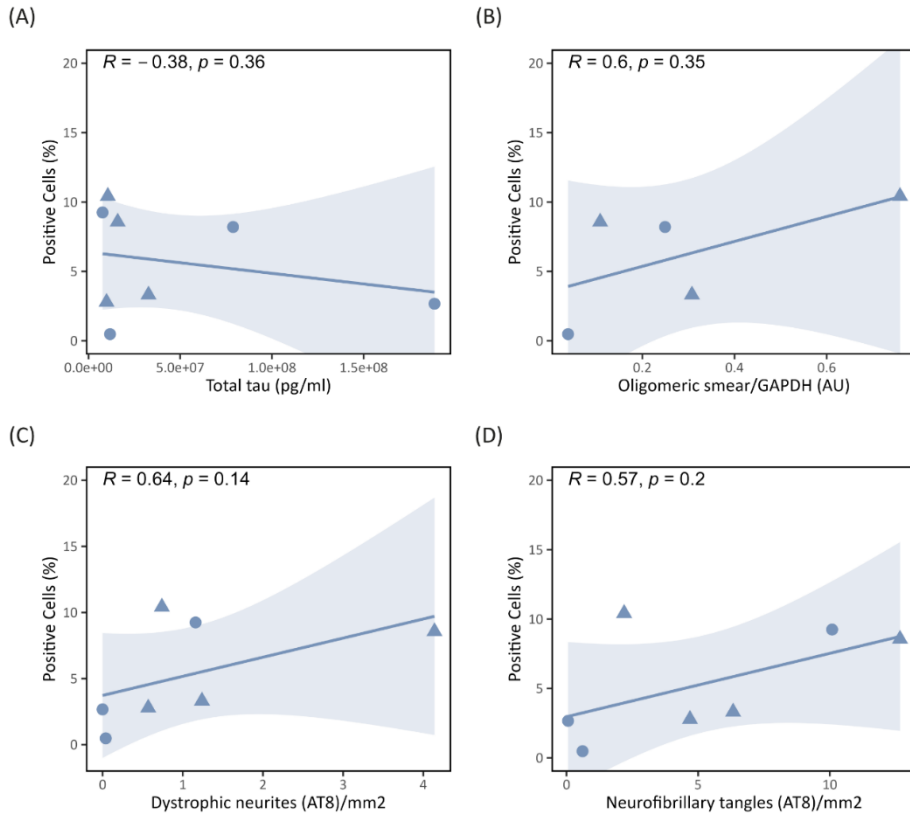


Figure S1. TSA is not associated with total tau pathology burden. Correlation plots of AD cases between the percentage of FRET positive cells and total tau (measured by ELISA, A), oligomeric smear (measured by WB, B), and the density of dystrophic neurites and NFTs (AT8 immunoreactivity in formalin fixed paraffin embedded samples, C-D). Spearman correlation results are shown in each condition. Data points refer to case means (females, circles; males, triangles). See Supplementary Table 1 for detailed information on the cases included in each analysis.

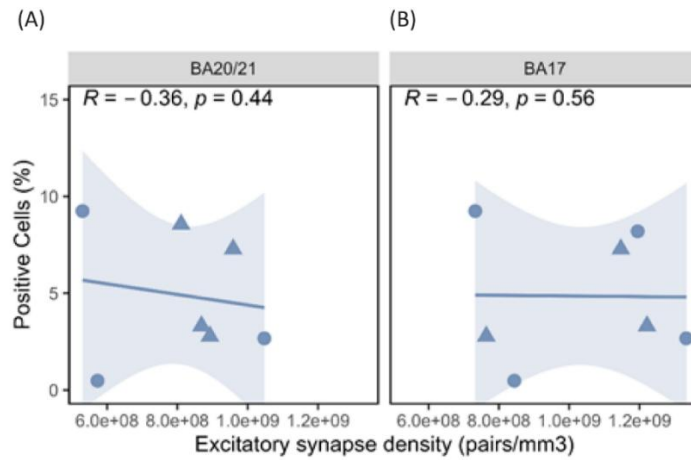


Figure S2. Relationship between TSA and excitatory synapse density. Correlation of TSA of AD cases in temporal cortex (BA20/21) and primary visual cortex (BA17) and excitatory synapse density. Spearman correlation results are shown in each condition. Data points refer to case means (females, circles; males, triangles).

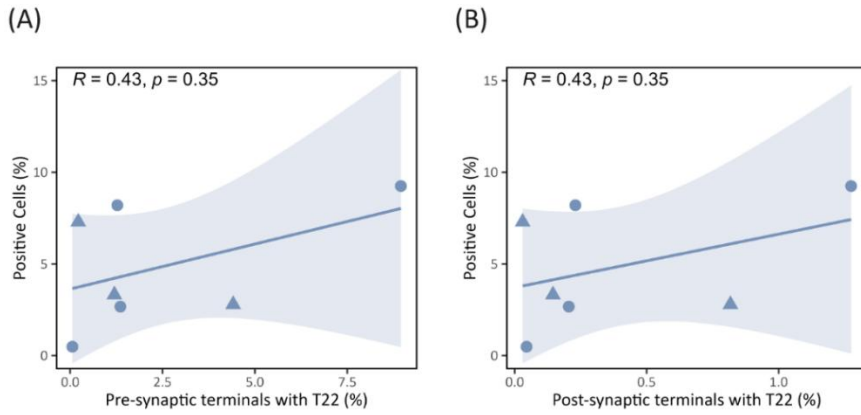


Figure S3. Correlations between seeding activity and the synaptic accumulation of T22 at the synaptic compartments in the primary visual cortex (BA17). (A) presynaptic terminals and (B) post-synaptic terminals. Spearman correlation results are shown in each condition. Data points refer to case means (females, circles; males, triangles).

CHAPTER 5 | DISCUSSION

5. STUDYING SYNAPTIC OLIGOMERIC TAU IN AD

In this thesis, we combined high-resolution microscopy techniques in human brain tissue in order to investigate the presence and synaptic distribution of oligomeric tau within the synapse, with the aim of better understanding the synaptotoxic role of these species (*Study 1 and 2*). In addition, we performed functional seeding assays *in vitro*, a widely used approach to assess tau seeding activity, to explore the relationship between tau seeding capacity and the accumulation of oligomeric tau at synapses (*Study 3*).

5.1 EARLY PRESENCE OF OLIGOMERIC TAU IN HUMAN SYNAPSES

In *Study 1*, we found that oligomeric tau, along with misfolded and phosphorylated tau species, are found at synaptic terminals of AD cases even when NFT burden is low. This finding is consistent with the hypothesis that loss of synapses represents an early event and a relevant localisation for tau accumulation (16,129,172).

This study, to our knowledge, represents the first comprehensive visualisation of oligomeric tau at synaptic terminals in human brain. Using AT, we imaged more than 1 million individual synaptic pairs in the inferior temporal cortex, together with different antibodies targeting tau species. Taking advantage of the high spatial resolution of this technique, we were able to identify oligomeric tau as the most common form of tau present at both pre- and postsynaptic compartments.

In prior studies it has been shown that synaptic loss is the strongest correlate of cognitive decline in AD (15), and that part of the synaptic dysfunction observed is associated with accumulation of tau (173,174). In this thesis we also described a marked synaptic excitatory loss in the AD cases compared to controls. In addition, we observed a significant reduction in synapses around neuritic plaques, in agreement with previous observations on the detrimental

effects of A β oligomers to the surrounding synapses of A β plaques. By contrast, the effect of tau seems to be more diffuse throughout the brain, as a decrease in excitatory synapses in the immediate environment of NFTs was not detected, which is consistent with the nature of this lesion as an intracellular pathology.

Additionally, oligomeric tau has been associated with morphological changes at the synapse, impairing synaptic plasticity and transmission, overall halting/disturbing the correct function of the synapse (77,80,153,175). Changes observed in memory function are restored when reducing oligomeric levels in models of tauopathy, highlighting the direct role of this species in synaptic function (157,176,177).

In line with observations made in animal models, where oligomeric tau is already present in areas with no overt tau pathology (75), we found that oligomeric tau was already accumulating in the AD synapses of the primary visual cortex, where there is low or no NFT presence. This result is in line with the amount of evidence that loss of synapses is an early event in AD and other neurodegenerative disorders, implicating oligomeric tau as a potential contributor to early synaptic damage, prior the formation of NFTs (178,179).

In parallel, we also evaluated the presence of early tau pathology using the AT180 antibody (180), and found that both oligomeric tau (T22) and phosphorylated tau (AT180) accumulates in AD presynaptic terminals compared to controls in the inferior temporal cortex. However, T22-labelled terminals were substantially more frequent than AT180-labelled terminals, supporting the idea that synaptic oligomeric tau is an early pathological feature.

Moreover, a recent study using expansion microscopy showed that synapses that contained oligomeric tau are more prone to be engulfed by microglia. This

result suggests that the presence of tau oligomers at synapses may signal increased glial-mediated synapse elimination and contribute to early functional impairment in AD (181).

We next confirmed the presence of tau oligomers at the synapse biochemically in synaptoneurosome preparations. We and others have previously reported the presence of tau in this compartment using biochemical isolation of synaptic terminals (163,168,182). However, these techniques do not allow the simultaneous investigation of multiple proteins within the same synapse and are therefore better suited for assessing different proteins across independent synapses rather than real co-localisation. For this reason, we also performed immunogold electron microscopy, DNA-PAINT and dSTORM, which further demonstrated the presence of pathological tau in both pre- and postsynaptic terminals.

Taken together, the presence and accumulation of oligomeric tau within synapses seems to be an early event in AD tau pathology, and because of the intricate composition and small size of the synapse, specialised techniques are needed to study synaptic pathology.

5.2 RESOLVING HUMAN SYNAPSES WITH AT AND dSTORM

In order to reliably resolve human synaptic terminals, we combined AT with dSTORM in *Study 1* and 2. These sub-diffraction techniques allowed the detailed characterization of synaptic tau in AD brains, enhancing an optimal lateral resolution for the study of synaptic tau pathology.

While in *Study 1* combination of AT and dSTORM was used mainly to confirm the presence of oligomeric tau at synapses of a single AD case, *Study 2* reflects a long process of optimisation of the combined protocol. As part of this work, we designed and developed in-house Python algorithms that enables the

detailed study of single synapses and is publicly available for other groups to use.

EM studies have shown that the synaptic cleft separates the pre- and postsynaptic compartments by approximately 20-50 nm (97,183,184). Here, by combining both techniques, we achieved nanoscale resolution, that allowed us to discriminate objects as close as 8 nm apart, reporting a mean width of the synaptic cleft of approximately 60 nm, which closely relates with the range reported with EM.

One factor that could affect the preservation of the ultrastructure in resin embedded samples is the PMI, with shorter intervals generally recommended (185). This represents a limitation when working with human post-mortem samples. However, when the protocol is well standardised even samples with long PMI can still show preserved ultrastructure (186). We validated our AT protocol by comparing cortical biopsy samples with AT control samples. The mean distance between synaptic compartments was not significantly different between the two groups, suggesting that PMI, or the AT sample preparation protocol, did not substantially affect synaptic ultrastructure. Nevertheless, as part of the analysis protocol, we continued to account for PMI as a potentially confounding variable.

Taking advantage of the improved lateral resolution, we further explored the presence of oligomeric tau within the synaptic compartments of sporadic AD cases. Oligomeric tau was closer to the centroid of the presynaptic terminal when compared to the postsynaptic density. We also found that oligomeric tau was larger than the pre and postsynaptic markers, consistent with their molecular composition being soluble oligomers 300–500 kDa, in contrast with synaptic proteins (187).

Moreover, we found that tau oligomers were larger when accumulated in the postsynaptic compartment in contrast to the presynaptic compartment, with length and eccentricity like results seen in primary cell cultures using SR microscopy (188). One possible scenario for the larger size of postsynaptic oligomeric tau is that this compartment favours the retention of tau species, as alterations on the ubiquitin-proteasome system has been associated with oligomeric tau (163). In contrast to the presynaptic compartment, where tau species may remain more involved in transfer mechanisms, the postsynaptic compartment may act as a receiving site, allowing oligomeric tau to accumulate into larger and more elongated assemblies.

Overall, AT+dSTORM proves to be an optimal technique to reliably resolve synaptic pathology in the human AD brain, accurately localizing oligomeric tau within synaptic terminals.

5.3 TRANS-SYNAPTIC SPREAD OF OLIGOMERIC TAU

Growing evidence suggests that tau could be spreading throughout the brain across connected synaptic circuits, rather than by spatial proximity (159,189). Data from this thesis (*Study 1*) support this hypothesis.

A relevant part of our analysis is that we restricted the results to paired synapses, where synaptic terminals had a partner within 0.5 μm (190). Although this restriction reduces the overall synaptic density analysed, it also lowers the risk of detecting false-positive synaptic pairs, making our inferences about synaptic oligomeric tau more reliable. In our data, oligomeric tau accumulates in both sides of the same synapses and seems to follow an anterograde direction, where it first accumulates at the presynapses followed by release and uptake by the postsynapses. Recent work on a living human brain slice culture model of PSP brains, have shown that oligomeric tau can enter live human postsynapses (191), supporting the anterograde transmission.

Although mechanisms of tau spread have been proposed in both directions (192–196), this observation suggest that, at least in the synaptic context, tau transmission may occur predominantly in an anterograde manner. Remarkably, oligomeric tau followed this pattern also in control brains, with the proportion observed in AD brains significantly higher. This suggest that the propagation of tau may be occurring both in health and in pathology, with the latter being at greater frequency.

This observation had been previously demonstrated in a mouse model with overexpression of human P301L mutant tau (197), where tau spreads through the synapse, from pre- to postsynaptic terminals, in the absence of degeneration. In *Study 1* we also included data from the same mouse model, that has synaptophysin tagged with GFP, showing that oligomeric tau can spread in an anterograde manner in mammalian brain.

We also found oligomeric tau more frequently in presynaptic than in postsynaptic terminals in the frontal cortex in *Study 2*. However, because our protocol of AT+dSTORM is limited to two fluorophores at a time, it does not allow visualization of terminals containing oligomeric tau at both synaptic compartments.

Taking together, we found indirect evidence that the trans-synaptic propagation of oligomeric tau is plausible. This statement should be made with caution due to the inherent descriptive cross-sectional nature of post-mortem studies.

5.4 SYNAPTIC ACCUMULATION OF OLIGOMERIC TAU CORRELATES WITH TSA

In the last years, TSA has emerged as a potential molecular driver of heterogeneity in AD patients (91,92), who differ in age of onset and clinical presentation, but also in the rate of cognitive decline. A recent study showed that TSA from brain fractions containing oligomers correlated most strongly

with disease aggressiveness, where patients classified as rapid progressors showed higher levels of oligomeric tau (198). Moreover, purified brain samples showed high heterogeneity, suggesting that different conformations of tau oligomers could contribute to the heterogeneity in disease progression. Another study reported that a lower TSA was associated with oligomers derived from patients that showed greater resilience to AD pathology (166). Based on this prior evidence, in *Study 3* we aimed to explore the relationship of TSA with the pathological aggregates present at the synapse. For this purpose, we used the same cohort as in *Study 1*.

Previous studies have reported that TSA does not reflect the total amount of tau pathology (199), and that it is more closely related to tau bioactivity (81,154,200,201). Our results are in line with this notion, as we did not observe correlations between TSA and either total tau levels (by ELISA) or tau pathology burden (by IHC).

Interestingly, we found a strong correlation between TSA and synaptic accumulation of oligomeric tau, especially in the presynaptic compartment. In contrast, no significant correlations were found with synaptic misfolded or hyperphosphorylated tau. This result is consistent with the observation made in *Study 1*, where oligomeric tau was the most prevalent tau form at synapses, and with previous reports suggesting that oligomeric tau represents one of the most bioactive forms of tau.

In combination, these findings support the idea that synaptic tau oligomers may be particularly relevant for AD pathology. Their association with TSA suggests that these species may not only accumulate at synapses but may also contribute to the propagation of tau pathology. Therefore, targeting synaptic oligomeric tau could be important not only to reduce local synaptic toxicity, but also to potentially limit tau spread and slow clinical progression.

5.5 SUMMARY

This thesis shows that synaptic oligomeric tau is an early event in the disease progression and a potential culprit in the spread of tau pathology through the AD brain, combining sub-diffraction techniques along with functional *in vitro* assays.

We found that oligomeric tau is present at both sides of the synapse, with a distribution that suggests an anterograde trans-synaptic spreading of tau oligomers. We also show that TSA correlates with synaptic accumulation of oligomeric tau, supporting that targeting synaptic oligomeric tau could potentially limit the spread of tau and disease progression.

CHAPTER 6 | CONCLUSIONS

The main conclusions of this thesis are:

1. Synaptic oligomeric tau is present in the synapses of AD brains and its accumulation is consistent with an early event.
2. From all tau species investigated in this thesis, oligomeric tau was consistently the most frequent form accumulated in synaptic terminals.
3. The combination of AT and dSTORM provides a novel and sensitive tool for resolving human synapses and for the study of synaptic pathology in human brain.
4. The gradient accumulation of synaptic oligomeric tau from pre- to postsynapses is consistent with an anterograde trans-synaptic spread.
5. TSA correlates with the synaptic accumulation of oligomeric tau in AD brains.

CHAPTER 7 | BIBLIOGRAPHY

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