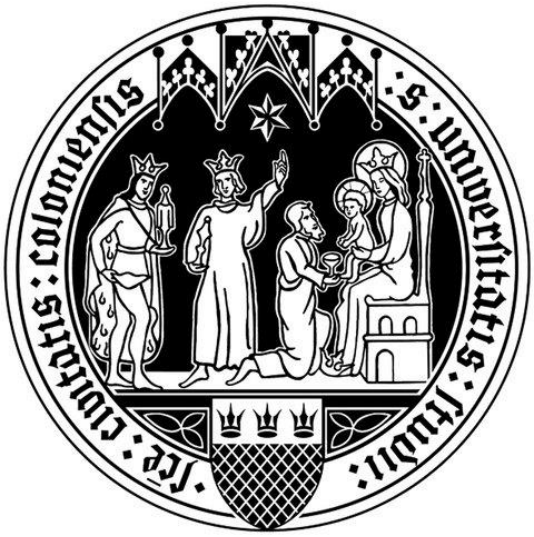
Investigating human TAU isoform-specific functions and their toxicity in Alzheimer's Disease and related Tauopathies



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List of Abbreviations

AD Alzheimer's Disease
AIS Axon initial segment
APP Amyloid precursor protein
ASOs Antisense oligonucleotides
Aβ Amyloid-beta
BBB Blood-brain barrier
CBD Corticobasal degeneration
COC Critical oligomer concentration
CSF Cerebrospinal fluid
CT Computed tomography
dimAβ Dimeric $A\beta_{40}$
DMTs Disease-modifying therapeutics
DNMT1 DNA methyltransferase 1
EB3 End-binding protein
fAD Familial Alzheimer's Disease
FDA U.S. Food and Drug Administration
FTD Frontotemporal dementia
FTDP-17 Frontotemporal dementia and parkinsonism linked to chromosome 17
HD Huntington’s disease
hiPSC Human induced pluripotent stem cell
HSV Herpes simplex virus
IF Immunofluorescence
iNs Human iPSC-derived neurons
KD Knockdown
KI Knockin
KO Knockout
LC Locus coeruleus
LOAD Late-onset Alzheimer's disease
MCI Mild cognitive impairment
mPNs Mouse primary neurons
MRI Magnetic resonance imaging
MTs Microtubules
NFTs Neurofibrillary tangles
PD Parkinson’s disease
PET Positron emission tomography
PiD Pick’s disease
PS1 Presenilin 1

PS2 Presenilin 2
PSD95 Postsynaptic density protein 95
PSP Progressive supranuclear palsy
sAD Sporadic Alzheimer's disease
shRNAs Small-hairpin RNAs
siRNA Small-interfering RNA
SRs Serine/arginine-rich splicing factors
TBI Traumatic brain injury
TDB TAU diffusion barrier
tg Transgenic
TTLL6 Tubulin-Tyrosin-Ligase-Like-6
vGLUT1 Vesicular glutamate receptor 1
WT Wildtype

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Data Availability Statement

The underlying data to support the findings of this thesis are available in the cited publications. Raw data is stored on the private servers of the research group Zempel, at the Institute of Human Genetics, University Hospital of Cologne and is available from the author(s) upon reasonable request.

Zusammenfassung

Die Alzheimer-Krankheit (AD) ist eine schwerwiegende, progressive neurodegenerative Erkrankung, die aufgrund ihrer steigenden Prävalenz - weltweit sind Millionen von Menschen betroffen - und des Mangels an wirksamen Behandlungsmöglichkeiten eine große Belastung für das moderne Gesundheitswesen darstellt. Die klinische Symptomatik umfasst die Abnahme kognitiver Funktionen, Gedächtnisverlust, Störungen der Sprache und des räumlichen Vorstellungsvermögens sowie Verhaltensveränderungen. Auf molekularer Ebene ist die Alzheimer-Krankheit durch die Akkumulation und Aggregation von Amyloid Beta (Aβ) und TAU in unlöslichen Proteinaggregaten im Gehirn gekennzeichnet. Die Aβ-Aggregation gilt als ein früher Faktor in der pathologischen Kaskade der Alzheimer-Krankheit. Jedoch korreliert die TAU-Pathologie besser mit dem Verlust von Synapsen und der Verschlechterung der kognitiven Fähigkeiten der Patienten, was die zentrale Rolle von TAU für das Fortschreiten der Krankheit unterstreicht. TAU, ein Mikrotubuli-assoziiertes Protein, stabilisiert neuronale Mikrotubuli (MTs), die für die Aufrechterhaltung der neuronalen Funktion wesentlich sind. Durch alternatives Spleißen des für TAU kodierenden *MAPT*-Gens entstehen sechs hirnspezifische TAU-Isoformen, die sich in der Anzahl der N-terminalen Inserts (0N, 1N oder 2N) und C-terminalen Repeats (3R oder 4R) unterscheiden. Auffallend ist, dass Veränderungen im Expressionsmuster der TAU-Isoformen allein ausreichen können, um neurodegenerative Krankheiten zu verursachen, die durch eine pathologische Anhäufung von TAU gekennzeichnet sind, wie z.B. Frontotemporale Demenz. Obwohl dies auf entscheidende Aufgaben der TAU-Isoformen bei neurodegenerativen Erkrankungen hindeutet, sind diese nicht hinreichend untersucht worden. Wir vermuten, dass humane TAU-Isoformen unterschiedliche Funktionen im gesunden und erkrankten Gehirn haben, und daher von therapeutischem Wert sein könnten.

Wir haben uns daher zum Ziel gesetzt, i) geeignete neuronale Zellmodelle zu etablieren, um die (isoformspezifischen) Funktionen von menschlichem TAU zu untersuchen, ii) die isoformspezifischen Eigenschaften von TAU in einem krankheitsrelevanten zellulären Kontext zu analysieren, iii) und die durch die TAU-Isoformen vermittelte Toxizität zu untersuchen, indem AD-ähnlicher Stress in menschlichen Neuronen modelliert wird, um neue potenzielle therapeutische Ziele für die Behandlung von AD zu identifizieren. Aus diesem Grund haben wir die Expression aller sechs menschlichen TAU-Isoformen mit Hilfe von Liposomen und Lentiviren optimiert, die Differenzierung menschlicher induzierter pluripotenter Stammzellen (hiPS) in glutamaterge Neurone auf der Grundlage der Doxycyclin-induzierten Expression des Transkriptionsfaktors Neurogenin 2 (NGN2) etabliert und eine *MAPT* KO hiPS Zelllinie mit Hilfe von CRISPR/Cas9 erzeugt, um die TAU-Isoformen unabhängig von endogenem TAU zu analysieren. Um die zellulären Auswirkungen in Abhängigkeit von den TAU-Isoform zu beurteilen, verwendeten wir

verschiedene Methoden, wie z.B. Immunfluoreszenzfärbungen zur Untersuchung der axodendritischen Verteilung von TAU, Beobachtung der MT-Dynamik in lebenden Zellen und Visualisierung von Kalzium-Oszillationen zur Untersuchung der neuronalen Aktivität.

Um die isoformspezifischen Eigenschaften von TAU in unseren etablierten Modellsystemen zu untersuchen, wurden einzelne TAU-Isoformen in menschlichen undifferenzierten SH-SY5Y-Neuroblastomzellen exprimiert und die MT-Dynamik mittels MT Plus End Tracking untersucht. Es wurden subtile Unterschiede zwischen den TAU-Isoformen in Bezug auf die Vermittlung der MT-Stabilität festgestellt, jedoch reduzierte die Präsenz der 4R-TAU-Isoformen die allgemeine Zellgröße. Die Beobachtung der Verteilung der TAU-Isoformen durch Immunfluoreszenzfärbungen in polarisierten murinen und menschlichen Neuronen zeigte, dass größere und reifere Isoformen, wie 2N4R und 1N4R, im Vergleich zu kleineren Isoformen weniger effizient in das Axon sortiert werden. Die Deletion von TAU in menschlichen Neuronen führte zu leichten phänotypischen Veränderungen, wie z.B. einem verringerten Neuritenwachstum und einer verringerten Länge des initalen axonalen Segments (AIS) in verschiedenen Entwicklungsstadien (sichtbar nach 7 bzw. 21 Tagen). Die Präsenz einzelner Isoformen reichte aus, um die Veränderungen in der Neuriten- und AIS-Entwicklung zu beheben. Spontane Kalzium-Oszillationen, die in lebenden Zellen mittels des Kalzium-sensitiven Fluoreszenzfarbstoffes Fluo4 beobachtet wurden und als Indikator für die neuronale Aktivität gelten, waren in Abwesenheit unterstützender Gliazellen leicht erhöht, blieben aber in *MAPT*-KO Neuronen weitgehend unverändert.

Um die durch die TAU-Isoformen vermittelte Toxizität zu untersuchen und die Isoform(en) zu identifizieren, die die Toxizität vermitteln, induzierten wir AD-ähnlichen Stress durch Exposition der Neurone gegenüber Amyloid Beta-Oligomeren (AβOs). Bei der Alzheimer-Krankheit verursacht die Bildung von AβOs vermutlich eine Hyperphosphorylierung von TAU, seine Dissoziation von axonalen MTs und eine Fehlsortierung von TAU, was zu Synaptotoxizität und kognitiver Beeinträchtigung führt. Die AβO-Exposition und das Anlagern an die Dendriten von menschlichen und murinen Neuronen führte zu einer TAU-Fehlverteilung in das Soma und die Dendriten (sichtbar in Immunfluoreszenz-Färbungen) und verringerte den Prozentsatz aktiver Zellen, was durch die Visualisierung von Kalzium-Oszillationen in lebenden Zellen bestimmt wurde. Vor allem aber waren *MAPT*-KO Neurone resistent gegen den Aβ-induzierten Verlust neuronaler Aktivität, was die Bedeutung von TAU in der pathologischen Kaskade, die durch toxische Aβ-Spezies in menschlichen Neuronen ausgelöst wird, unterstreicht. Darüber hinaus stellte die Re-Expression der 1N4R-Isoform, nicht aber anderer Isoformen, die neuronale Vulnerabilität für AD-ähnlichen Stress vollständig wieder her.

In der vorliegenden Arbeit haben wir mehrere Modelle entwickelt, die sich für die Untersuchung der Funktion der menschlichen TAU-Isoformen unter gesunden und

pathologischen Bedingungen eignen. Unsere Ergebnisse liefern Beweise dafür, dass: i) menschliche TAU-Isoformen in reifen Neuronen unterschiedlich verteilt sind, ii) einzelne TAU-Isoformen den MT-Aufbau und die MT-Stabilität in ähnlichem Ausmaß in undifferenzierten Zellen vermitteln können, iii) die Regulierung der Neuriten- und AIS-Entwicklung neue Funktionen von TAU darstellen, die von allen sechs humanen spezifischen TAU-Isoformen vermittelt werden können, iv) die Deletion von TAU menschliche Neuronen vor dem Aβ-induzierten Verlust der neuronalen Aktivität schützt, was die Schlüsselrolle von TAU bei der Vermittlung der Aβ-induzierten Synaptotoxizität zeigt, und v) dass eine spezifische TAU-Isoform, 1N4R, für die frühe TAU-vermittelte Toxizität verantwortlich ist, was künftige Therapieansätze für diese derzeit noch unheilbare Krankheit erheblich verbessern könnte.

Zusammenfassend liefert diese Studie den ersten Beweis für isoform- und kompartmentspezifische Funktionen von TAU in menschlichen Neuronen, legt nahe, dass TAU eine Schlüsserolle in der Aβ-induzierten synaptischen Dysfunktion spielt, und hebt eine differenzierte Rolle der TAU-Isoformen bei der AD-Progression hervor, wobei die TAU-Isoform 1N4R die wichtigste Isoform bei der frühen TAU-vermittelten Toxizität ist. Darüber hinaus legt diese Studie den Grundstein für zukünftige Studien, in denen das Potenzial der TAU-Isoformen als potenzielle therapeutische Ziele für die Alzheimer-Krankheit und verwandte neurodegenerative Erkrankungen untersucht wird.

Summary

Alzheimer's disease (AD) is a devastating, progressive neurodegenerative disorder that constitutes a major burden for modern health care due to its rising prevalence, with millions of people affected worldwide and the lack of effective treatments. Clinical symptoms include a decline in cognitive function, memory loss, impairments of language and visuospatial function, and behavioral changes. On the molecular level, AD is characterized by the accumulation of amyloid beta (Aβ) and TAU in insoluble protein aggregates in the brain. While Aβ aggregation is considered an upstream factor in the pathological cascade causing AD, TAU pathology correlates better with synapse loss and cognitive decline in patients, demonstrating the key role of TAU in driving disease progression. TAU, a microtubule-associated protein, stabilizes neuronal microtubules (MTs), which are essential for maintaining neuronal function. Alternative splicing of the TAU-encoding *MAPT* gene generates six human brain-specific TAU isoforms, which differ in the number of N-terminal inserts (0N, 1N, or 2N) and C-terminal repeats (3R or 4R). Strikingly, changes in the abundance of the TAU isoforms alone can be sufficient to cause neurodegenerative diseases characterized by the pathological accumulation of TAU, such as Frontotemporal dementia. Although this implies crucial functions for the TAU isoforms in disease, they have not yet been thoroughly examined. We hypothesize that human TAU isoforms have differential functions in health and disease and may be of therapeutic value.

Therefore, we aimed here to i) establish suitable neuronal cell models to study (isoform-specific) functions of human TAU, ii) analyze isoform-specific properties of TAU in a disease-relevant cellular context, iii) and investigate TAU-isoform mediated toxicity by modeling AD-like stress in human neurons to identify novel potential therapeutic targets for the treatment of AD. Therefore, we optimized the expression of all six human TAU isoforms using liposome- and lentivirus-based delivery, established the differentiation of human induced pluripotent stem cells (hiPSCs) into glutamatergic neurons based on the doxycycline-induced expression of transcription factor Neurogenin 2 (NGN2), and generated a *MAPT* KO hiPSC line using CRISPR/ Cas9 to analyze the TAU isoforms independent of endogenous TAU. To assess the cellular effects of the individual TAU isoforms, we used several methods, such as immunofluorescence stainings to investigate the axodendritic distribution of TAU, live-cell tracking of MT dynamics, and calcium imaging to investigate neuronal activity.

To investigate isoform-specific properties of TAU in our established model systems, single TAU isoforms were introduced into human undifferentiated SH-SY5Y neuroblastoma cells, and MT dynamics were assessed by live-cell MT plus end tracking. Only subtle differences were observed between the TAU isoforms with regard to mediating MT stability, however, the presence of 4R TAU isoforms reduced general cell size. Further, TAU isoform distribution assessed by immunofluorescence stainings in polarized murine and human neurons showed

that larger and more mature isoforms, such as 2N4R and 1N4R, are less efficiently sorted into the axon comparted to smaller isoforms. TAU depletion in human neurons resulted in mild phenotypic changes, such as reduced neurite outgrowth and axon initial segment (AIS)length at different developmental stages (apparent after 7 and 21 days, respectively). Re-introduction of individual isoforms was sufficient to rescue the changes in neurite and AIS development. Spontaneous calcium oscillations measured by Fluo4-based live-cell imaging as a readout of neuronal activity were slightly increased in the absence of supporting glia but remained unchanged in *MAPT* KO neurons.

To investigate TAU-isoform mediated toxicity and identify the isoform(s) mediating toxicity, we induced AD-like stress using exposure to amyloid beta oligomers (AβOs). In AD, the formation of AβOs supposedly causes TAU hyperphosphorylation, its dissociation from axonal MTs, and TAU missorting, resulting in synaptotoxicity and cognitive impairment. AβO exposure of human and murine neurons resulted in dendritic targeting of AβOs, induction of TAU missorting to the soma and dendrites (apparent in immunofluorescence stainings), and decreased the percentage of active cells assessed by live-cell calcium imaging. Most importantly, *MAPT* KO neurons were resistant to Aβ-induced loss of neuronal activity demonstrating the importance of TAU in the pathological cascade induced by toxic Aβ species in human neurons. In addition, re-introduction of the 1N4R isoform, but not other isoforms, fully restored neuronal vulnerability to AD-like stress.

Here, we established several models suitable for studying the function of human TAU isoforms under healthy and pathological conditions. Our results provide evidence that: i) human TAU isoforms are differentially distributed in mature neurons, ii) all TAU isoforms can mediate MT assembly and stability in an isolated manner and to a similar extent in undifferentiated cells, iii) the regulation of neurite and AIS development represent novel functions of TAU that can be mediated by all six human specific TAU isoforms, iv) TAU depletion protects human neurons from Aβ-induced loss of neuronal activity demonstrating the key role of TAU in mediating Aβ-induced synaptotoxicity, and v) that one specific TAU isoform, 1N4R, mediates early TAU-mediated toxicity, which could significantly improve future treatment strategies for this currently untreatable and detrimental disease.

In conclusion, this study provides the first evidence for isoform- and compartment-specific functions of TAU in human neurons, suggests that TAU is a key mediator of Aβ-induced synaptic dysfunction, and highlights a differential role of TAU isoforms in AD progression with the TAU isoform 1N4R being the most important isoform in early TAU-mediated toxicity. Furthermore, this study lays the foundation for future studies evaluating the potential of TAU isoforms as potential therapeutic targets for Alzheimer's disease and related neurodegenerative disorders.

1. Introduction

With over 55 million people affected worldwide in 2020 and an estimated increase of 10 million cases per year, dementia-related syndromes represent a huge burden for those affected, their families, caretakers, and society [1]. By 2050 about 113 million people will be affected by dementia[2]. Furthermore, the risk of developing dementia increases dramatically with age, with an incidence of 1 in 100 individuals per year aged over 65 and 4 in 100 individuals per year aged over 80[3]. Alzheimer's disease (AD) is the most common form of dementia, contributing to up to 70% of all dementia cases[4]. Individuals with AD are usually only diagnosed in the later disease stages when they show noticeable memory and behavioral impairments, have problems in their daily life (e.g., problems getting dressed, problems recognizing friends and family, difficulty speaking, etc.), and depend on others for their care. However, pathological changes in the brain occur already years or even decades before the clinical onset of symptoms, making early diagnosis and timely treatment of AD especially challenging [4]. Besides existing symptomatic treatment, two disease-modifying therapeutics (DMTs) were recently approved by the U.S. Food and Drug Administration (FDA), targeting pathological amyloid-beta (Aβ) species that are a major contributor to AD pathology [5,6].

1.1. Hallmarks of Alzheimer's Disease

AD is a slowly progressive disease characterized in later stages by impairments of memory, language, visuospatial, and executive functions. Even though individuals in early phases do not display any phenotypic changes and are cognitively normal, subtle changes in the brain can be noted years to decades before the onset of symptoms [7,8]. In these preclinical phases, decreased abundance of amyloid beta 42 ($A\beta_{42}$) in the cerebrospinal fluid (CSF) is one of the first detectable changes, next to enhanced signals of Aβ in positron emission tomography (PET) scans and decreased Fluorodeoxyglucose (FDG) PET signals reminiscent of glucose hypometabolism [9,10] . Furthermore, a variety of alterations occur on the cellular level, such as astrocyte and microglial activation, increased neuroinflammation, blood vessel alterations, dysfunction of the glymphatic system, and the blood-brain barrier (BBB) that contribute to disease progression [7,11–15]. Individuals in intermediate disease stages show mild cognitive impairment (MCI) that does not interfere with everyday activities. Increased levels of total and phosphorylated TAU can be detected in the CSF, and enhanced signals of TAU are observed in PET scans. In severe stages of AD, individuals are not able to function in their daily life, and most require full-time care. Symptoms include memory and learning impairments, behavioral changes (such as aggression and agitation), and impairments of language and visuospatial function[4]. Besides elevated levels of TAU in the CSF and increased PET signals for Aβ and TAU, brain atrophy is visible by structural magnetic

resonance imaging (MRI) or computed tomography (CT) scans and correlates with cognitive impairment[8–10].

On the molecular level, major hallmarks of AD [7,8,16,17] are then detectable in the brain (see Figure 1):

A) The accumulation of Aβ peptides in insoluble extracellular amyloid plaques and intracellular aggregation of hyperphosphorylated TAU protein in neurofibrillary tangles (NFTs) and neuropil threads.
B) Synapse loss and neuronal cell death leading to brain atrophy.
C) Loss of blood-brain barrier integrity, activation of microglia and astrocytes, and subsequent neuroinflammation.

The pathological changes observed in the brain of AD patients result in severe synaptic and neuronal loss, which correlates with the onset of cognitive impairment[7,8]. Based on the drastic changes in the brain and the development of prognostic biomarkers, the A/T/N classification has been implemented by the National Institute on Aging and Alzheimer's Association (NIA-AA) for the definition of AD stages ("AD continuum")[18]. While A and T are based on amyloid and TAU pathology (PET and CSF levels) and define if an individuum has AD, N is a marker for neurodegeneration (analyzed by FDG PET, anatomical MRI) and defines disease severity[18]. Taken together, eight different A/T/N biomarker profiles can be defined, which help classify and stage AD cases.

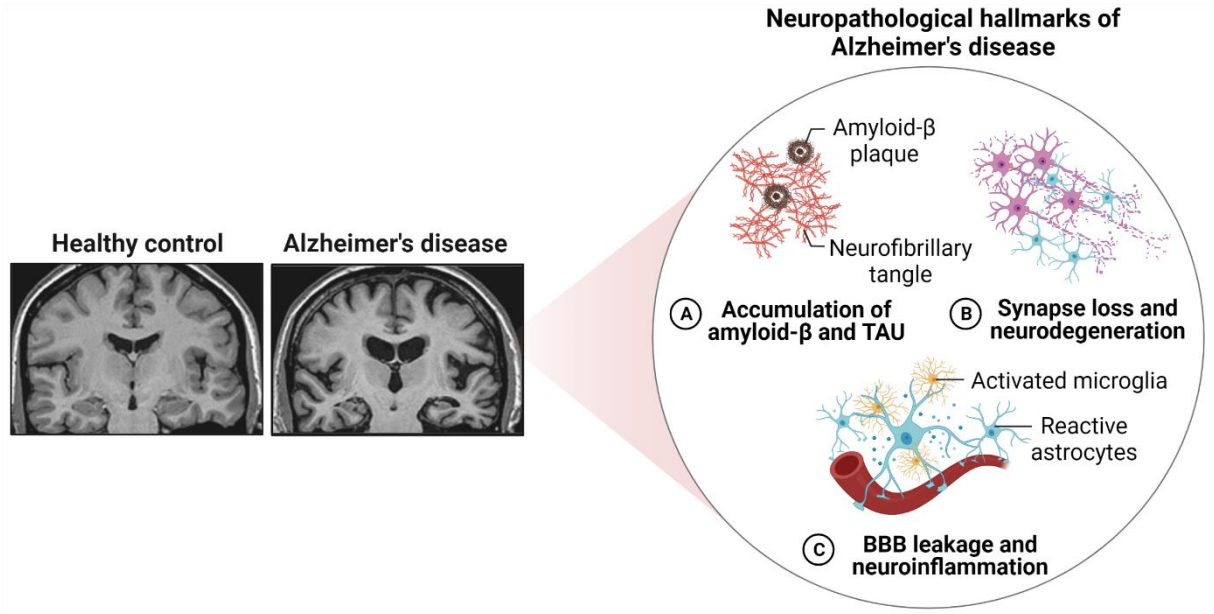

Figure 1 Neuropathological hallmarks of Alzheimer's disease. In the late stages of AD, hippocampal and cortical atrophy are observed in structural MRIs that correlate with neuron loss and cognitive symptoms (left side). In addition, on the molecular level, the following neuropathological hallmarks are present (right side): (A) Extracellular accumulation of Aβ in insoluble plaques and intracellular accumulation of TAU in neurofibrillary tangles, (B) massive synapse loss and neurodegeneration resulting in brain atrophy, (C) significant neuroinflammation (driven by microglia and astrocytes) and blood-brain barrier (BBB) dysfunction [7,8,16,17]. Figure created with BioRender.com.

1.2. Familial and sporadic Alzheimer's Disease

The majority of AD cases occur sporadically and at an age above 65 years (late-onset (LOAD)/ sporadic AD (sAD)). However, a small subset of cases (<5%) is autosomal dominantly inherited and causes an early disease onset (between the age of 30-65 years). Pathogenic variants causing familial AD (fAD) can be found in the genes encoding for the amyloid precursor protein (*APP*) or proteases responsible for the processing of APP (*PSEN1 or PSEN2*) and cause increased production of aggregation-prone Aβ peptides or directly affect their aggregation propensity (see 1.4) [7,19,20].

1.3. Risk factors for sporadic Alzheimer's Disease

Besides early-onset fAD, caused by inherited variants in the *APP*, *PSEN1*, or *PSEN2* genes, more than 40 alleles have been implied as susceptibility factors for late-onset sAD[7]. The *APOE* ε4 allele, the most prominent risk allele, increases the AD risk up to 15 times when present on both alleles[21]. Other risk genes, such as *TREM2*, *SORL1*, *ABCA7*, *BIN1*, *CD33*, and *CLU*, are primarily associated with APP (APP) processing, immune response, microglial function, cholesterol lipid dysfunction, endocytosis, and vascular factors, implying central roles of these pathways for disease pathology [22–25]. Interestingly, variants in the TAU protein-encoding gene *MAPT* are not associated with AD, although several of TAU's interaction partners, such as *BIN1*, *FERMT2*, and *PTK2B,* have been identified as risk factors [7,24].

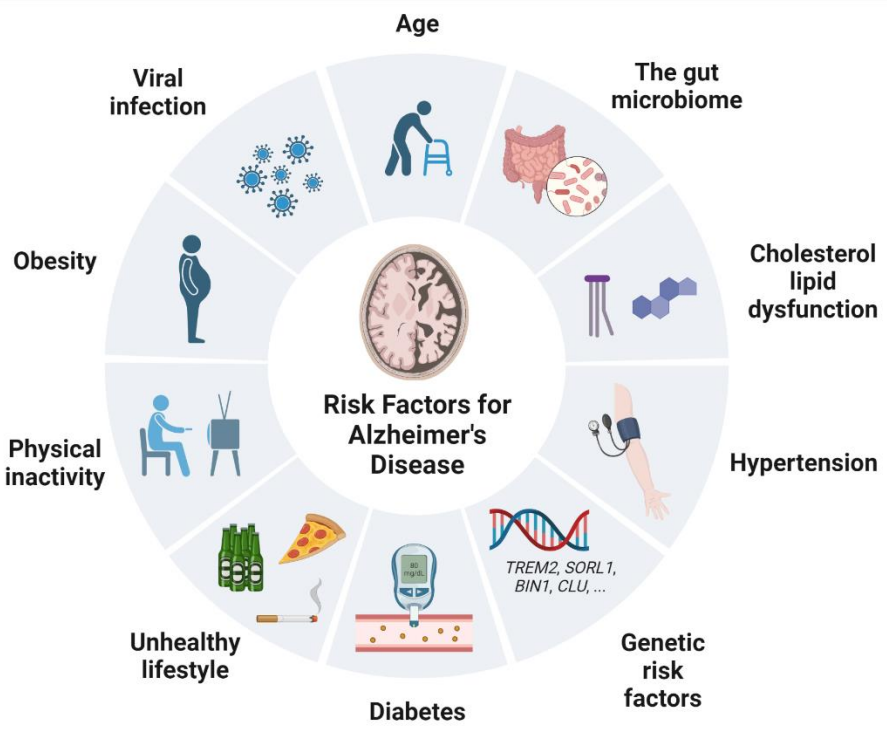

Figure 2 Risk factors for sporadic Alzheimer's disease. The majority of AD cases (>95%) are sporadic and various risk factors increase the risk of developing sAD. Besides advanced age, several genetic risk factors have been associated with AD, such as *TREM2*, *SORL1*, *ABCA7*, *BIN1*, *CD33*, and *CLU*. Further, medical conditions such as diabetes, hypertension, viral infections and head injury, and environmental and lifestyle factors (e.g., air

pollution, diet, physical activity, smoking, etc.) mainly contribute to the disease risk [4,7,8,17,26]. Figure created with BioRender.com.

The most significant risk factor for developing AD, however, is advanced age, indicated by the fact that the majority of people having AD are older than 65 years and that the incidence of overall dementia increases from less than 1% in people aged 65-69 years to about 27% in individuals >90 years [3,27]. In addition, other comorbidities, such as diabetes mellitus, hypertension, obesity or hearing loss, traumatic brain injury (TBI), or depression, can significantly increase the risk of developing AD and other forms of dementia. Further factors influencing AD risk include an unhealthy lifestyle (e.g., smoking, alcohol abuse, sleep deprivation), low physical activity, environmental factors (e.g., air pollution), and changes in the gut microbiome (see Figure 2) [7,8,28–30]. Recently, infections with herpes simplex virus (HSV) or influenza have been implied to significantly increase the risk of developing neurodegenerative diseases, including AD [26].

On the other hand, several factors that reduce the risk of developing AD are known. Besides maintaining a healthy lifestyle, several protective alleles have been identified, including, for example, the *APOE* ε2 allele leading to two times reduced risk of AD [31,32]. In addition, the rare Icelandic APP mutation (A647T) has been shown to protect against cognitive decline by reducing the production of $A\beta_{42}$ by up to 40% [33]. Remarkably, a homozygous mutation in APOE ε3 (R136S, “Christchurch”), identified in one individual with an inherited *PSEN1* variant, protected against neurodegeneration and cognitive decline for decades after the expected onset of disease symptoms [34].

1.4. Amyloid pathology in Alzheimer’s disease

The aggregation of misfolded Aβ peptides in insoluble extracellular plaques is a key hallmark of AD pathology (see Figure 1). The $A\beta_{40}$ and $A\beta_{42}$ peptides derive from the sequential cleavage of APP by the β-secretase and the γ-secretase complex (see Figure 3) [35,36]. Under physiological conditions and at low concentrations, these Aβ peptides promote synapse formation and synaptic signaling, and the processing and release of Aβ peptides are tightly coupled to synaptic activity [37–42]. At higher concentrations, however, Aβ peptides, especially $A\beta_{42}$, are prone to aggregate into β-sheet confirmations like oligomers and (proto-) fibrils, causing a variety of downstream processes, including neuronal dysfunction (mainly by interacting with NMDA and insulin receptors), TAU hyperphosphorylation, and synapse loss, leading to neurodegeneration [40,43–45]. Furthermore, several APP mutations are associated with fAD, e.g., the Swedish (K670_M671delinsNL), the London (V717I), or the Indiana (V717F) mutation, causing increased production of amyloidogenic Aβ peptides or structural changes enhancing the aggregation propensity of the peptides (see Figure 3)[35]. In addition, variants of *PSEN1* or *PSEN2* encoding for presenilin 1 (PS1) and 2 (PS2) that are part of the

γ-secretase complex lead to a dominant-negative effect on γ-secretase activity and cause changes in the $A\beta_{42}$ to $A\beta_{40}$ ratio [46,47].

Extracellular deposition of insoluble Aβ is a successive progress that can be first observed in the neocortex. From there, it spreads in an anterograde manner to the regions in which neocortical neurons project, starting in allocortical regions and progressing to diencephalic and basal ganglia structures, and in late stages to the brain stem and eventually the cerebellum[48,49]. For the spreading of Aβ pathology, a prion-like seeding model is suggested, in which small Aβ assemblies ("seeds") function as templates for the formation of larger neurotoxic aggregates [50,51].

Of note, the non-amyloidogenic processing of APP by α- and γ-secretases produces several peptides, such as sAPPα, αCTF, AICD, and p3, that are implicated in neuroprotective functions (see Figure 3) [52,53].

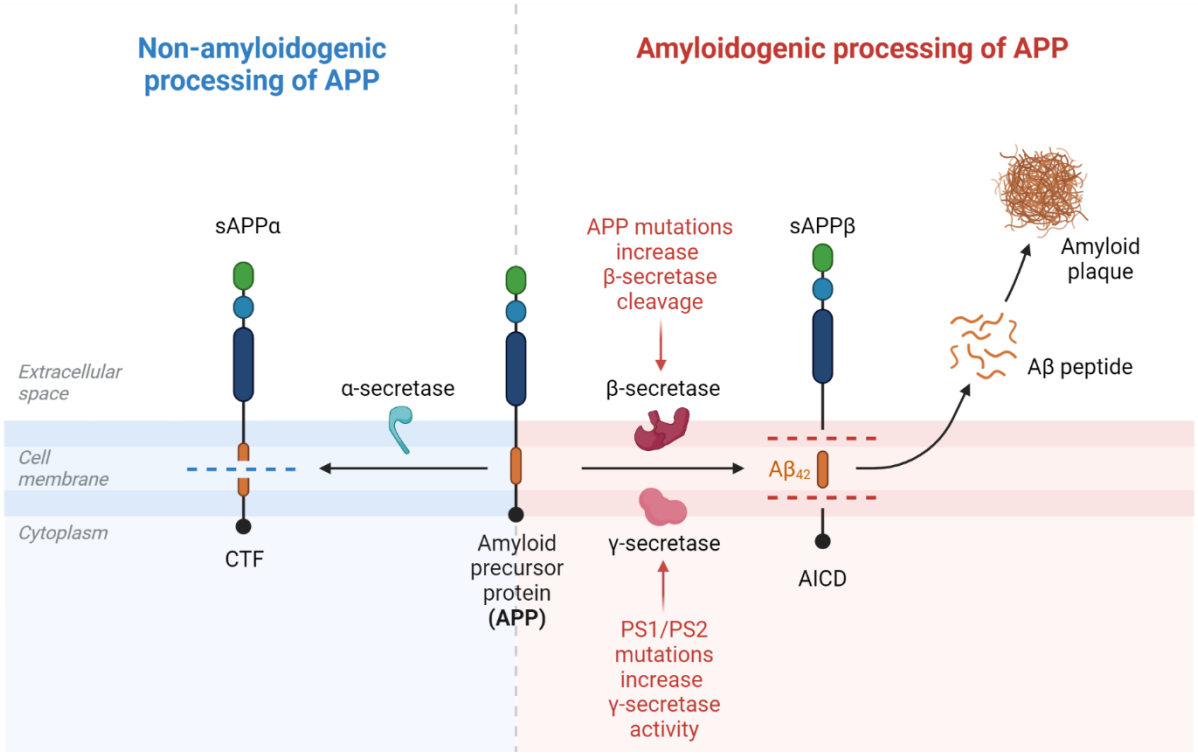

Figure 3 Proteolytic processing of the amyloid precursor protein (APP). Under physiological conditions, APP is proteolytically processed at several sites by the α-secretase (non-amyloidogenic pathway, left), or β-secretase (amyloidogenic pathway, right), followed by further cleavage by the γ-secretase complex. Under pathological conditions, amyloidogenic processing by the β- and γ-secretases is favored, leading to increased secretion of aggregation-prone $A\beta_{42}$ peptides and the formation of insoluble amyloid plaques. In addition, pathogenic variants in *APP*, *PSEN1*, or *PSEN2*, associated with familial AD, increase β-secretase processing or γ-secretase activity, resulting in higher levels of pathogenic Aβ peptides. Figure adapted from ref. [35]. Figure created with BioRender.com.

1.5. TAU pathology in Alzheimer's disease

Unlike Aβ, which aggregates in insoluble plaques in the extracellular space, TAU pathology presents as intracellular NFTs, neuropil threads, and dystrophic neurites. In the healthy brain, TAU is a natively unfolded protein that is efficiently sorted into the axon, where it binds

and stabilizes axonal microtubules (MTs). However, under pathological conditions, e.g., in the presence of pathological Aβ species in AD, TAU gets abnormally phosphorylated, dissociates from axonal MTs due to decreased MT-binding affinity, and translocates from the axon into the soma and dendrites [44,54–59]. As a MT stabilizing protein, TAU is highly involved in the regulation of axonal transport of proteins and organelles by influencing cytoskeletal motor proteins such as dynein and kinesin [60]. Posttranslational modification (PTM) of TAU leads to decreased MT-binding affinity, dissociation from axonal MTs, and MT destabilization (see Figure 4). Furthermore, pathological TAU directly inhibits the binding of motor proteins to axonal MTs, causing axonal transport deficits, and accumulation of organelles and other proteins in the soma, resulting in synaptic dysfunction [61]. In addition, pathological missorting of TAU into the somatodendritic compartments and especially into dendritic spines causes local destabilization of microtubules and synapse loss driving cognitive dysfunction. MT destabilization is caused by TAU-mediated missorting of the Tubulin-Tyrosin-Ligase-Like-6 (TTLL6) into the dendrites where TTLL6 polyglutamylates MTs resulting in spastin-mediated MT severing [62–65]. Of note, dendritic TAU further recruits the tyrosine kinase Fyn to the postsynaptic NMDA receptor complex, which leads to the phosphorylation of NMDA receptor subunits and subsequently to excitotoxicity [66,67]. In addition, post-translation modifications of TAU, such as phosphorylation and acetylation increase the dendritic localization of TAU and its aggregation propensity, resulting in synaptic impairments [67–69]. The therapeutic potential of MT-stabilizing drugs demonstrated in AD models further supports the importance of TAU-mediated MT dynamics in AD [70–73]. Besides the detrimental impact of the loss of TAU function on MT stability and synaptic integrity, TAU hyperphosphorylation leads to an increased aggregation propensity, resulting in the accumulation of TAU assemblies in NFTs (see Figure 4)[59,61,74]. Two hexapeptide motifs, PHF6 ($_{306}$VQIVYK$_{311}$) and PHF6*($_{275}$VQIINK$_{280}$), within the MT-binding domain of TAU, have been identified that significantly increase the self-assembly of TAU into larger oligomers and filaments. The PHF6 sequence, for example, can form β-sheet conformations similar to that of TAU fibrils *in vitro*, and its deletion prevents TAU assembly [75]. TAU aggregation indirectly causes further MT destabilization, impaired cytoskeleton dynamics, and axonal transport deficits, causing synaptic dysfunction and neuronal cell death by the loss of physiological TAU function [16,59,61].

TAU deposition in the brain can be observed first in the Locus coeruleus (LC), from where it spreads to the entorhinal cortex, hippocampus, and in late stages throughout the whole cortex [49,76–78]. The specific and time-dependent progression of TAU pathology was first described by Braak and Braak (1991) and is commonly used to classify the disease stage [76,79,80]. As described for Aβ, TAU pathology also spreads to anatomically connected brain regions in a prion-like manner: TAU aggregates either synthetically prepared or derived from AD brains induce pathological TAU aggregation in mouse models of AD[81–87].

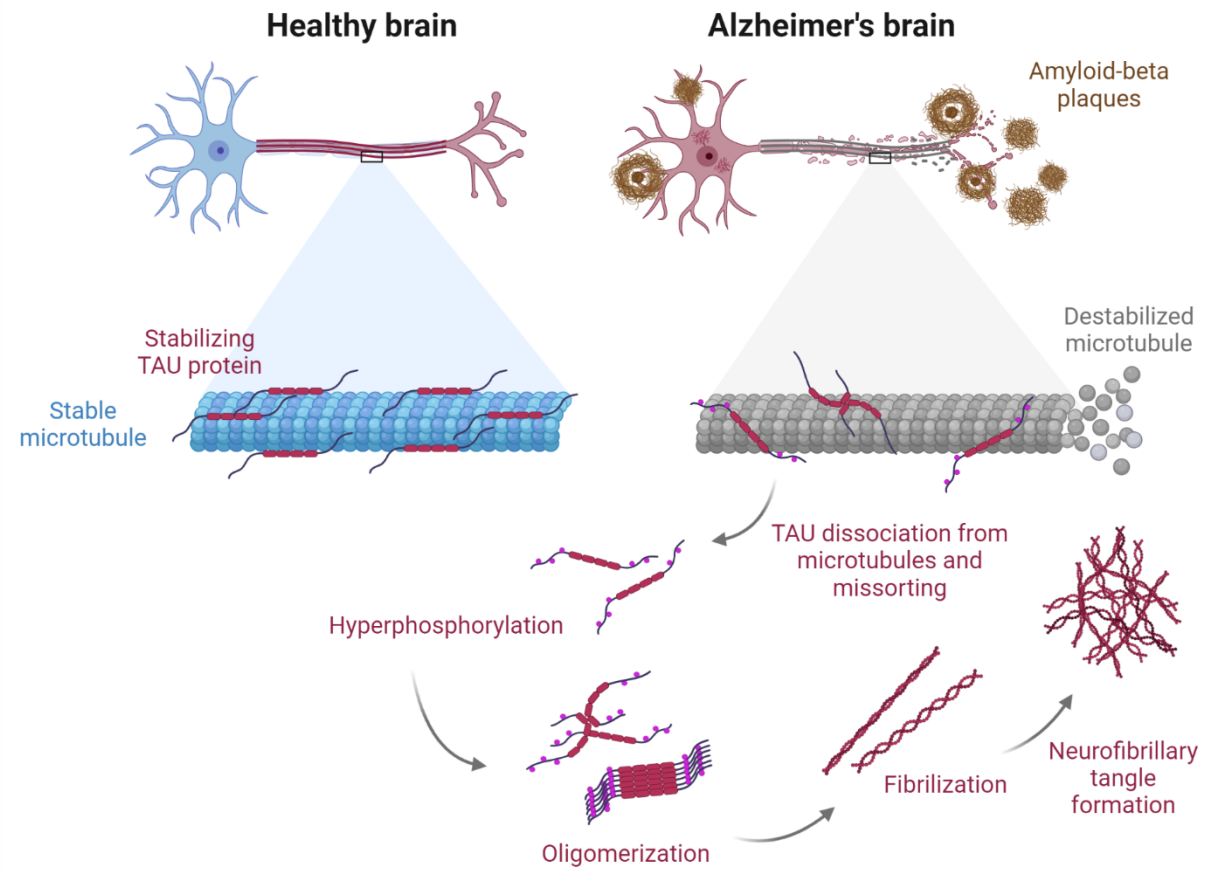

Figure 4 TAU pathology in Alzheimer's disease. In healthy neurons, TAU is enriched in the axon, where it binds and stabilizes microtubules (MT). However, under pathological conditions, such as upon accumulation of amyloid-beta plaques, TAU dissociates from axonal microtubules, translocates to the somatodendritic compartment and gets hyperphosphorylated. Due to the phosphorylation, TAU changes its conformation, which increases its aggregation propensity and results in the accumulation of TAU assemblies in NFTs. Loss of the stabilizing function of TAU causes MT disintegration and impairments of downstream functions, such as axonal transport, which ultimately drives synaptic dysfunction and neuronal cell death. Furthermore, TAU missorting into the dendrites causes postsynaptic spine loss and NMDA receptor mediated excitotoxicity driving cognitive decline [65,88,89]. Figure created with BioRender.com.

Over 20 neurodegenerative diseases are characterized by the pathological accumulation of TAU (see 1.7). While some tauopathies are directly associated with pathogenic variants in the TAU-encoding gene *MAPT*, no variants have been reported in AD cases. In contrast to Aβ, TAU accumulation correlates well with cognitive impairment, however, co-occurring Aβ pathology is necessary to drive TAU accumulation and disease progression [90–94]. In addition, the role of TAU in disease progression is further highlighted by the absence of cognitive symptoms in individuals with Aβ pathology and the protection of TAU-depleted animals from Aβ-induced memory deficits [95–101]. Besides the loss of MT stability, dendritic TAU, PTMs, and TAU aggregation strongly contribute to axonal transport defects and synaptic dysfunction, thereby mediating Aβ-induced neurotoxicity and driving the cognitive decline.

1.6. The microtubule-associated protein TAU

TAU is a microtubule-associated protein and a key driver of pathology in various neurodegenerative diseases. These so-called tauopathies are usually characterized by extensive phosphorylation of TAU (hyperphosphorylation), translocation (or missorting) of TAU from the axon into the somatodendritic compartment, and accumulation of TAU in insoluble NFTs (see Figure 4) [59,102]. Under basal conditions, the primary function of TAU is stabilizing axonal microtubules, thereby contributing to a plethora of MT-associated functions, including axonal outgrowth, cargo transport, neuronal polarization, synaptic plasticity, and other essential cellular functions [59,65,103,104]. In AD, TAU is an essential mediator of Aβ-induced neurotoxicity, and reduction or depletion of TAU is neuroprotective [64,66,95,96,105–107].

1.6.1. *MAPT* alternative splicing and TAU isoforms

The *MAPT* gene on chromosome 17q21.31 encodes for the human TAU. Alternative splicing results in a variety of isoforms in the human central and peripheral nervous system[108–111]. Out of 16 exons, mainly exons 2, 3, 4a, and 10 are alternatively spliced, however, over 30 different isoforms could be produced in total. Of note, from these predicted isoforms, only exon 6+ and intron 12+ splice-isoforms have been detected in human tissues [112–118].

In the adult human brain, six TAU isoforms are present, which differ in the number of N-terminal inserts (0, 1, or 2 N) and C-terminal repeat domains (3R or 4R) based on the differential splicing of exons 2, 3, and 10 of the *MAPT* gene (see Figure 5) [108]. The abundances of the isoforms change during brain development and vary in neuronal subtypes[119]: During neurogenesis only the shortest isoform, 0N3R, is present. The isoform composition shifts during neuronal maturation to bigger isoforms, especially 2N (which includes all isoforms having two N-terminal inserts, namely 2N3R and 2N4R) and 4R (which includes all isoforms having four repeats, namely 0N4R, 1N4R, and 2N4R), leading to a one-to-one ratio of 3R and 4R TAU isoforms in adult brains [119,120]. However, TAU protein abundance and transcript splicing differ in brain regions and are cell-specific [121–124].

A variety of factors have been described to influence *MAPT* splicing. Regulation of splicing has been focused on exon 10 due to its significant involvement in familial tauopathies, such as progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)[125–127]. Specifically, the boundary between exon 10 and intron 10 is a hotspot for pathogenic variants that result in the missplicing of exon 10 and a shift in the isoform ratio of 3R and 4R TAU [125,126,128–134]. More than ten different serine/arginine-rich splicing factors (SRs) have been associated with the splicing of exon 10 and exons 2/3 [117,125,126]. In addition, exon 10 splicing is regulated indirectly by miRNAs (e.g., miRNA-132), phosphorylation of

kinases that regulate splicing factor activity, e.g., PKA, GSK-3β, or by DNA methylation (e.g., a CpG island is located in exon 9) [117,126,135–140].

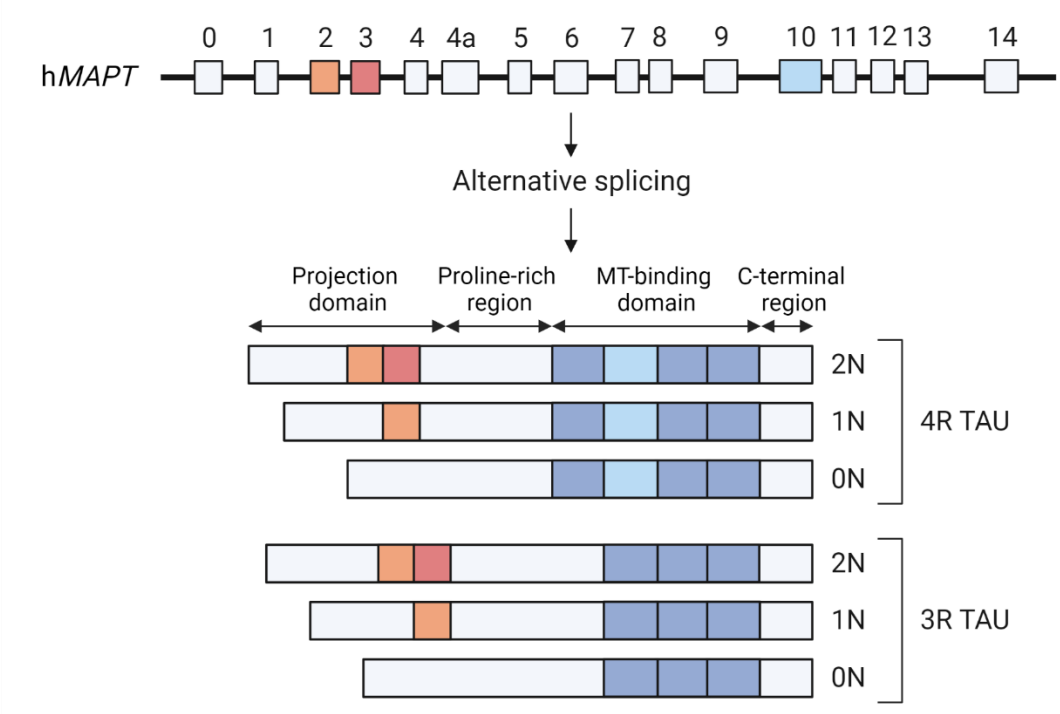

Figure 5 Alternative splicing of the human *MAPT* gene. The human TAU-expressing *MAPT* gene encodes for six brain-specific TAU isoforms based on alternative splicing of exons 2, 3 and 10. The TAU protein can be structured into four different domains. The N-terminal projection domain, which projects away from the microtubules (MT) acts as a spacer and interacts with components of the plasma membrane. The proline-rich region is involved in cellular signaling by interacting with Src-familiy kinases such as Fyn. The MT-binding domain mediates MT polymerization and stability. The C-terminal region additionally contributes to MT polymerization and TAU's interaction with the plasma membrane [59]. Figure adapted from ref.[141]. Figure created with BioRender.com.

Besides the mentioned splicing factors, two major haplotypes influence the expression and alternative splicing of the *MAPT* gene [142]: The H1 haplotype, observed in approx. 75% of the European population leads to increased inclusion of exon 10 and an increased risk for tauopathies, including Parkinson's disease (PD), PSP, and CBD [142–147]. In contrast, the H2 haplotype, characterized by a ~970 kb inversion spanning the whole *MAPT* locus, has a neuroprotective effect an leads to increased inclusion of exon 3 [148,149]. This haplotype is nearly exclusively present in the European population and has a frequency of about 25% [150].

The coding sequence of TAU is relatively conserved in mammals, however, its splicing pattern changed significantly between phylogenetic groups [59,109,135,151]. While in adult human and primate brains six TAU isoforms are present (0-2N and 3/4R), only up to four TAU isoforms are observed in mice: Consistent with humans, 0N3R is produced during murine neuronal development. However, the isoform composition changes to exclusively 4R TAU isoforms (0N4R, 1N4R, and 2N4R) in adult mouse brains [122,151–154]. These differences may also explain the vulnerability of humans to splice deficits in *MAPT* and the absence of TAU pathology in transgenic mouse models of AD (e.g., overexpressing mutant APP; see also 1.8) [59,155].

1.6.2. The TAU protein: structure, functions, and interactions

The TAU protein is a natively unfolded, soluble protein, which is structured into four different domains, depending on their interactions[156,157]: i) the N-terminal projection domain, ii) a proline-rich region, iii) the MT-binding domain, and iv) the C-terminal region (see Figure 5). During brain development, especially during neuronal polarization, TAU is efficiently sorted into the axon. However, in the adult brain, a small fraction is also observed in the somatodendritic compartment and the nucleus [158–163]. Axonal targeting of TAU is mediated by various processes, such as the presence of a TAU diffusion barrier (TDB) at the axon initial segment (AIS), which prevents retrograde diffusion of TAU[164,165]. Furthermore, microtubule-binding affinity might be higher in the axon, likely accomplished by the presence or absence of PTMs, such as phosphorylation and acetylation [69,166,167]. The microtubule-binding affinity further depends on the number of C-terminal repeats encoded by exon 10, as shown *in vitro* [168–171]. By binding to tubulin, TAU promotes the assembly and stability of MTs, acts as a spacer, and links them to the neuronal plasma membrane [172–174]. Through its interaction with axonal MTs, TAU supports axonal differentiation, morphogenesis, outgrowth, branching, cargo transport, and neuronal plasticity [62,161,175–178]. Of note, knockout (KO) of TAU does not lead to severe phenotypes in mice due to the compensatory upregulation of other microtubule-associated proteins, such as MAP1A[179,180]. However, some mild (age-related) phenotypic changes occur, e.g., changes in sleep-wake behavior, motor deficits (parkinsonism), and mild memory impairments, pointing towards novel roles of TAU within these pathways [97,181–188].

Besides its traditional role in stabilizing MTs, TAU has been shown to interact with various other proteins and mediate diverse cellular processes [59,189,190]. For example, TAU regulates synaptic plasticity at the postsynapse via its interactions with NMDA and AMPA receptors and other postsynaptic density components, such as PSD95), Fyn kinase, and GSK-3β [66,67,191–193]. A recent study in human induced pluripotent stem cell (hiPSC) derived neurons found that TAU interactions are enriched for cytoskeletal, pre-synaptic vesicle, proteasomal, RNA-binding, and mitochondrial proteins, implying diverse functions of TAU [194]. Interestingly, the interactome differed between N- and C-terminally tagged TAU, indicating subdomain-specific interactions that could be altered upon alternative splicing of TAU.

The interactome and functions of TAU have been studied primarily in pathological conditions or using mutant TAU (TAU variants from frontotemporal dementia (FTD), see also 1.8), and have been associated with several detrimental functions, such as impairment of the proteasomal system, reducing protein synthesis by interacting with ribosomal components, mediating excitotoxicity by interaction with components of the NMDA receptor complex and spine loss by destabilizing local MTs [64,66,67,190,193,195–201]. In addition, nuclear localization of TAU has been observed, and while under physiological conditions, TAU protects RNA and

DNA, the interaction of TAU with RNA-binding proteins and RNA can also enhance its aggregation propensity [154,202–207]. In human neurons derived from an immortalized neural progenitor cell line (ReN VM), specific TAU interactions with non-muscle myosins that are important regulators of dendritic spine morphology were ablated upon the presence of mutant TAU^{P301L}, which highlights the importance of TAU in maintaining dendritic spines [208–211]. Furthermore, in human iPSC-derived neurons, presence of TAU^{P301L} or TAU^{V337M} decreases TAU interactions with ribosomal and mitochondrial proteins, leading to impairments in mitochondrial biogenesis. In addition, decreased abundance of the abolished TAU interacting proteins found *in vitro* was observed in AD brains and correlated with AD progression [194].

Of note, data on isoform-specific interactions and functions of TAU is scarce. Recently, differential intracellular localizations of TAU isoforms have been described in primary neuronal cultures and mouse brains, suggesting a differential contribution to TAU-associated function [165,212]. While smaller 0N isoforms are efficiently sorted into the axon, 1N and 2N isoforms show enrichment in dendrites and cell bodies. In line with the differential localization of the isoforms, co-immunoprecipitations of endogenous murine 0N, 1N, and 2N4R TAU revealed distinct isoform-specific interactions and found that proteins preferentially binding to 2N TAU are associated with neurological disease, suggesting a critical role of 2N TAU in disease pathology [213]. In a *Drosophila melanogaster* model, overexpression of 0N3R led to a reduced life span and axonal transport defects, while the 0N4R isoform caused neurodegeneration and impairments in learning and memory [214]. Further, differences in the phosphorylation pattern and aggregation propensity of the TAU isoforms have been demonstrated: While 3R TAU seeds can recruit both 3R and 4R monomers, 4R isoform seeds contain only 4R monomers [215–217]. These results further imply isoform-specific functions of TAU under basal and pathological conditions, suggesting that depending on the disease studied, different isoforms could mediate the observed neuronal toxicity of TAU.

In sum, besides its traditional role of stabilizing axonal MTs, TAU is involved in various cellular functions and processes. Under basal conditions, loss of TAU can be compensated by other microtubule-associated proteins. However, under pathological conditions, changes in the intracellular localization of TAU due to PTMs, its abundance and/or splicing, and protein misfolding cause a shift in interaction partners, an increase in detrimental functions, and aggregation of TAU, leading to neuronal dysfunction and cell death. The importance of TAU in the deleterious cascade of AD is further supported by data from TAU-depleted neurons and animals that are protected from Aβ-induced neurotoxicity. However, most studies used animal models and/or relied on the presence of mutant TAU to study TAU-associated functions. Further research is necessary to validate that the proposed functions of TAU are not limited to the studied model system.

1.7. Tauopathies

Neurodegenerative diseases accompanied by TAU pathology are characterized by the mislocalization and accumulation of hyperphosphorylated TAU in the somatodendritic compartment [135]. These so-called tauopathies, a heterogeneous group of over 20 neurodegenerative diseases, can be classified according to the presence of co-pathologies, if they are inherited, or based on which TAU isoform is present in aggregates and the confirmation of TAU filaments [59,102,218,219]. Classical primary tauopathies are diseases in which TAU pathology is the major contributing factor, such as PSP, CBD, PiD, and FTDP-17. In contrast, AD, PD, and Huntington's disease (HD) are secondary tauopathies characterized by the aggregation of an additional amyloidogenic protein (Aβ, α-synuclein, and huntingtin, respectively). The underlying causes for the sporadic development of tauopathies are not fully understood, but changes in splice factors, endo-lysosomal and mitochondrial dysfunction, aberrant ROS production, and epigenetic dysregulations together with certain risk factors, such as TAU haplotype (see 1.6.1), an unhealthy lifestyle (e.g., alcohol abuse, smoking, overweight), cardiovascular factors (such as hypertension or diabetes) and head injuries have been implicated in the disease pathogenesis [61,88,102,220].

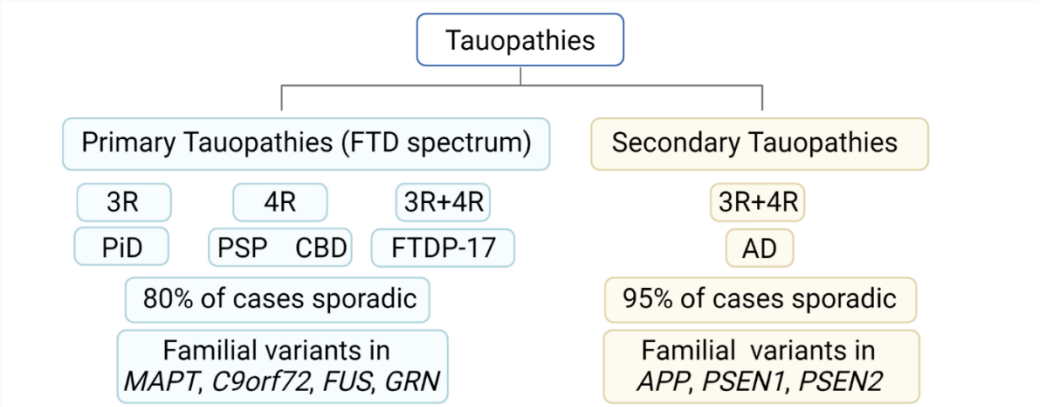

Figure 6 Classification of Tauopathies. Tauopathies can be classified into primary and secondary tauopathies depending on the major contributing factor to disease pathology. While TAU pathology is present in both cases, aggregates of other proteins can be observed in secondary tauopathies. Further, depending on the TAU isoform present in the neuronal pathology, tauopathies can be classified into three-repeat (3R) tauopathies, (e.g., Pick's Disease (PiD)), four repeat (4R) tauopathies (e.g., progressive supranuclear palsy (PSP), corticobasal degeneration (CBD)), or mixed 3R+4R tauopathies (e.g., AD, frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)). Most tauopathies are idiopathic, however a small percentage (5-20%) is genetically inherited and caused by pathogenic variants, e.g., in *MAPT*, *C9orf72*, *FUS*, *GRN*, *APP*, *PSEN1*, *PSEN2*. Figure adapted from ref. [221]. Figure created with BioRender.com.

While most tauopathies occur sporadically, some are caused by inherited pathogenic variants, e.g., in *MAPT*, *C9orf72*, *FUS*, or *GRN* [220]. More than 50 variants in *MAPT* are associated with FTD spectrum disorders and account for up to 20% of familial cases [59,222]. The mutations are centered around exons 9-12 or are intronic and often result in impaired splicing of exon 10, which leads to an imbalance in the isoform ratio of 3R to 4R TAU (e.g., ΔK280, ΔN296, P301L, P301S)[132,223]. Other missense mutations or deletions cause a

decrease in MT binding affinity and reduce the ability of TAU to promote MT assembly (e.g., P301L, P301S, V337M), increase the aggregation propensity of the protein (e.g., ΔK280 and P301L), disturb its interaction with other proteins (e.g., R406W), or change its phosphorylation pattern (e.g., P301L, V337M, G272V) [59,223–226]. Furthermore, in sporadic tauopathies, dysfunction of other proteins, has been associated with missplicing of TAU, leading to an increased abundance of 4R isoforms [227,228]. Depending on the altered splicing, tauopathies can also be classified as 3R or 4R tauopathies: While PiD is characterized by tangles containing 3R isoforms (0N3R, 1N3R, and 2N3R), 4R-TAU (0N4R, 1N4R, and 2N4R) accumulates, e.g., in PSP and CBD, and all isoforms are present in the aggregates of AD patients [59,229]. Of note, the aggregation propensity of TAU relies highly on its PTMs and the underlying isoform, and recently it has been shown that tauopathies are characterized by distinct TAU filament structures[219,230–233]. These results suggest that disease pathogenesis differs remarkably between tauopathies and that disease-modifying therapies need to account for these differences.

1.8. Experimental models of Alzheimer's disease

Experimental models of tauopathies are required to gain insights into the underlying disease mechanisms and to identify and test novel therapeutic strategies. Most of these models are based on transgenic (tg) mice expressing pathological variants of *APP*, *PSEN1*, or *MAPT*. Further animal models include aged non-human primates and other model organisms such as *Drosophila melanogaster*, *Danio rerio*, or *Caenorhabditis elegans*. In addition, recent advantages have led to the development of novel human-based neuronal model systems, such as the generation of iPSCs and neurons from AD patient fibroblasts and the generation of 2D and 3D culture models and human brain organoids [234–239]. The following paragraphs will shortly describe commonly used models and their advantages and limitations.

1.8.1. Animal models

Although less than 5% of AD cases are familial (see 1.2), most animal models rely on the overexpression of human APP, PSEN1, or TAU variants associated with fAD or FTD. Currently, the Alzforum database lists 214 rodent models of AD (02/2023), with transgenic (tg) mice representing the majority of models (197/214) [240]. In *APP* tg mice, Aβ pathology and plaque formation are age-dependent and recapitulate Aβ pathology as observed in human brains. However, depending on the model used, pathological onset can vary from a very young age (3 months) to aged animals (>12 months), and more importantly, unlike in humans, cognitive decline correlates well with the Aβ plaque load [241–243]. Another major drawback of these models is the lack of TAU pathology unless human mutant TAU is overexpressed [244]. Therefore, one of the most commonly used mouse model for AD is the triple tg mouse (3xTg), expressing $APP^{K670\_m671delinsNL}$ (Swedish mutation), $PS1^{M146V}$, and TAU^{P301L}, which develops an age-dependent, progressive pathology including Aβ plaques

and NFTs[245]. Although this model recapitulates the phenotypic and pathological changes observed in humans, it relies on heavy overexpression of human mutant proteins, which is not associated with fAD. Of note, tangle pathology has been observed in a transgenic rat model of AD (TgF344-AD) lacking the overexpression of mutant TAU [246], which might be explained by a more human-like TAU isoform composition compared to mice [247]. To overcome the differences in the isoforms present in mice and humans, humanized TAU knockin (KI) mouse models have been established expressing the human *MAPT* gene in murine *Mapt* KO background [248,249]. Interestingly, the hTAU mouse generated by Andorfer and colleagues develops age-associated TAU pathology, likely due to the imbalance of 3R and 4R TAU isoform abundance observed in this model, which is associated with several tauopathies (see 1.7) [249,250]. In contrast, humanization of the entire murine *Mapt* locus did not result in any phenotypic changes in the absence of mutant APP [248].

Besides using transgenic animals, induction or seedings models have been established: For example, intracerebral injections of Aβ or TAU aggregates generated *in vitro* or isolated from mouse brain or postmortem tissue induce pathological deposition of endogenous or transgenic proteins that closely mimic AD inclusion pathology [83,251–255]. Besides rodent models, non-human primates are attractive models since Aβ accumulations occur naturally in an age-dependent manner similar to humans. In addition, there is up to 100% sequence homology of *APP* and *MAPT* between humans and non-human primates [256]. However, although robust Aβ pathology has been observed in rhesus monkeys, great apes, and other species, TAU pathology is rare or limited to hyperphosphorylation-positive neurites [256,257]. Other popular model organisms, such as *Drosophila melanogaster*, *Danio rerio, Caenorhabditis elegans*, or *Saccharomyces cerevisiae*, have been used additionally to study aspects of AD-associated neurodegeneration, however the transfer of those studies is limited due to the lack of genetic homology, absence of Aβ generation (e.g., in flies and worms), and lack of behavioral complexity. Nevertheless, easy genetic manipulation and short lifespan are beneficial for high throughput genetic or drug screenings [257,258].

In sum, animal models can be used to study aspects of AD and neurodegeneration, but several limitations must be considered, especially when working with murine models: i) Mice do not develop age-dependent amyloid pathology, ii) the isoform composition is different compared to human brains, iii) development of full AD pathology depends on overexpression of mutant proteins and iv) there is a lack of translatability of preclinical studies and human clinical trials, often leading to failures of potential drug candidates[155,242,258]. These factors suggest that additional models or a combination of several models are necessary to further understand disease pathology and develop therapeutic strategies.

1.8.2. Cell-based models

Besides rodent-derived primary cells, immortalized cell lines have been widely used to study TAU and AD pathogenesis. For example, the human neuroblastoma-derived SH-SY5Y cell line can be easily differentiated into several neuronal subtypes, expressing immature markers of cholinergic neurons in the undifferentiated state and neuronal markers as well as neuronal polarity [259,260]. Another advantage of this cell line is that it expresses all six human-specific TAU isoforms and shows efficient TAU axonal sorting [261]. However, these cells lack the electrophysiological characteristics of mature neurons [262]. In contrast, the immortalized neural precursor cell line ReN VM, derived from the ventral mesencephalon region of the human fetal brain, can be easily differentiated into dopaminergic neurons by the depletion of growth factors [263]. Using this cell line, a 3D-culture model of neurons that forms Aβ plaques and accumulates insoluble hyperphosphorylated TAU upon overexpression of $APP^{K670N/M671L/V717I}$ and $PS1^{\Delta E9}$ has been established that was further developed into a tri-culture system of neurons, microglia, and astrocytes to better resemble the organization of the human brain [235,264,265]. Of note, this model was the first cell culture model showing accumulation of Aβ and TAU pathology *in vitro* [257].

Since the discovery of the Yamanaka factors in 2006 and the development of hiPSCs derived from human fibroblasts, advances have been made in generating and characterizing human iPSCs-derived neurons from fAD and sAD patients [266–270]. A major advantage of hiPSCs is that they can be differentiated into various neuronal subtypes and cell types, enabling the study of AD-associated phenotypes in neurons and astrocytes, microglia, or co-culture models. In addition, 3D models of the human brain, e.g., forebrain organoids or spheroids, can be derived from hiPSCs and neural precursors [238,271]. Of note, the limitations of these models include the lack of full maturity to express all human-specific TAU isoforms and robust spine formation as observed for primary neuronal cultures. To overcome some of these limitations, xenograft models were developed, in which iPSC-derived neurons are transplanted into rodent brains for further maturation and spine formation and to study the selective vulnerability of human neurons *in vivo*[272,273]. Furthermore, direct reprogramming of fibroblasts into functional neurons has been used to overcome limitations of stem cell generation: During the reprogramming of human fibroblasts into hiPSCs, cells undergo an epigenetic and functional reset, leading to rejuvenation of the cells (such as an extension of telomere size, reset of gene expression, and changes in DNA methylation) [274–278]. Directly converted neurons preserve age-associated signatures, such as DNA damage, methylation patterns, and mitochondrial dysfunction, and additionally have a more mature TAU isoform composition, including 4R and 2N isoforms [237,279–283].

Taken together, both animal and human cellular models exhibit advantages and limitations. These models often recapitulate the Aβ pathology observed in AD patients' brains. However,

the development of TAU pathology relies on the overexpression of mutant TAU associated with FTD and does not resemble sporadic AD (which accounts for over 95% of AD cases). In addition, these models usually do not consider changes in TAU splicing and differences between TAU isoforms. Therefore, developing suitable models recapitulating TAU pathology is necessary to study TAU (isoform)-associated functions in human cellular context and fully understand its role in AD pathogenesis.

1.9. Therapeutic strategies for Alzheimer's disease

Modifiable risk factors of AD include hypertension, obesity, diabetes, depression, smoking, and physical inactivity (see 1.3). Reducing these factors by following a particular lifestyle, such as eating healthy, being physically active, not smoking, and reducing alcohol consumption, can substantially reduce the risk of developing AD [284]. Nevertheless, lifestyle changes cannot prevent AD development entirely and are ineffective in familial AD. Therefore, seven drugs are currently approved for AD treatment by the FDA. Most (5/7) of these drugs (donepezil, rivastigmine, galantamine, memantine, and the combination of memantine with donepezil) treat symptoms of the disease but do not improve cognition or prevent disease progression [4]. Recently, two amyloid-targeting antibodies, aducanumab (Biogen) and lecanemab (Eisai and Biogen), have been approved as disease-modifying therapeutics. Of note, both drugs have been approved under the accelerated approval pathway of the FDA, which is based on a "surrogate endpoint" likely to predict a positive impact on cognition [5,6]. To receive full approval by the FDA, both drugs must provide evidence for their clinical benefit in further trials.

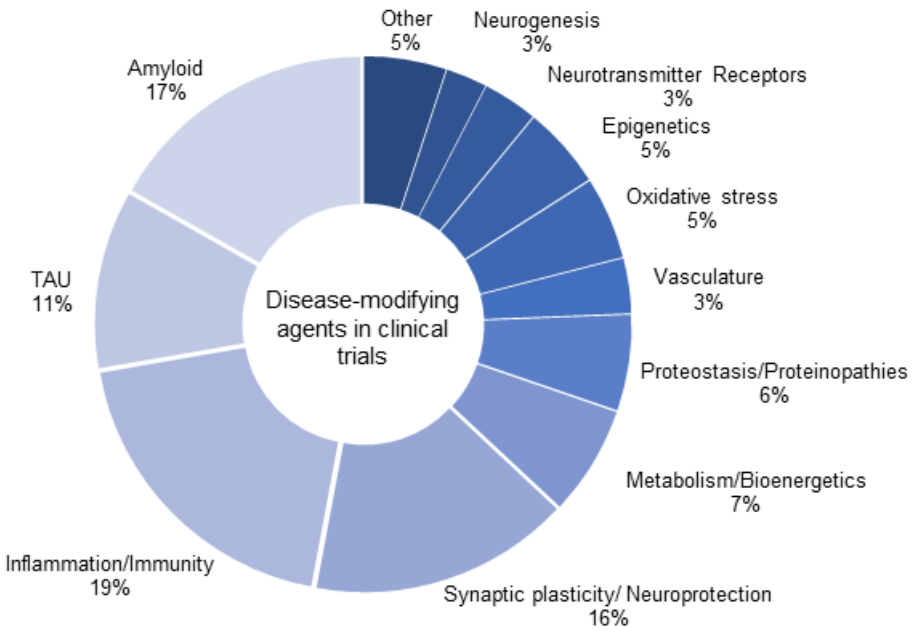

Figure 7 Targets of disease-modifying agents for AD currently in clinical trials. Currently, 119 agents are tested in clinical trials (Phase 1-3) as DMTs for treating AD. Most of the drugs target (neuro-)inflammatory or neuroprotective pathways or the key proteins of AD pathology, TAU, and Aβ. Figure adapted from ref. [285].

In the last decades of AD research, therapeutic development focused on the key proteins accumulating in the disease, namely Aβ and TAU. Aβ, as an upstream factor for TAU accumulation, was targeted by many therapeutic agents and trials. Unfortunately, most failed due to severe adverse events (e.g., microhemorrhages) and low-to-no cognitive benefit or effect on disease progression demonstrated in the clinical trials. Furthermore, due to missing diagnostic precision (e.g., biomarkers), patients with other neurodegenerative diseases or advanced symptoms have been recruited to these studies [17]. As outlined before, however, molecular changes already occur up to two decades before the onset of symptoms and might be specific to AD (see 1.1)[286]. In addition, although mouse models show many similarities with human disease progression, they do not perfectly recapitulate the characteristics observed in humans and often rely on the artificial expression of mutated proteins, which does not resemble the changes occurring in late-onset sporadic AD (see 1.8). All these factors might contribute to the failure of most clinical trials. Nevertheless, due to the tremendous socio-economic impact of AD, over 100 agents are currently being investigated for treating AD, most of them (83.2%) focusing on disease modification and mainly targeting Aβ, TAU, synaptic plasticity, and (neuro-) inflammation (see Figure 7) [285].

2. Aims of this study

The MT-associated protein TAU plays a major role in the pathogenesis of AD and other tauopathies. Considering the distinct involvement of TAU isoforms in those disorders, it is crucial to investigate the contribution of the six major human brain isoforms to the manifold functions described under healthy and pathological conditions. Most of the previous studies performed on TAU were conducted in tauopathy mouse models (which rely on overexpression of mutant recombinant human TAU) and in primary rodent neuronal cultures. However, in adult rodent brains only three brain-specific TAU isoforms are present [287], so that many described functions of TAU might be limited to the model system and do not resemble the situation in the human brain.

Therefore, in this project, we aimed to

1) Establish suitable neuronal models and methods to study (isoform-specific) functions of human TAU,

2) characterize isoform-specific properties of TAU in the established model systems, and

3) model AD-like stress in human cells, investigate TAU-isoform mediated toxicity, identify the isoform(s) mediating toxicity, and identify novel potential therapeutic targets for the treatment of AD.

Our results will help to understand the neurobiological, and molecular mechanisms of TAU physiology in disease-relevant human neuronal cultures, which is crucial for developing successful therapeutic approaches for AD, and associated tauopathies.

3. Publications and preprints

The findings of this doctoral thesis have been published as three independent studies in peer-reviewed journals. In addition, one original manuscript and two protocols are publicly available on preprint servers. Further, both protocols have also been accepted for publication in "Tau protein: Methods and Protocols" as part of the Methods in Molecular Biology book series published by Springer.

The following paragraphs will summarize the main findings of the corresponding studies, their contribution to the aims of this thesis and, if several first authors are listed, my specific contributions to the results and the writing of the article.

3.1. Publication 1: Differential Effects of the Six Human TAU Isoforms: Somatic Retention of 2N-TAU and Increased Microtubule Number Induced by 4R-TAU

3.1.1. Overview

Authors:	Sarah Bachmann, Michael Bell, Jennifer Klimek, and Hans Zempel
Journal:	*Frontiers in Neuroscience*
Impact Factor	5.152
Date of publication:	25. May 2021
DOI:	https://doi.org/10.3389/fnins.2021.643115

3.1.2. Key findings relevant to the thesis

As outlined above (see 1.6.2), the role of the six human brain-specific TAU isoforms under healthy and pathological conditions remains unclear. While differences in the intracellular sorting have been previously described for murine TAU isoforms, the first part of this study aimed to investigate the intracellular localization of human TAU in neuronal cultures. For this, primary mouse neurons (mPNs) were transfected with individual TAU isoforms and axonal enrichment was analyzed. In line with previous reports, the biggest isoforms, especially 2N, were partially retained in the soma of the neurons, while the smallest isoform, 0N3R, which is highly abundant during neuronal maturation and polarization, was most efficiently sorted into the axon[165,212]. The other 3R and 4R isoforms did not show significant differences in axonal targeting, indicating a sorting mechanism that depends on the N-terminal inserts and is independent of the repeat domains.

Based on the observed differences in the MT-binding affinity and assembly of the TAU isoforms *in vitro* [168–171], the second part of the study aimed to investigate MT dynamics in dependence of individual TAU isoforms. The experiments were performed in naïve undifferentiated SH-SY5Y cells to avoid bias due to differential axodendritic TAU distribution in polarized cells. For this, live-cell MT plus-end tracking was performed in SH-SY5Y cells transfected with individual TAU isoforms using a fluorescently labeled (by tdTomato) end-

binding protein (EB3) that binds to growing MTs [288]. MT numbers were slightly increased by 4R TAU isoforms compared to 3R TAU isoforms, confirming the enhanced assembly observed *in vitro* [168–171]. Further, cell size was changed for 4R-expressing cells, indicating that TAU isoforms might also differentially regulate neurite growth in neurons. Of note, MT dynamics such as MT stability, length, or growth were not altered upon the presence of individual TAU isoforms. However, due to the differential sorting of TAU isoforms demonstrated, a compartment-specific effect on MT dynamics could be possible in neurons.

In sum, this study suggests differential sorting and subtle differences in MT parameters for individual TAU isoforms, which is in line with previous studies. Of note, this is the first time, MT dynamics were assessed in human cells in dependence of human TAU isoforms and thereby contributing to Aims 1+2 of this thesis. Further experiments in human neurons, however, were necessary to better study sorting- and isoform-dependent functions of TAU especially in human neurons and more disease-relevant models.

3.1.3. Individual contribution

The experiments performed in this publication have been planned and performed by me. In addition, I analyzed and visualized the results and drafted the complete manuscript, which was later reviewed and edited by all co-authors.

3.2. Publication 2: DNA Methyltransferase 1 (DNMT1) Shapes Neuronal Activity of Human iPSC-Derived Glutamatergic Cortical Neurons

3.2.1. Overview

Authors:	Sarah Bachmann*, Jenice Linde*, Michael Bell, Marc Spehr, Hans Zempel[#], Geraldine Zimmer-Bensch[#] (*,[#] equal contribution)
Journal:	*International Journal of Molecular Sciences*
Impact Factor	6.208
Date of publication:	18.02.2021
DOI:	https://doi.org/10.3390/ijms22042034

3.2.2. Key findings relevant to the thesis

This study assessed the impact of DNA methylation, mediated by DNA methyltransferase 1 (DNMT1), on neuronal activity in hiPSC-derived excitatory neurons. For this, a small-interfering RNA (siRNA) to specifically knockdown (KD) *DNMT1* was designed and tested for efficacy in HEK293 cells. Afterwards, siRNA was transfected into hiPSC-derived neurons (iNs), and neuronal activity was assessed by live-cell calcium imaging. Our results indicate a positive correlation between DNA methylation and neuronal network activity and a regulatory function of DNMT1 in excitatory human neurons. The study describes for the first time our differentiation protocol to generate excitatory neuronal cultures from human iPSCs, using doxycycline-induced *Ngn2* expression and includes a basic characterization of these neurons by immunofluorescence (IF) stainings of dendritic maker MAP2, axonal marker TAU and synaptic markers vGLUT1 and synaptophysin [289,290]. This study was particularly important in establishing suitable cellular models and methods to study TAU-associated functions (Aim 1). The human iNs have been used, for live-cell calcium imaging, immunofluorescence stainings, biochemical studies and the generation of *MAPT* KO hiPSCs by CRISPR/Cas9 in follow up studies (see 3.4).

3.2.3. Individual contribution

While our collaborator designed, tested, and provided the siRNA constructs, I performed the majority of experiments described in this study. I established and performed the neuronal differentiation of the hiPSCs and conducted the stainings for their basic characterization (Fig. 1). In addition, I transfected the neurons with either a control siRNA or a siRNA targeting *DNMT1* and analyzed neuronal activity in the cultures using live-cell calcium imaging (Fig. 2b-g). Further, I contributed to the manuscript by visualizing all the results (Fig. 1 and 2), describing the methods used, and reviewing and editing the manuscript draft.

3.3. Publication 3: Endo-lysosomal Aβ concentration and pH trigger formation of Aβ oligomers that potently induce TAU missorting

3.3.1. Overview

Authors:	Marie P. Schützmann*, Filip Hasecke*, Sarah Bachmann*, Mara Zielinski, Sebastian Hänsch, Gunnar F. Schröder, Hans Zempel[#], and Wolfgang Hoyer[#] (*,[#] equal contribution)
Journal:	*Nature Communications*
Impact Factor	17.694
Date of publication:	30. July 2021
DOI:	https://doi.org/10.1038/s41467-021-24900-4

3.3.2. Key findings relevant to the thesis

In AD, toxic Aβ species and AβO formation induce endo-lysosomal dysfunction, which is recognized as a key driver of AD pathology [44,291–301]. Amyloidogenic APP processing by β- and γ-secretases into aggregation-prone Aβ peptides mainly occurs within endo- and lysosomes[35,36,53,302]. Furthermore, released Aβ, taken up via endocytosis, can exacerbate AD pathology, as they aggregate within endo-or lysosomal vesicles, resulting in further lysosomal dysfunction, leakage into the cytosol or transmission to other cells [53,298,302–304]. AβO formation *in vitro*, however, requires high unphysiological concentrations of Aβ peptides and is very slow at physiological pH (pH 7.8) [303,305]. This study demonstrates that the formation of AβOs is accelerated at endo-lysosomal pH (between pH 4.5- 5.5), while the concentration needed to form oligomers (critical oligomer concentration, COC) is reduced, which enables the formation of oligomers at physiological Aβ concentrations. In addition, oligomers formed by dimeric $Aβ_{40}$ (dimAβ) [306], bind to dendritic spines, induce pathological TAU missorting, and decrease neuronal activity without causing extensive cell death, demonstrating both the disease-relevance and suitability of these oligomers to study AD pathomechanisms. In this study, I optimized the experimental protocols for mPNs cultivation and IF stainings, which are publicly available as preprints (see 3.5 and 3.6). In addition, I developed a suitable assay for treating the neurons with AβOs, finding the optimal concentration to induce pathological missorting of TAU without causing extensive cellular toxicity. Furthermore, I implemented a readout for neuronal activity, based on live-cell calcium imaging of spontaneous calcium oscillations with a fluorescent calcium sensor. This protocol has also been optimized for use in hiPSCs and has been published in several studies [307–309] (see 3.4). In sum, this publication was highly relevant to achieve the aims of this thesis: It contributed mainly to the methodology establishment to evaluate TAU-mediated toxicity by inducing AD-like stress in our model systems and by implementing a functional readout by live-cell calcium imaging (Aims 1+3).

3.3.3. Individual contribution

Our collaborators provided us with purified dimAβ and $Aβ_{40}$ control peptides to study the effect of AβOs formed by these Aβ species on neuronal viability and activity. For this, I cultivated mPNs, and treated them with preformed AβOs for two different time points. Figures 2, 3 and 4 are derived from these experiments: To assess binding of AβOs to postsynaptic spines and investigate cellular toxicity, IF stainings were performed for spines and nuclei. In addition, neurons were stained for TAU to investigate pathological missorting of TAU (Fig. 3) and live-cell calcium imaging was used to assess neuronal activity after AβO treatment (Fig. 4). I thus contributed to this study by providing three out of nine total main figures. I designed, performed, analyzed, and visualized the described experiments. I contributed to the manuscript by describing the protocols used, writing the results part for our dataset, and including relevant literature in the introduction and discussion.

3.4. Preprint 1: The TAU isoform 1N4R restores vulnerability of *MAPT* knockout human iPSC-derived neurons to Amyloid beta-induced neuronal dysfunction

3.4.1. Overview

Authors: Sarah Buchholz, Michael Bell-Simons, Mohammed Aghyad Al Kabbani, Lena Kluge, Çağla Çakmak, Jennifer Klimek, and Hans Zempel
Preprint Server: *Research Square*
Date of upload: 21. Nov. 2022
DOI: https://doi.org/10.21203/rs.3.rs-2277268/v1

3.4.2. Key findings relevant to the thesis

To investigate TAU (isoform)-associated functions and their contribution to TAU-mediated toxicity, we generated a *MAPT* KO/ TAU depletion hiPSC cell line using CRISPR/Cas9 (Aim 1). Using doxycycline-induced expression of *Ngn2*, wildtype (WT) and *MAPT* KO hiPSC were differentiated into excitatory neurons (iNs) and characterized for morphological features, such as neurite length and AIS development, as well as neuronal function, assessed by live-cell calcium imaging. In line with observations from TAU KO mice [179,310], TAU KO iNs showed only mild phenotypic changes, like decreases in neurite and AIS length (~30% and ~20%, respectively). Of note, these changes were directly connected to TAU depletion since the reintroduction of TAU isoforms restored the AIS and neurite length to WT levels. In the second part of the study, we induced AD-like stress by treating the iNs with AβOs (as established before, see 3.3) to investigate the effect of individual TAU isoforms on AβO-mediated cellular toxicity (Aim 3). Again, in line with the data from animal models [95,96,98,105,107], TAU depletion protected human iNs from AβO-induced reduction of neuronal activity. Of note, only the reintroduction of 1N4R fully restored the vulnerability of TAU KO iNs to WT levels. These results suggest that 1N4R-TAU is a key driver of TAU-mediated toxicity. All in all, this study describes for the first time the generation of a human neuronal *MAPT* KO hiPSC line and its basal characterization. Impairments in neurite and AIS development upon TAU depletion suggest putative novel roles of TAU in neuronal development. All six isoforms restored the WT levels, indicating that mediating neurite and AIS dynamics is a universal function of all isoforms. Furthermore, our results identified 1N4R TAU as the most important isoform in the mediation of Aβ-induced neurotoxicity and highlight its potential as a target for future therapeutic strategies of AD.

3.4.3. Individual contribution

As the first author, I planned and performed the majority of experiments leading to this preprint. In addition, I analyzed and visualized the results and drafted the entire manuscript, which was later reviewed and edited by the co-authors.

3.5. Preprint 2: An update to: Tracking TAU in neurons: How to grow, fix and stain primary neurons for the investigation of TAU in all developmental stages

3.5.1. Overview

Authors: Sarah Buchholz, Michael Bell-Simons, Natja Haag, and Hans Zempel
Preprint Server: *Protocol Exchange*
Date of upload: 05. December 2022
DOI: https://doi.org/10.21203/rs.3.pex-2085/v1

3.5.2. Key findings relevant to the thesis

This protocol describes the preparation and cultivation of pre- and postnatal mPNs. For prenatal (E13.5) preparations, the whole cortex is used, while for postnatal (p0-3) preparations, either the hippocampus or the cortex is used. The protocol was initially established by Zempel and Mandelkow (2017) [311], further optimized in our laboratory as well as updated to match the legal requirements for the killing of pregnant animals and including postnatal dissections. In addition, it describes our standard IF staining protocol for fixed cells, now optimized for the characterization of TAU in primary and human iNs and used in several of our publications and preprints[308,309,312,313]. Furthermore, a troubleshooting guide is included in the preprint based on the experiences with our cultures and stainings.

3.5.3. Individual contribution

As first author, I contributed majorly to the optimization and establishment of the brain dissection and the culture conditions of the protocol. In addition, I drafted the entire manuscript, which was edited and reviewed later by all co-authors.

3.6. Preprint 3: An update to: Tracking TAU in neurons: How to transfect and track exogenous TAU in primary neurons

3.6.1. Overview

Authors: Sarah Buchholz, Michael Bell-Simons, and Hans Zempel
Preprint Server: *Research Square*
Date of upload: 27.01.2023
DOI: https://doi.org/10.21203/rs.3.pex-2145/v1

3.6.2. Key findings relevant to the thesis

Usually, non-dividing and primary cells, such as neurons, are hard to transfect with liposome-based approaches[314]. Therefore, introduction of exogenous proteins is usually achieved by viral-based delivery, which requires higher safety precautions and is often time-consuming. However, transduction efficiencies can reach >90%. However, this efficiency is not suitable for tracking proteins in individual axons, dendrites, or spines. Therefore, in this protocol we optimized a liposome-based transfection to reach up to 10% transduction efficiency in mPNs depending on the age of the cultures. The protocol was adapted from Zempel and Mandelkow [315] and was initially optimized to investigate the distribution and trafficking of exogenous TAU. However, this protocol is not limited to the transfection of TAU and can be adapted to any other protein of interest. This protocol has also been successfully used in Article 1 (see 3.1)[312].

3.6.3. Individual contribution

The protocol was adapted and optimized by myself with the help of Michael Bell-Simons, who I supervised during that time. In addition, I drafted the entire manuscript, which was reviewed later by all co-authors.

4. Discussion

Alzheimer's disease (AD) and related neurodegenerative disorders constitute a major burden for modern societies. Despite the tremendous need for therapy, no curative treatments are available to date. One key towards effective therapies is a better understanding of the underlying pathophysiology. In AD, two major pathological hallmarks are observed, i) the extracellular aggregation of Aβ peptides and ii) the intracellular missorting and aggregation of the MT-associated TAU protein. Strikingly, TAU pathology is observed in numerous neurodegenerative diseases called tauopathies. While the role of TAU as a key driver of neurodegeneration in AD and other tauopathies is well established in the field, the contribution of the six human brain specific TAU isoforms remains enigmatic. This lack of knowledge is remarkable since many tauopathies exhibit predominant aggregation of specific isoforms (e.g., 3R or 4R tauopathies, see 1.7), and numerous tauopathies are only caused by changes of the isoform expression pattern [59,125,171,218]. Both observations emphasize the striking relevance of certain TAU isoforms in both, normal function, and disease pathology. Indeed, previous studies in animal models and rodent primary neurons identified differences in intracellular localization of TAU isoforms and a varying abundance depending on the brain region and cell type in rodent brains [119–124,152]. In addition, isoform-specific intracellular distribution, and interactions (e.g., murine 2N4R primarily interacts with proteins associated with neurological disease [213]) underline the spectrum of TAU isoform-specific functions in health and disease [165,190,194,211,213]. Isoform abundance changes during brain development and switches from the predominant expression of the smallest isoform, 0N3R, to the expression of larger isoforms, especially 4R and 1N/2N [119,120]. These observations clearly implicate isoform-specific functions of TAU under normal and pathological conditions.

Considering the current knowledge of TAU and its role in AD pathogenesis, it is crucial to investigate the contribution of the different isoforms to the manifold functions of TAU under healthy and pathological conditions. While literature provides evidence for TAU isoform-specific roles in health and disease, the conducted studies are characterized by several limitations, such as: i) Rodents generally express a different set of TAU isoforms compared to humans and adult mice lack 3R TAU isoforms [152], ii) animal models often rely on overexpression of mutant TAU to model tauopathies and therefore do not resemble the majority of AD and tauopathy cases that occur sporadically and in the absence of mutated TAU (see 1.7 and 1.8), iii) TAU-associated functions are often studied exclusively in pathological contexts, iv) differences between TAU isoforms are not considered since often only the largest TAU isoform, 2N4R is studied [190].

To overcome these limitations, we aimed to establish suitable neuronal cell models to study (isoform-specific) functions of human TAU (Aim 1). Therefore, i) we optimized the expression of all six human TAU isoforms (Publication 1, Preprint 1 and 3), ii) developed an efficient and

reliable protocol for the differentiation of human iPSCs into homogenous glutamatergic neuronal cultures based on the doxycycline-induced expression of the transcription factor *Ngn2* (Publication 2), iii) and generated a *MAPT* KO hiPSC line suitable for analyzing the TAU isoforms individually and independent of endogenous TAU (Preprint 1). Furthermore, we established several protocols to assess diverse functions of TAU, such as immunofluorescence stainings to investigate axodendritic distribution of TAU, live-cell tracking of MT dynamics, and calcium imaging to investigate neuronal activity (Publication 1-3, Preprint 1 and 2).

Next, we characterized isoform-specific properties of TAU in the established model systems (Aim 2). We showed that only subtle differences between the TAU isoforms exist in regard to mediating MT stability in human SH-SY5Y neuroblastoma cells (Publication 1), suggesting that all TAU isoforms can mediate MT assembly and stability similarly. TAU localization in polarized neurons, however, depended on the isoform and specifically larger and more mature isoforms, such as 2N4R and 1N4R are less efficiently sorted into the axons of mPNs and human iNs (Publication 1 and Preprint 1). These results further promote the hypothesis that TAU isoforms have distinct and likely compartment-specific functions apart from stabilizing axonal MT in the human brain.

TAU depletion in human neurons mediated by gene editing using CRISPR/Cas9 resulted in mild phenotypic changes, such as a reduction of neurite and AIS length, which did not affect neuronal activity (Preprint 1). This is in line with data obtained from *Mapt* KO primary neurons and TAU KD in neuroblastoma cells that show delayed neuronal maturation, axonal and dendritic outgrowth deficits and changed AIS dynamics [175,179,310]. Re-introducing individual TAU isoforms restored the neurite and AIS length to WT levels, providing further evidence for a role of TAU in neuronal maturation and neurite and AIS development.

As an early hallmark of AD, upon the presence of toxic Aβ species, TAU dissociates from axonal MTs and accumulates in the soma and dendrites of neurons resulting in postsynaptic spine loss and neurodegeneration. Furthermore, Aβ accumulation causes synaptotoxicity and cognitive impairment via various pathways, including the induction of neuroinflammation, endoplasmic reticulum and oxidative stress, and mitochondrial and endo-lysosomal dysfunction [44,291–297]. APP processing to generate toxic Aβ species occurs in endo-lysosomal compartments, and endo-lysosomal dysfunction is recognized as a key hallmark of AD [16,35,301,316,317]. However, how Aβ oligomer formation happens *in vivo* is not well understood. Here, we demonstrate that toxic AβOs are formed readily at an acidic pH in endo- or lysosomes and induce early pathological hallmarks of AD, including the targeting of AβOs to postsynapses, TAU missorting into the soma and dendrites and a reduction of neuronal activity in mPNs (Publication 3), suggesting that neurotoxic AβOs could be particularly formed in the endo-lysosomal compartment, leading to lysosomal leakage and cellular

damage. Exposure of human iPSC-derived neurons to AβOs induced early pathological hallmarks of AD such as pathological TAU missorting and impairments in neuronal activity (Preprint 1), suggesting that these neurons are a suitable model system to investigate human TAU-isoform mediated toxicity, identify the isoform(s) mediating toxicity, and identify novel potential therapeutic targets for the treatment of AD (Aim 3). Remarkably, *MAPT* KO neurons were resistant to Aβ-induced changes in neuronal activity that are observed in WT neurons, demonstrating the key role of TAU in mediating the synaptotoxicity induced by toxic Aβ species. Furthermore, the re-introduction of 1N4R TAU restored neuronal vulnerability to Aβ-mediated neuronal dysfunction, suggesting that 1N4R is not only involved but the critical isoform in early TAU-mediated toxicity and a potential target for future therapeutic strategies for AD.

The following sections will provide a more detailed discussion of the main findings underlying this work.

Differential regulation of microtubule dynamics by TAU isoforms

The major canonical function of the MT-associated protein TAU is the regulation of neuronal MT stability. Dynamic assembly and disassembly of axonal MTs is crucial for neuronal function, as it enables the formation of axons, axonal cargo transport, synapse formation, and neuronal plasticity [318,319]. The differential intracellular localization of TAU isoforms suggests that MT dynamics are controlled in an isoform-specific manner. *In vitro* studies already revealed differences in the MT binding affinity, tubulin assembly rate, and efficiency of 3R and 4R TAU isoforms [168–170]. If and how MT dynamics are altered upon the presence of different TAU isoforms has not been addressed in a human cell model and isoform-dependent manner.

To rule out compartment-specific effects due to differential intracellular sorting of TAU that was demonstrated in mouse brains and mPNs [165,212], we assessed MT dynamics in the human neuroblastoma-derived SH-SY5Y cell line (Publication 1). This cell line is widely used to study TAU-related aspects of neuronal function since they can be easily differentiated into neuron-like cells, express in principle all human brain-specific TAU isoforms, and develop axonal polarity with proper axonal sorting of TAU comparable to mPNs [259,260].

To assess the effect of TAU isoforms on MT dynamics, individual human TAU isoforms were transfected into SH-SY5Y cells. MT-dynamics were analyzed using a live-cell EB3 comet assay. While the presence of 4R TAU (2N4R, 1N4R, and 0N4R) isoforms increased MT number and significantly reduced the cell size, MT dynamics were not significantly different when the individual TAU isoforms were compared to each other, suggesting that all TAU isoforms can mediate MT assembly and stability in an isolated manner and to a similar extent in unpolarized cells with even isoform distribution. Nevertheless, due to the presence of

endogenous TAU in naïve SH-SY5Y cells [259,260,312], minor differences between the individual isoforms could also be maksed by the activity of endogenous TAU. Analysis of the abundance of exogenous TAU revealed a four-fold increased level compared to endogenous TAU (normalized to the TAU abundance in differentiated SH-SY5Y cells), which could also lead to a saturation effect and may mask subtle differences between the isoforms.

Previous *in vitro* studies revealed an increased MT binding affinity for 4R TAU isoforms, which due to the inclusion of exon 10 have an additional repeat domain [59]. These 4R isoforms assembled MTs faster and more efficiently than 3R TAU isoforms [168–170]. While this is the first time MT dynamics were assessed in human living cells in a TAU isoform-specific manner, our results contradict the *in vitro* findings and indicate that as an essential function of TAU, MT stability can be mediated efficiently by all TAU isoforms. This uniform regulation of MT dynamics by all isoforms is further underlined by the differences observed between human and murine *MAPT* splicing, resulting in differences in the TAU isoform pattern: In adult murine brains, only 4R isoforms are present, while in the adult human brain six TAU isoforms are expressed [122,151–154]. The additional presence of 3R TAU isoforms indicates an evolutionary benefit [59] but also implies isoform-specific functions besides the regulation of MT stability. Our results suggest that all TAU isoforms are capable of regulating MT dynamics, which would go in line with the fact that all six isoforms are present in the adult human brain. However, in the human brain, 0N and 2N TAU isoforms each make up only up to 20% of all TAU isoforms, while 1N isoforms account for ~50% [121]. In this study, we did not control for differences in the isoform expression, which could be a technical limitation that might affect the observations regarding the regulation of MT dynamics. Furthermore, differences in the abundance of TAU isoforms between brain regions and cell types point towards cell-specific functions, which add further complexity to TAU functions [120–122,124].

In primary neurons and the brain, TAU isoform localization is compartment-specific, and TAU is highly modified by PTMs, which could in combination lead to a different regulatory pattern and isoform-specific regulation of MT dynamics [69,166,167]. Further, post-translational modifications of tubulin such as detyrosination, acetylation, and polyglutamylation influence MT dynamics and could be altered depending on the TAU isoform abundance [320–322]. Therefore, the unpolarized character of the undifferentiated SH-SY5Y is a limitation of the system since it does not account for differences in the intracellular sorting of TAU, compartment-specific effects and for PTMs which are likely specific for differentiated neurons. Besides tubulin, TAU interacts with actin, thereby linking MTs and the actin cytoskeleton to neurite outgrowth and growth cone dynamics [323–326]. The changes in cell size observed here in SH-SY5Y cells for 4R TAU isoforms could also point towards changes in the interaction with actin and indicate that in polarized neurons neurite outgrowth, growth

cone dynamics, and synapse formation could be differentially regulated by the TAU isoforms based on their interaction with actin.

While our results so far do not suggest significant differences between the TAU isoforms regarding the regulation of MT dynamics, in contrast to previous *in vitro* studies this essential TAU function must be assessed in human neurons to control for the differential intracellular localization of TAU, its PTMs, PTMs of tubulin and isoform abundance, that could affect MT-binding affinity. Nevertheless, because undifferentiated SH-SY5Y cells lack the complexity of neurons (e.g., compartmentalization, PTMs, neuronal activity) that might interfere with the regulatory functions of TAU and increases the difficulty of data interpretation, they provide a valuable system to study MT dynamics in dependence of TAU in an unbiased and isolated approach.

HiPSC-derived neurons as a potent model system to study TAU-associated functions

Even though SH-SY5Y cells are a suitable model to study TAU-associated functions in some respects, they also have limitations: i) Differentiation capability and efficiency (ranging between 40-80%), highly depends on the quality of the original undifferentiated culture [327] and is influenced by the differentiation protocol used [313]. ii) Neuronal identity is variable, and cultures contain a mixture of neuronal subtypes [260]. iii) Electrophysiological properties of neurons are largely missing. iv) Protein introduction via liposome-based transfection is difficult, and gene editing can be challenging due to chromosomal abnormalities [260,328,329].

To overcome the mentioned limitations and to further assess isoform-specific functions of TAU in human neurons, we established a neuronal differentiation protocol derived from human iPSCs, based on the doxycycline-inducible expression of the murine transcription factor Neurogenin 2 (NGN2). Expression of *Ngn2* in hiPSCs was shown to induce neuronal differentiation with efficiencies above 90% [289,290,330,331]. Neuronal cultures derived from *Ngn2* overexpression mainly consist of glutamatergic excitatory cortical neurons, which are electrophysiologically active and responsive to AMPA receptor antagonists, indicating AMPA-mediated excitatory neurotransmission [289,331,332]. Strikingly, stable integration of the *Ngn2* transgene into the AAV1 safe harbor locus, ensures rapid, scalable, and reproducible neuronal differentiation [289,331]. Thus, iPSC-derived neurons derived from *Ngn2* expression (induced neurons (iNs)) represent a suitable model for studying human TAU and its associated functions.

To validate our model system and compare it with previous studies, we characterized our neurons derived from doxycycline-induced *Ngn2* expression and analyzed the presence and localization of neuronal and glutamatergic markers (Publication 2). After two weeks, the presence of MAP2 in soma and dendrites, together with axonal enrichment of TAU, indicate efficient neuronal development and maturation [333,334]. The presence of vesicular glutamate

receptor 1 (vGLUT1) and its co-localization with PSD95 confirmed the glutamatergic identity of the neurons and the establishment of functional synapses [289,290]. Furthermore, neuronal activity was assessed by live-cell calcium imaging and revealed the presence of spontaneous calcium oscillations, clearly demonstrating neuronal activity and functionality [335]. In line with previous reports, this spontaneous activity was further enhanced by co-cultivation with murine primary astrocytes [239,290,336–338]. We further assessed the validity of the neuronal model system in a collaborative manner, in which the capability of RNA interference (RNAi)-mediated KD of *DNMT1*, a gene associated, e.g., with hereditary neuropathy [339,340], was tested. Using live-cell calcium imaging as a readout of neuronal function, we showed that DNA methylation via DNMT1 positively correlated with neuronal activity and proposed a regulatory role of DNMT1 in excitatory human neurons. Thus, these neurons are a valid tool for studying human neurologic diseases.

All in all, the differentiation of hiPSCs into iNs by doxycycline-inducible expression of *Ngn2* established in this study efficiently generates homogeneous cultures of excitatory glutamatergic neurons. Besides the presence of neuronal markers TAU and MAP2, neurons form functional synapses and exhibit spontaneous activity, making them a suitable model for investigating TAU-associated and isoform-specific functions. Even though this model is suitable to study TAU functions in neurons, it is an isolated system, that does not fully resemble the situation of the brain, in which a complex interplay between several cell types, such as different neuronal subtypes, astrocytes, oligodendrocytes, microglia and endothelial cells, maintains neuronal function and viability. Therefore, it is important to address TAU-associated functions also in mixed neuronal cultures or brain organoids that better model the *in vivo* situation (see also 5. Significance, limitations, and outlook).

All six human TAU isoforms mediate neurite growth and AIS length but are differentially localized in murine and human neurons

Although our initial results obtained in undifferentiated SH-SY5Y cells did not reveal differences in MT dynamics, 4R TAU isoforms increased the overall MT number and decreased cell size (Publication 1). We next assessed TAU-associated functions in polarized neurons considering the described differences in intracellular localization in literature. Isoform-specific interactions have been already described for murine 0N, 1N, and 2N4R TAU isoforms [213]: Proteins binding to 0N TAU were enriched in cellular homeostasis, the cellular respiration pathway, and glycolysis and include α- and β-synuclein, synapsin-1 and synaptogyrin-3. 1N-TAU binding proteins, such as ATPase, neuromodulin, calmodulin, and sodium- and chloride-dependent GABA transporter 3, were enriched in glycolysis, while proteins that preferentially bind to 2N Tau were involved in the ATP biosynthetic process and synaptic transmission, including e.g., ApoA1, ApoE, Synaptotagmin. Notably, such isoform-

specific interactome studies have not been conducted in human neurons or brain lysates to date.

Immunohistochemical findings in rodent brains demonstrated a differential localization of murine-specific TAU isoforms in axons, dendrites, and somata, that correlates well with the described distinct interactions [212]: While 0N and 2N TAU isoforms are enriched in CA3 mossy fiber region that contains axonal projections from dentate granule cells, especially 1N cannot be detected in these axons. In addition, 1N and 2N isoforms are enriched in the dendritic fractions, which could be explained by the stronger interaction of these isoforms with dendritic or postsynaptic proteins [212] . Expression of human Dendra2-tagged TAU isoforms in mPNs indicated an isoform-specific intracellular sorting: While smaller 0N isoforms are efficiently sorted into the axon, 1N and 2N isoforms show enrichment in dendrites and cell bodies [165]. However, the TDB at the AIS has a size-dependent filter with a cut-off of approximately 70 kDa [164] , and the use of the Dendra2c tag increasing the protein size by approximately 26 kDa might interfere with efficient axonal sorting of TAU isoforms.

Therefore, we assessed the intracellular sorting of the six human TAU isoforms in mPNs using the influenza hemagglutinin (HA)-tag with a relatively small size of only nine amino acids (~1.1 kDa) to better understand the intracellular sorting of TAU (Publication 1). While any tag may change the function of a protein, the HA-tag allows for identical staining of all isoforms independent of possible differential antibody affinities for the different isoforms, and independent of endogenous Tau. All isoforms showed robust axonal targeting, however we observed that especially 1N, 2N, and 4R containing human TAU isoforms were less efficiently sorted into the axon and are partially retained in the somatodendritic compartment of mPNs. These results are in agreement with previous reports, indicating that human 2N TAU is partially retained in the axon [165,212].

Notably, TAU isoform distribution was not analyzed in human neurons so far. To overcome the possible influence of endogenous TAU on the axonal sorting of exogenous TAU and to further characterize TAU-associated functions, we generated a *MAPT* KO/ TAU depletion hiPSC line based on the above described doxycline-inducible *Ngn2* cell line, using the CRISPR/Cas9 system (Preprint 1). TAU depletion did not affect iPSC morphology or pluripotency, as validated by the presence of stem cell markers, Oct3/4 and Sox2. In differentiated *MAPT* KO neurons, TAU loss resulted in a decrease in neurite and axon initial segment (AIS) length, however, these changes did not affect neuronal differentiation capacity, neuronal function, or cellular survival. These observations are in line with data from *Mapt* KO mice and derived mPNs, showing mild axonal and dendritic growth deficits and mild age-dependent phenotypes, including changes in sleep/wake cycle and mild motor deficits [95,179,180,310,341]. In contrast to *Mapt* KO mice [179,342,343], *MAPT* KO iNs did not show compensatory upregulation of MAP1A, suggesting that in human neurons, several MAPs,

e.g., MAP1 and MAP2 [333,343], orchestrate MT dynamics and compensate for TAU loss rather than one individual protein.

Our results provide first evidence for a positive role of TAU in neurite and AIS development and outgrowth in human neurons. The observed changes in neurite outgrowth and AIS length could indicate a delayed neuronal maturation due to TAU loss, since normally, TAU protein levels and abundance correlate well with neuronal maturation [179,344]. KD of TAU reduced axonal outgrowth and disturbed growth cone dynamics in primary neurons, leading to the identification of TAU as a linker of MT and actin networks [323,325]. Changes in the actin and MT network and their interplay could explain the reduction in neurite outgrowth upon TAU deletion. Therefore, growth cone dynamics and axonal outgrowth, in addition to MT and actin dynamics, should be further assessed in these neurons.

To investigate the capability of TAU isoforms to rescue the observed AIS and neurite phenotypes and to validate that the observed effects on neurite outgrowth and AIS dynamics are directly linked to TAU depletion, we re-introduced individual TAU isoforms early during differentiation in *MAPT* KO neurons. All six isoforms restored AIS and neurite length to WT levels individually, indicating that i) these effects were directly associated with TAU loss, and ii) that regulation of neurite extension and AIS length may be essential functions of TAU that can be fulfilled by all isoforms. In adult human and murine brains, six and three isoforms are present, respectively [119,121,152] that could have overlapping functions and can all compensate for TAU loss. However, as described above, differences in the abundance of the TAU isoforms [121] need to be considered in follow-up studies to rule out saturation effects due to the amount of exogenous TAU present in the neurons.

Taken together, our results indicate that all six human isoforms can take over basic TAU functions such as MT stabilization (Publication 1) and mediate neurite and AIS development (Preprint 1) in human cells but suggest that due to differential intracellular enrichment, TAU might mediate additional compartment- and isoform-specific effects.

Further, assessment of individual isoform distribution in TAU depleted iNs, demonstrated that axonal sorting of human TAU is isoform-specific and negatively correlates with isoform size (Preprint 1), thereby in principle confirming the previous results obtained from primary neurons (Publication 1 and ref. [165]). In human *MAPT* KO iNs and WT mPNs, smaller isoforms of TAU, such as 0N3R, are efficiently sorted into the axon, while larger isoforms, especially 1N and 2N4R, are more abundant in the somatodendritic compartment. Axonal trafficking of TAU depends on the presence of the TBD at the AIS, which has a size-dependent filter with a cut-off of approximately 70 kDa [164]. This is one possible explanation why TAU is generally more efficiently sorted into the axon than other MAPs, that range between sizes of 72-280 kDa [164,165]. Even though TAU is considered an axonal protein, small amounts of TAU have been detected in the dendrites of primary neurons and immunohistochemical stainings of

murine brains before [66,212]. Furthermore, endogenous murine 1N TAU was not detected in axons of the CA3 mossy fiber region, and both, 1N and 2N TAU were enriched in dendritic fractions of murine brains [212]. These findings correlate well with our observations of human TAU in mPNs and iNs, showing that larger isoforms (especially 1N4R and 2N4R) are retained in the somatodendritic compartment (Publication 1 and Preprint 1), hinting towards additional functions besides MT stabilization for these isoforms. While in the fetal human and murine brain, only 0N3R TAU is present, the isoform composition shifts to higher molecular weight isoforms having additional N-terminal inserts (1N, 2N) and an additional repeat domain (4R) [59,119,120,152]. These observations suggest that 0N3R is especially important for axonal elongation during neuronal maturation and that with increasing maturity of the neurons, the functions of TAU might shift from mainly mediating MT dynamics at the axon towards other regulatory functions such as synapse maintenance, mediated by differential subcellular enrichment of TAU and by more mature TAU isoforms (e.g., 1N4R and 2N4R) [67,211,345,346]. This hypothesis is supported by the finding that overexpression of human 2N4R TAU increased dendrite length and accelerated dendritic spine maturation in WT mPNs [165]. Depletion or KD of TAU in primary and iPSC-derived neurons resulted in delayed neuronal maturation and neurite outgrowth deficits, further underlining the critical role of TAU in these processes (Preprint 1 and ref. [95,179,180,310,341]). Although during neuronal development, only 0N3R is present, all six human brain-specific TAU isoforms were able to rescue the observed neurite, and AIS changes upon loss of TAU in human neurons, suggesting that upon TAU depletion, all isoforms can take over basic TAU functions such as MT binding, neurite extension, and AIS length regulation. While in *Mapt* KO mice, re-introduction of human 2N4R TAU increased neurogenesis and neuronal survival in brains and restored axonal outgrowth deficits in primary cultures derived from these animals [341], other isoforms were not investigated so far.

Besides the size-dependent and isoform-specific intracellular localization of TAU observed in this study, additional factors have been identified in animal models that contribute to axonal targeting of TAU: i) Prevention of retrograde diffusion of TAU from the axon into the soma by the TDB at the AIS [164], ii) increased binding affinity to axonal MTs, accomplished by the presence or absence of PTMs of TAU such as phosphorylation and acetylation and depending on the number of C-terminal repeats (with 4R TAU having a higher MT-binding affinity) [69,166–170], iii) active targeting or local translation of TAU mRNA at the axon, iv) selective degradation of somatodendritic TAU [347–352]. The great amount of possible phosphorylation sites (over 80 are described)[353] plus other PTMs, together with the different domains and interaction partners of the six TAU isoforms give rise to an enormous complexity of regulatory mechanisms for TAU-associated functions [59]. The majority of phosphorylation sites are localized at the regions flanking the MT-binding domain, thereby negatively regulating the binding affinity of TAU for MTs[59,166,353]. Furthermore,

phosphorylation regulates the subcellular localization of TAU, as pseudophosphorylation of several epitopes increased dendritic localization of TAU in mPNs and different phosphorylated species of TAU can be found depending on the subcellular compartment [354]. Phosphorylation of TAU also regulates its interaction with the axonal transport machinery [355], its association with the plasma membrane [103,356], and with other described interaction partners [59]. Acetylation of 4R TAU has been additionally demonstrated to inhibit MT-binding and increases the aggregation propensity of TAU [357]. While 4R TAU isoforms have a higher MT-binding affinity due to the presence of an additional MT-binding domain, this additional domain could increase the amount of PTMs in that region, thereby negatively influencing its association with axonal MTs and increasing its dendritic localization. Of note, phosphorylation patterns of TAU are developmentally regulated, which could additionally contribute to the observed differential localization of TAU isoforms [358]. Furthermore, the N-terminal projection domain is essential for the interaction of TAU with the plasma membrane and was shown to regulate neurite outgrowth [359,360]. Interaction with annexin A2, which is enriched in growth cones and axonal branches further regulates axonal localization of TAU [361].

All in all, our results point towards a size-dependent regulation of TAU isoform distribution, which indicates isoform- and compartment-specific functions of TAU. TAU axonal sorting is mediated early during neuronal development, and our results suggest that efficient sorting of TAU depends on its size and is influenced by the number of N-terminal inserts. Nevertheless, several other factors, such as local translation, PTMs, and protein-protein interactions might influence axonal targeting or somatic retention of certain TAU isoforms. Therefore, further experiments, such as the use of truncated, phospho-mimicking or phospho-dead mutants of TAU, as well as the investigation of compartment-specific interactions of TAU (see also 5. Significance, limitations, and outlook), are necessary to investigate the mechanism regulating isoform-specific intracellular distribution of TAU and to assess isoform-specific functions considering their different subcellular localization.

AβOs formed in the endo-lysosomal compartment induces pathological missorting of TAU in murine neurons

As an early hallmark of AD, upon the presence of toxic Aβ species, TAU dissociates from axonal MTs and accumulates in the soma and dendrites of neurons resulting in postsynaptic spine loss and neurodegeneration. Besides induction of TAU hyperphosphorylation and missorting, AβOs, formed by $Aβ_{40}$ and $Aβ_{42}$, mediate synaptotoxicity and cause cognitive impairment by inducing neuroinflammation, endoplasmic reticulum and oxidative stress, and mitochondrial and endo-lysosomal dysfunction [44,291–297]. AβO formation *in vitro* requires high unphysiological concentrations of Aβ peptides and is very slow at physiological pH [303,305]. To

better understand how AβOs can be formed under physiological conditions, a dimeric variant of $A\beta_{42}$ (dimAβ) was developed by our collaborator, providing higher local Aβ concentrations to promote oligomer formation [306]. *In vitro* assays using dimAβ demonstrated that the formation of AβOs is accelerated at endo-lysosomal pH (~8000-fold), while the concentration needed to form oligomers is reduced compared to a cytosolic pH, which enables the formation of oligomers at physiological Aβ concentrations (Publication 3). Aβ generation, especially of $A\beta_{42}$, mediated by the processing of APP by the γ-secretase complex is performed in the endo-lysosomal compartments [35,317]. Furthermore, endo-lysosomal dysfunction has been demonstrated as a key hallmark of AD, and Aβ accumulation can be observed in these organelles [16,301,316]. Therefore, the results obtained during this study indicate that neurotoxic AβOs could be particularly formed in the endo-lysosomal compartment, leading to lysosomal leakage and cellular damage.

To assess the disease relevance of the oligomers formed by dimAβ, we analyzed the cellular toxicity of dimAβOs, the capability of binding to dendritic spines, Tau missorting, and neuronal function in mPNs after treatment with dimAβOs. DimAβOs co-localized with dendritic spines and induced pathological TAU missorting over time (up to 24 hours). Furthermore, neuronal activity was analyzed as a readout for neuronal function and revealed that treatment with dimAβOs significantly reduced neuronal function. Of note, the observed effects did not cause extensive cellular toxicity indicating that dimAβOs instead mediate neuronal dysfunction than directly promoting neuronal death. In previous studies, different $A\beta_{40}$ to $A\beta_{42}$ ratios were tested for their potential to form oligomers *in vitro* and were later assessed for their neurotoxic potential in mPNs [64,362]. Treatment of primary neurons with a preparation of AβOs in a ratio of 7:3 of $A\beta_{40}$ to $A\beta_{42}$, which reflects the composition of Aβ in AD (70% $A\beta_{40}$ and 30% $A\beta_{42}$), resulted in pathological TAU missorting, hyperphosphorylation of TAU, and leads to MT and synapse loss [64,362]. These findings were replicated by the treatment with dimAβOs. However, differences in the time course of pathological TAU missorting (12 [64,362] and 24 hours (Publication 3), respectively) indicate increased kinetic stability of dimAβOs. Our results demonstrate that dimAβOs are a highly disease-relevant system suitable for studying AD pathomechanisms. The prolonged effects of dimAβO treatment might also represent a model of chronic instead of acute stress, which could be beneficial in order to study the downstream consequences of TAU missorting, as it better recapitulates the low-level, long-term stress neurons are exposed to in AD patients' brains [7,17,363]. While these AβOs might be less toxic compared to other oligomers, their increased kinetic stability could also result in a prolonged stress induction, thereby being even more toxic at later timepoints. To further study the suitablilty of dimAβOs to model AD-like stress, neuronal cultures should be closely monitored regarding viability, cell death, neuronal activity, and TAU pathology upon long-term exposures to dimAβOs (> 24 hours).

All in all, our results demonstrate that AβOs, likely formed in endo-lysosomes, demonstrate a disease relevant species that localize to dendrites and induce early pathological hallmarks of AD, including TAU missorting and a reduction of neuronal activity in mPNs without causing excessive neuronal death. However, further investigations are necessary to clarify, if their kinetic stability is advantageous for the use as a AD-stress inducer in neuronal cultures and later also *in vivo*.

TAU depletion protects human neurons from Aβ-induced loss of neuronal activity

To model AβO-induced TAU pathology and study downstream effects in human neurons, we treated WT iNs with a preparation of AβOs in a ratio of 7:3 of $Aβ_{40}$ to $Aβ_{42}$ [64,362] (Preprint 1). Pathological missorting of TAU into the somatodendritic compartment was observed after three hours of treatment with AβOs. This effect was transient, reversible, and did not cause neuronal death, which has been assigned to the formation of larger non-toxic AβOs over time in previous studies [64,362]. In addition, live-cell calcium imaging was performed to analyze the acute effect of AβOs on synapse integrity and neuronal activity. A significant reduction of neuronal activity was observed for WT iNs after incubation with AβOs. Neuronal activity and function highly dependent on calcium signaling and changes in neuronal calcium homeostasis are associated with aging and AD, and impairments in calcium signaling have been shown to drive synaptic dysfunction, Aβ deposition, and TAU hyperphosphorylation [335,364,365]. Our results indicate that AβO treatment of iNs leads to early hallmarks of AD, such as TAU missorting and impairments in neuronal activity, suggesting that iNs are a suitable model system to study AD-associated neurotoxicity.

Various studies have shown that despite a detectable amyloid plaque burden, Aβ alone is insufficient to drive cognitive decline in humans. Notably, cognitive symptoms correlate well with TAU pathology but not Aβ pathology [91–94,99,100]. Studies in rodents demonstrated that TAU depletion is protective against Aβ-induced neurotoxicity and cognitive impairments, strongly implying a key role of TAU in the Aβ-induced pathological cascade [95,96,98–100,105,107]. Therefore, we assessed how AβO treatment affects spontaneous calcium oscillations of TAU depleted iNs as a readout of neuronal function (Preprint 1). While one hour of AβO treatment was already sufficient to reduce spontaneous calcium oscillations in WT iNs, no effect on neuronal activity was observed for TAU-depleted neurons, indicating a protective effect of TAU depletion on AβO-induced neurotoxicity. This protective function further supports the hypothesis, that TAU is the main driver of neurodegeneration in AD. Nevertheless, as outlined previously, upstream factors, such as endo-lysosomal and mitochondrial dysfunction, neuroinflammation, and metabolic changes, e.g., insulin resistance, which occur even earlier could trigger the downstream accumulation of Aβ and TAU and contribute significantly to disease progression [7,20,44,65]. Therefore, it is essential to further evaluate how

TAU mediates Aβ-induced synaptotoxicity and to identify factors which protect TAU-depleted neurons from these detrimental consequences.

The protection of TAU depleted iNs from AβO-induced synaptotoxicity clearly demonstrates a key role of TAU in mediating the pathological cascade induced by toxic Aβ species leading to the rapid cognitive decline observed in AD patients. Further assessment of the mechanisms, how TAU contributes to disease progression and why TAU depleted neurons are protected from these effects is vital to better understand the disease and to identify suitable therapeutic targets for its treatment.

The TAU isoform 1N4R mediates Aβ-induced neuronal dysfunction in human neurons

Besides stabilizing axonal MTs, TAU is involved in various cellular functions and processes [59,61,67,366]. Our results underline compartment- and isoform-specific functions of TAU, while basic functions of TAU, such as the regulation of MT stability and neurite or AIS growth, can be mediated by all six isoforms in human neurons (Publication 1 and preprint 1). Further, we demonstrate that TAU is a key mediator of Aβ-induced neuronal toxicity in human neurons since *MAPT* KO neurons are protected from Aβ-induced neuronal dysfunction (Preprint 1).

We next assessed the effect of TAU isoforms on AβO-induced neuronal dysfunction using TAU depleted iNs in which we re-introduced individual TAU isoforms. Neurons expressing 0N4R, 2N4R, or one of the 3R TAU isoforms showed only a mild reduction of neuronal activity, indicating that the neurons remained partially protected from the AβO-induced decrease in neuronal activity that was observed for WT iNs after treatment with AβOs. Only 1N4R induced a reduction in spontaneous calcium oscillation indicating impaired neurotransmission and a restoration of the vulnerability of TAU depleted neurons to AβO-mediated neuronal dysfunction. These observations clearly suggest that the Aβ-induced impairment of neuronal activity is mediated specifically by 1N4R TAU. While in WT iNs, only immature TAU (mainly 0N3R and 0N4R) is present during neuronal maturation, we re-introduced TAU isoforms only after seven days in *MAPT* KO iNs, when synapses are already formed, and neurons developed axonal polarity. Nevertheless, the response of both, WT and 1N4R TAU expressing neurons to AβOs was comparable, which suggests that acute TAU presence in general rather than its effect on neuronal development is important for AβO-induced toxicity. Therefore, it is important to further test, how an acute introduction of TAU, e.g., by switch-on experiments, or a prolonged TAU presence starting from the first day of differentiation would affect neuronal function after exposure to AβOs.

As outlined before, we observed that especially 1N4R and 2N4R TAU isoforms were localized to the somatodendritic compartment (Publication 1 and Preprint 1, see Figure 8). Dendritic TAU has been found to play a vital role in synapse formation and post-synaptic spine morphology [165,190,191,211]. In primary rodent neurons, e.g., presence of dendritic TAU

isoforms, such as 0N4R, 1N4R and 2N4R resulted in enhanced spine maturation and dendritic elongation, and TAU regulates dendritic MT stability [165]. Further, TAU interacts with non-muscle myosins and filamentous actin, which further underlines a role of TAU in regulating spine morphology [211]. Through its interaction with Fyn kinase and PSD95, TAU regulates synaptic activity via NMDA receptors [191]. The importance of TAU for neurotransmission is further highlighted by *Mapt* KO mice, which exhibit defects in long-term depression and hippocampal synaptic plasticity [367,368]. Notably, translocation of TAU to the postsynaptic compartment in an activity-dependent manner has been demonstrated under healthy conditions [369].

While in adult mice, only 4R TAU isoforms are present that fulfill all TAU-associated functions, in humans, six isoforms exist [119,152]. It is likely that the smaller or less mature ones (e.g., 0N3R and 0N4R) mediate axonal functions, while larger and more “mature” isoforms, such as 1N4R, that localize to soma and dendrites could be essential for dendritic functions and synapse maintenance. This model is further supported by the differential axonal enrichment of the isoforms observed in several models here and in previous studies (Publication 1, Preprint 1, ref. [165,212]). As outlined above, TAU localization is influenced by several mechanisms, such as PTMs and protein-protein interactions. The different domains of TAU might also explain the differences between the isoforms in regard to mediating AβO-induced synatotoxicity. The N-terminal projection domain has been implemented in the interaction of TAU with components of the plasma membrane and further exhibits a primate-specific sequence, which specifically interacts with synaptic and plasma membrane proteins, such as annexin A5, synapsin-1 and neurogranin [360] . In addition, specific regions in the MT-binding domain of TAU have been associated with the binding of TAU to lipid bilayers, underlining that several domains of TAU are important for membrane binding [361,370]. The association with the plasma membrane is further regulated by the phosphorylation status of TAU [371]. As the longest TAU isoform, 2N4R contains two N-terminal inserts and an additional C-terminal repeat domain, we hypothesize that this isoform is primarily bound to the neuronal plasma membrane. In contrast, 0N4R, which might have a reduced affinity to the plasma membrane and is more efficiently sorted into the axon compared to 1N4R and 2N4R, which suggests that it is rather bound to axonal MTs.

Spine loss and synaptic dysfunction are key hallmarks of AD [372,373], to which TAU contributes significantly. Under pathological conditions, TAU dissociates from axonal and dendritic MTs, leading to their destabilization, resulting in the breakdown of axonal transport and loss of dendritic spines [63,64,68,374]. TAU missorting results in the missorting of TTLL6 into the dendrites where it polyglutamylates MTs, thus leading to MT destabilization via spastin-mediated severing [62–64]. In addition, post-translational modification of TAU, such as phosphorylation and acetylation, increase the dendritic localization of TAU and its

aggregation propensity, resulting in synaptic impairments [67–69]. Furthermore, 1N and 2N isoforms show increased aggregation propensity, and the presence of four repeat domains (4R) reduces the critical concentration to form fibrils *in vitro* [168,231]. TAU further contributes to neuronal dysfunction through a gain of toxic function mechanism leading to changed or increased interactions of unbound TAU to synaptic components [65,67]. For example, TAU mediates excitotoxicity by recruiting Fyn kinase to the postsynaptic NMDA receptor complex, which leads to the phosphorylation of NMDA receptor subunits and subsequent activation of toxic downstream pathways [65,67].

In this study, we identified 1N4R TAU as a main driver of Aβ-induced neuronal dysfunction. While in mice, the 2N4R interactome is associated with neurodegenerative processes [213], it is important to mention that AD does not naturally occur in rodents. In disease models (using external stimuli such as overexpression of mutated human proteins), often “full-length TAU” (=2N4R) is studied [190] , however, in the human brain, 1N TAU isoforms are the predominantly expressed isoforms (~ 50% of whole TAU protein expression) [121]. Of note, heterozygous deletion of exon 10, encoding for the fourth repeat domain, in mice resulted in a one-to-one ratio of 3R and 4R murine TAU isoforms, mimicking the physiological abundance of TAU in human brains [375]. However, these mice develop age-dependent sensorimotor deficits. Furthermore, KI of human TAU in *Mapt* KO mice, results in increased dendritic localization of TAU, caused pathological phosphorylation and accumulation of TAU in these animals [249]. Furthermore, the TAU interactome of mice and humans differs significantly, highlighting the differential functions of TAU between these two species [190].

Due to its primarily somatic and dendritic localization, 1N4R could mediate toxic functions via specific interactions with synaptic proteins. It has been demonstrated for example that dendritic TAU is essential for targeting Fyn kinase to NMDA receptors, leading to their phosphorylation and induction of excitotoxicity in the presence of Aβ pathology [66] . TAU interacts also with PSD95, acting as a scaffolding protein [191]. Hyperphosphorylation of TAU could ablate this interaction and result in defects in synapse maintenance. Furthermore, dendritic TAU has been shown to recruit TTLL6 to the dendrites, where it polyglutamylates MTs and leads to MT severing [64]. While under normal conditions, TAU can translocate from dendrites to the postsynapse in an activity-dependent manner, this translocation is ablated upon exposure to AβOs and results in pathological accumulation of TAU in dendrites [369]. Phosphorylation of the KxGS motifs (within the MT-binding domain) could further contribute to local disruption of MT stability and contribute to synapse loss[376,377]. Alternatively, or in combination with that, hyperphosphorylated and missorted TAU, which accumulates in the soma and dendrites, may scavenge 1N4R, leading to its accumulation and inhibiting its essential dendritic functions. Furthermore, TAU has been shown to interact with RNA binding proteins and ribosomal subunits [190], and TAU oligomers formed by 1N4R induce a

translational stress response and inhibit translation, resulting in stress granule formation and changes of dendrite length and morphology that further drive neuronal dysfunction [201,207,378,379]. Notably, other tauopathies are characterized by changes in the splicing of *MAPT*, resulting in an increased or decreased production of 4R TAU [59,229]. This further highlights that TAU isoform abundance is well balanced in healthy brains and that disruption of this balance is an essential driver of neurodegeneration. Of note, a prion like propagation of TAU pathology is assumed for the intercellular spreading of disease-associated TAU [61]. Interneuronal spreading of TAU requires mature synapses, suggesting a trans-synaptic TAU propagation mechanism[380]. In this model, prion-like TAU seeds would interact again with postsynaptic TAU and could trigger its accumulation and downstream processes. This mode of action is further underlined by the fact that extracellular TAU promotes translocation of intracellular TAU into dendrites [67,381].

Taken together, dendritic 1N4R could mediate AβO-induced synaptotoxicity via several pathways: by destabilizing local MTs via TTLL6, interaction with Fyn kinase, PSD95, and/ or non-muscle myosins, inhibition of translation, and trans-synaptic spreading, thereby mediating synaptic dysfunctin and driving neurodegeneration [66,67,191–193,211] (see Figure 8). The differences between human and rodents regarding *MAPT* splicing and the absence of AD pathology in rodents, underline the urgency to further evaluate human TAU isoforms in human neuronal model systems. It is crucial to evaluate how 1N4R might drive the early pathological response to AβOs, especially by assessing its interactome in human neurons under basal and pathological conditions (see 5. Significance, limitations, and outlook). Based on the reduced neuronal activity observed here (Paper 3 and Preprint 1), we can speculate that the proposed synaptic functions of TAU play essential roles in disease pathogenesis (see Figure 8).

Together with the proposed functions of dendritic TAU under pathological conditions and the findings described here, showing i) a differential intracellular distribution of TAU and ii) a specific mediation of Aβ-induced neuronal dysfunction by 1N4R TAU, we hypothesize that somatic and dendritic 1N4R TAU, is the first mediator of the AβO-induced neurotoxicity observed in AD. Even though not assessed in an isoform-specific manner, dendritic TAU has been shown to mediate synaptic plasticity via its interaction with NMDA receptors, postsynaptic density components, and non-muscle myosins [66,67,191–193,211]. Under pathological conditions, dendritic interactions of TAU might be ablated or changed due to changes in post-translational modifications (e.g., phosphorylation at the KxGS motifs resulting in post-synaptic actin disruption [64]), resulting in spine loss and excitotoxicity. Our results indicate that TAU-mediated neuronal dysfunction induced by AβOs is isoform-specific and likely mediated by dendritic 1N4R, making this isoform a promising novel target for the treatment of AD.

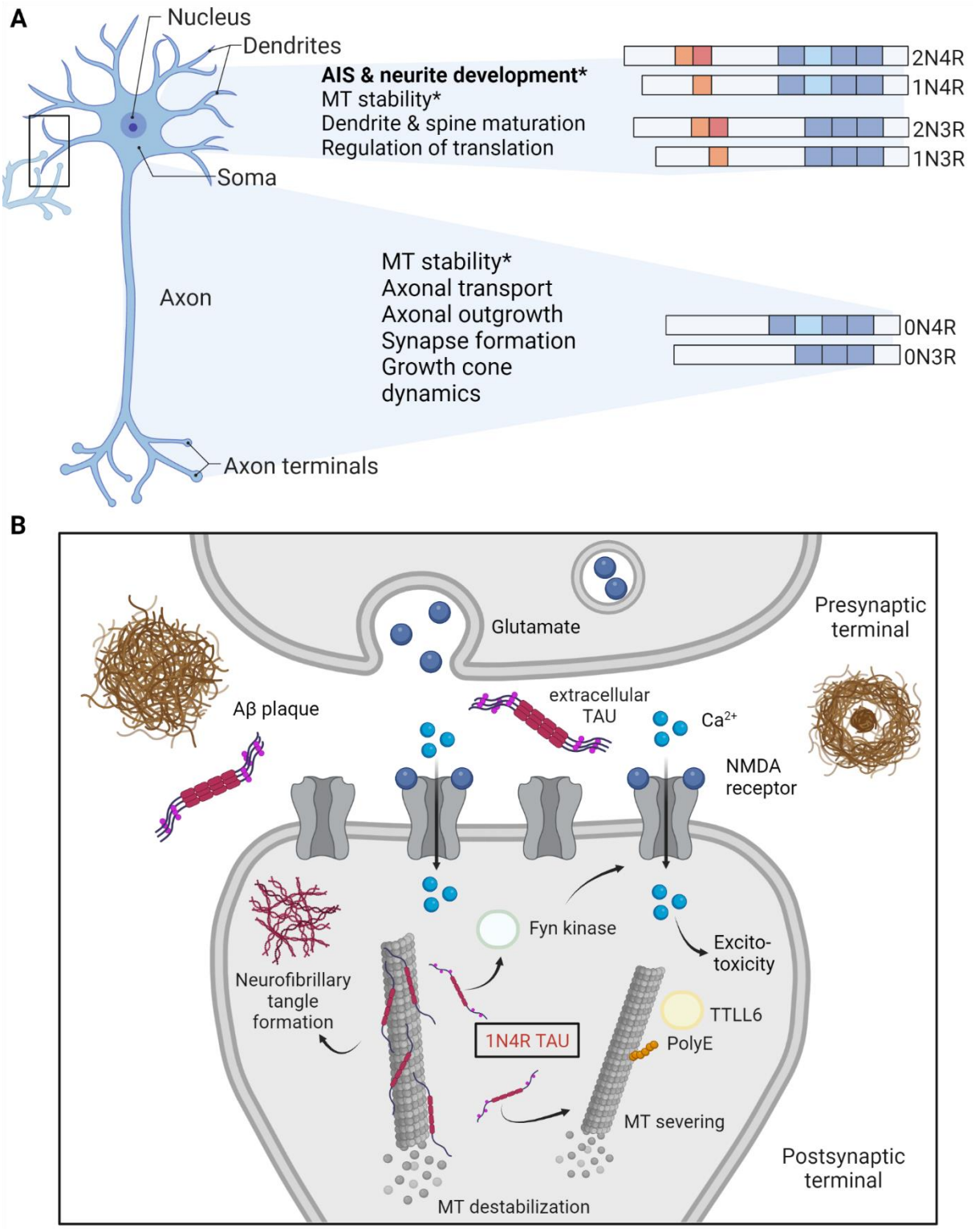

Figure 8 Differential localization of TAU isoforms, TAU functions and proposed mechanisms of 1N4R-mediated synaptotoxicity. (A) Schematic representation of the differential intracellular localization of TAU isoforms observed in this study and proposed functions of TAU. Smaller isoforms of TAU, such as 0N3R and 0N4R are efficiently targeted to the axon. In contrast, bigger isoforms, especially 2N4R and 1N4R are retained in the somatodendritic compartments. We demonstrate here that all six TAU isoforms likely mediate basic functions of TAU such as MT stability. In addition, we propose that TAU is involved in neuronal development by regulating neurite growth and AIS length independent of the isoform present. Asterisks indicate the functions of TAU demonstrated in this study. **(B)** In this study, 1N4R TAU was identified as a key driver of early neuronal dysfunction induced by AβOs. Dendritic 1N4R TAU likely acts via several pathways: i) Dissociation from local MTs upon phosphorylation of TAU leads to MT destabilization. ii) TAU recruits TTLL6 to the postsynapse, where it

polyaglutamylates MTs, causing MT severing by spastin. iii) Interaction with Fyn kinase leads to NMDA receptor-mediated excitotoxicity and other interactions with non-muscle myosins and PSD95 (not shown) could further drive synapse loss. iv) Trans-synaptic spreading of TAU could induce aggregation of TAU in tangles at the postsynapse. Figure created using BioRender.com.

5. Significance, limitations, and outlook

TAU is a key driver of AD pathology, but the contribution of the TAU isoforms is unclear. Within this study, we could show that under basal conditions, AD-like TAU missorting can be induced with AβOs and that TAU isoforms show a differential axodendritic localization in murine and human neurons. While the presence of individual TAU isoforms leads to small effects on cell size, neurite outgrowth and AIS length, in an AD paradigm (induced by AβO treatment) only one isoform, 1N4R, conferred TAU toxicity. By creating and characterizing a *MAPT* KO iPSC line and by studying TAU isoforms in several settings, we have thus not only expanded our understanding of basic TAU in neuronal cell biology, but may have also identified a new therapeutic target for AD.

Based on our results and published findings by others, a plethora of additional functions have been suggested for TAU. For example, TAU-depleted human and murine neurons show some phenotypes of delayed neuronal maturation, such as reduced neurite and AIS length, which suggest an essential role of TAU during early neuronal development (Preprint 1 and ref. [179,310]). However, the observed phenotypes are mild, implying that other neuronal proteins, such as other MAPs, compensate for TAU loss. Investigating transcriptional and proteomic changes during neuronal differentiation in WT and *MAPT* KO human neurons will lead to further functional insights into the role of TAU in neuronal development and maturation and the compensatory mechanism ensuring proper neuronal function upon TAU depletion. To assess neurite, and specifically, axonal outgrowth in detail and in the dependence of TAU isoforms, microfluidic chambers can be used, providing exclusive access to different parts of the neurons to study axon growth and guidance independently of their cell bodies [382]. In addition, these microfluidic devices can be used for studies on axonal transport, which highly depends on functional MT dynamics [383]. Of note, within this study, no isoform-specific differences were observed for MT dynamics in the human neuroblastoma cell line SH-SY5Y (Publication 1), however, given the differences in the subcellular localization of TAU isoforms, these parameters must be assessed in human *MAPT* KO neurons (in a compartment-specific manner).

While a variety of interactions have been identified for TAU, limited data is available on isoform-specific interactions, especially on human TAU. To investigate the interactome of TAU isoforms, co-immunoprecipitation experiments with individual TAU isoforms can be performed in *MAPT* KO human neurons. Correlating these results to the observed subcellular localization and the already described interactors of TAU can thereby lead to the identification of novel TAU interactions and provide insights into isoform- and compartment-

specific roles of TAU. Of note, under basal and pathological conditions, TAU is highly modified by post-translational modifications. Besides the differences in TAU isoform interactions and intracellular localization, differences in PTMs could further increase the complexity of TAU functions. Furthermore, axonal sorting of TAU seems to be a tightly regulated process involving components of the AIS, and depending on isoform size, certain domains, PTMs, and MT binding affinity of TAU (Publication 1, Preprint 1, and ref. [64,164,313,347]). In addition, active targeting of TAU mRNA, its selective translation in axons, or degradation of somatodendritic TAU are proposed mechanisms [347–352]. The generated *MAPT* KO iNs provide a suitable model system to investigate further how TAU is sorted into the axon and why larger isoforms such as 1N4R and 2N4R are partially retained in the somatodendritic compartment.

The stabilization of neuronal MTs is a key function of TAU. It is very likely that TAU, by influencing axonal microtubule dynamics, is involved in downstream processes such as axonal elongation, neurite branching, and synaptic plasticity, which are crucial to maintaining neuronal function. Of note, TAU has been found to play a vital role in synapse formation and postsynaptic spine morphology [165,190,191,211]. Besides MT destabilization and TAU hyperphosphorylation, spine loss and synaptic dysfunction are key hallmarks of AD [372,373]. This study shows that neuronal activity is directly affected by AβO-induced stress and is mediated specifically by TAU and especially 1N4R TAU (Preprint 1). Therefore, further experiments are necessary to understand how 1N4R mediates neuronal dysfunction and whether this effect depends on its intracellular localization and its role in synaptic plasticity. However, one limitation of our human neuronal model system is the absence of postsynaptic spines, as seen in primary rodent neurons, although proper synapse formation is present, and neurons are electrophysiologically active. To promote spine formation and neuronal maturation, either culture conditions must be improved, or alternative models must be established in order to fully understand TAU's role in synapse and spine formation.

Hallmarks of AD include the destabilization of axonal microtubules and the breakdown of axonal transport, which contributes to synaptic dysfunction and neuronal death [16,20,310]. In this study, we modeled AD-like stress in our model systems by treatment with AβOs, which induced pathological missorting of TAU and a reduction in neuronal activity (Publication 2 and Preprint 1). Of note, relatively high concentrations of Aβ are necessary to form oligomers *in vitro* [303,305]. However, AD is a slowly progressive disease in which Aβ accumulation occurs over years to decades before the onset of cognitive symptoms [7,8,17]. Therefore, to better model the situation in the human brain and provide a low-level persistent stress instead of an acute insult, neurons can be exposed to physiological concentrations of secreted Aβ (e.g., secreted by helper cells [363]). Alternatively, mutated APP associated with fAD can be introduced into neuronal cultures via lentivirus and adeno-associated virus (AAV)-based

delivery or by introducing the pathogenic variant into the corresponding gene using CRISPR/Cas9 to model AD-like stress and further assess TAU-mediated neurotoxicity.

Region- and cell-specific differences in the abundance of TAU isoforms have been observed in human and murine brains [121–124]. Together with the highly reproducible spreading of TAU pathology in AD, beginning in the Locus coeruleus and the entorhinal cortex and extending to the hippocampus and finally the whole cortex, these observations point towards a selective vulnerability of certain brain regions or neuronal subtypes to TAU pathology [76,78]. The ability to differentiate hiPSCs into different neuronal subtypes, mixed neuronal cultures and other brain-specific cell types, and our results suggesting that AβO treatment is a suitable stressor to model AD hallmarks in iPSC-derived neurons could be combined to investigate the selective vulnerability of different neuronal subtypes to TAU pathology [384]. Besides neurons, astrocytes and microglia play a vital role in AD pathology [16,385]. Within this study, we exclusively assessed the role of TAU in an isolated manner and exclusively in neurons. Recent advances have been made in establishing model systems, including neurons, astrocytes, and microglia to better model the physiological environment of the brain [235,273]. Notably, our *MAPT* KO neurons or neurons re-expressing TAU isoforms could be integrated into these model systems to further investigate TAU-associated functions under pathological conditions. Furthermore, this study suggests that *MAPT* KO human iNs are protected from Aβ-induced neuronal dysfunction. Comparing its transcriptome and proteome with WT neurons might identify protective modifiers or drivers of disease pathology, which could reveal novel putative candidates for the treatment of AD.

By re-introduction of individual TAU isoforms, we identified an isoform-specific mechanism of Aβ-induced TAU-mediated neurotoxicity. Notably, the model system used in this context is based on the expression of individual TAU isoforms. However, this does not resemble the situation of the human brain, in which 3R and 4R TAU isoforms are abundant in equal ratios, and all N-terminal splice forms of TAU are present [119,120]. Therefore, future studies should be aimed at generating a human neuronal system in which all six human TAU isoforms are present at physiological levels. This could be achieved by further neuronal maturation, the use of 3D models or organoids, or re-introduction of the isoforms by virus-based delivery. As an alternative, a humanized *MAPT* KI mouse that was recently generated (on a mouse *Mapt* KO background) [248,386] could be used. Further, this mouse model was crossed with an *App*$^{NL-G-F}$ KI mouse (expressing murine App with the Swedish, Iberian (I716F), and Arctic (E639G) mutations) to induce amyloid pathology and model the effect of human TAU on disease progression [248,386]. To better understand the role of the TAU isoforms in AD pathology, individual TAU isoforms could be targeted in this model by an AAV-mediated KD approach using small-hairpin RNAs (shRNAs). Although complete depletion of TAU is protective against Aβ-mediated spine loss and neurodegeneration in human neurons (Preprint 1) and

mouse models of AD, these animals are more susceptible to Traumatic Brain Injury and Parkinsonism and show impairments in synaptic plasticity, pointing towards an essential function of TAU for neuronal survival also under pathological conditions [95,96,107,179,387]. In addition, aged *Mapt* KO animals display small non-pathological deficits in muscle function, sleep/awake behavior, fear conditioning, and reduced long-term potentiation [97180,186,187,388]. The Koolen–De Vries syndrome, caused by a microdeletion of 17q21.31 including the *MAPT* gene, is characterized by severe symptoms such as mental retardation, developmental delay, and facial abnormalities [389,390]. Therefore, complete reduction of *MAPT* expression by passive or active immunization, RNAi, or antisense oligonucleotides (ASOs), could lead to severe adverse events in humans. Our results indicate that it might be sufficient to KD specifically 1N4R-TAU, which makes up to 20% of the total TAU protein amount [121] in order to reduce or even prevent the neuronal toxicity mediated by TAU, without affecting other essential functions of TAU. While targeting of 1N4R specifically is not possible with current biotechnological approaches, it is possible to suppress specifically 1N- or 4R-TAU isoform production, e.g., using RNAi, ASOs, targeted protein degradation, or indirectly by targeting splice factors, which must be tested in future studies for their therapeutic potential.

In conclusion, this study provides further insights into isoform- and compartment-specific functions of TAU and describes a differential intracellular sorting of TAU in human neurons. Furthermore, this study demonstrates that human iPSC-derived neurons treated with AβOs are a suitable and reliable model for studying TAU-mediated neurotoxicity, showing hallmarks of neurodegeneration, such as pathological missorting of TAU and impairments in neuronal activity. The protection of *MAPT* KO iNs from AβO-induced synaptotoxicity clearly demonstrates a key role of TAU in mediating the pathological cascade induced by toxic Aβ species in human neurons. This work provides further evidence that besides a loss-of-function in regard to axonal MT stabilization, a gain-of-toxic function mechanism exists in AD in which specifically dendritic TAU dissociates from MTs, leading to changes in its interactome, its aggregation propensity, and pathological interactions, driving MT destabilization, spine loss and neurodegeneration[16,61,64,66,67,190,193,195–201]. Furthermore, TAU-associated functions are isoform-specific and may change during neuronal maturation and under pathological conditions. Hence targeting one specific TAU isoform, such as 1N4R, might be sufficient to reduce TAU-mediated neurotoxicity without interfering with the basal functions of TAU, which could significantly improve future treatment strategies for this currently untreatable and detrimental disease. While this study and former results provide evidence for a differential role of TAU isoforms in AD progression, several open questions that need to be addressed in future studies remain. Understanding the differential contribution of TAU and its isoforms to neurodegenerative processes will strongly enhance the basic knowledge of TAU and help to develop more targeted treatment strategies for AD and related neurodegenerative diseases.

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Eidesstattliche Erklärung

Gemäß der Promotionsordnung vom 12. März 2022.

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Die Ergebnisse dieser Dissertation wurden in 3 unabhängigen Veröffentlichungen publiziert:

1. Schützmann MP*, Hasecke F*, **Bachmann S***, et al. Endo-lysosomal Aβ concentration and pH trigger formation of Aβ oligomers that potently induce Tau missorting. *Nature Communications.* 12, 4634 (2021). https://doi.org/10.1038/s41467-021-24900-4
2. **Bachmann S**, Bell M, Klimek J und Zempel H. Differential effects of the six human TAU isoforms: Somatic retention of 2N-TAU and increased microtubule number induced by 4R-TAU. *Frontiers in Neuroscience*. 15:547. https://doi.org/10.3389/fnins.2021.643115
3. **Bachmann S***, Linde J*, Bell M, et al. DNA methyltransferase 1 (DNMT1) shapes neuronal activity of human iPSC-derived glutamatergic neurons. *International Journal of Molecular Sciences.* 2021; 22(4):2034. https://doi.org/10.3390/ijms22042034

   (*gleichwertiger Beitrag)

Köln, den 17.03.2023

Sarah Maria Buchholz (geb. Bachmann)