Domain requirements and mechanisms of axonal Tau sorting

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List of abbreviations

0N/1N/2N	Number of N-terminal inserts
3R/4R	Number of C-terminal repeat domains
A/Ala	Alanine
AAVS1	Adeno-associated virus integration site 1
ACTR2	Actin-related protein 2
AD	Alzheimer's disease
AIS	Axon initial segment
AMPA	α-amino-3-hydroxy-methyl-4-isooxazoleproprionic acid
ANKG	Ankyrin-G
APEX2	Ascorbate peroxidase 2
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ARP2	*ACTR2*-encoded protein
AT8	Phosphorylation motif inside the proline-rich region 2 (S199, S202, T205)
ATAT1	α-tubulin N-acetyltransferase 1
Aβ	Amyloid-beta
BDNF	Brain-derived neurotrophic factor
BirA	Bacterial biotin ligase BirA
CDC42	Cell division control protein 42
CDK5	Cyclin-dependent kinase 5
CELR3	*CELSR3*-encoded protein
CELSR3	Cadherin EGF LAG seven-pass G-type receptor 3
CSF	Cerebrospinal fluid
CTE	Chronic traumatic encephalopathy
Cterm	Tau C-terminal half
DNA	Deoxyribonucleic acid
DOCK7	Dedicator of cytokinesis protein 7
e.g.	exempli gratia
E/Glu	Glutamic acid
F-actin	Filamentous actin
FTD	Frontotemporal dementia
FTDP-17	FTD with parkinsonism linked to chromosome 17
GFP	Green fluorescent protein
GO	Gene ontology
GSK-3β	Glycogen synthase kinase 3-beta
HA	Hemagglutinin tag
HNRNP	heterogeneous nuclear ribonucleoprotein
HSP110	*HSPH1*-encoded protein
HSPA1A/B	Heat shock protein 1A/B
HSPH1	Heat shock protein 105 kDa
i.e.	id est
iPSC	Induced pluripotent stem cell
kDa	kilodalton
KO	Knock out
KXGS	Phosphorylation motif inside the repeat domains (S262, S293, S324, S356)
LUZP1	Leucine zipper-containing protein 1
MAPT	Microtubule-associated protein Tau
Mapt	Microtubule-associated protein Tau (murine gene)
MARCKS	Myristoylated alanine-rich C kinase substrate
MARK1-4	Microtubule affinity-regulating kinases 1-4

mRNA	Messenger ribonucleic acid
MT	Microtubules
NeuN	Neuronal nuclei
NFT	Neurofibrillary tangles
Ngn2	Neurogenin 2 (murine gene)
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
Nterm	Tau N-terminal half
o-GlcNAc	O-linked β-N-acetylglucosamine
PD	Pick's disease
PEX14	Peroxisomal membrane protein 14
PEX5	Peroxisomal targeting signal 1 receptor
PP2A/5	Protein phosphatase 2A/5
PRC2A	*PRRC2A*-encoded protein
PRR	Proline-rich region
PRRC2A	Proline-rich and coiled-coil A2
PSD95	Postsynaptic density 95
PSEN	Presenilin
PSP	Progressive supranuclear palsy
PTM	Posttranslational modification
RA	Retinoic acid
RAB11A/B	Ras-related protein 11 A/B
RALA	Small Ras-related GTPase A
ROS	Reactive oxygen species
S/Ser	Serine
SEC8	Exocyst complex component 4
SH3	Src-homology 3
SH3PXD2B	SH3 and PX domain-containing protein 2B
SH-SY5Y	Human neuroblastoma cell line SH-SY5Y
SNARE	SNAP Receptors
SPD2B	*SH3PXD2B*-encoded protein
T/Thr	Threonine
TAI	Traumatic axon injury
TBI	Traumatic brain injury
TJP1	Tight junction protein 1
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRIM46	Tripartite motif-containing protein 46
TTLL6	Tubulin tyrosine ligase-like 6
UV	Ultra violet
VGLUT1	Vesicular glutamate transporter 1
WNT/PCP	Wnt/planar cell polarity
WTC11	Human iPSC line WTC11
ZCCHC3	Zinc finger CCHC domain-containing protein 3
ZCHC3	*ZCCHC3*-encoded protein
ZO1	*TJP1*-encoded protein

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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) stellt eine große Belastung für die modernen Gesundheitssysteme dar. Pathologische Veränderungen des Mikrotubuli-bindenden Proteins Tau sind ein Hauptmerkmal der Alzheimer-Krankheit und vieler anderer Erkrankungen, die als Tauopathien zusammengefasst werden. Unter gesunden Bedingungen reguliert Tau in Abhängigkeit von seinem Phosphorylierungszustand den dynamischen Aufbau der axonalen Mikrotubuli-Filamente und beeinflusst damit wesentliche neuronale Funktionen. Die somatodendritische Fehlsortierung von Tau ist ein frühes Anzeichen der Tau-Pathologie bei Alzheimer. Trotz der komplizierten Rolle der intrazellulären Tau-Fehllokalisierung unter pathologischen Bedingungen sind die Mechanismen der axonalen Tau-Sortierung und der somatodendritischen Fehlsortierung noch immer kaum verstanden. Es wurden mehrere Prozesse vorgeschlagen, die eine effiziente axonale Tau-Sortierung beeinflussen, darunter der anterograde Proteintransport oder die retrograde axonale Retention, aber ihr Beitrag ist nach wie vor nicht klar. Auch über Tau-Domänen, Motive oder zelluläre Interaktionspartner, die für eine erfolgreiche Tau-Sortierung erforderlich sind, ist wenig bekannt. Mehr Wissen über die zugrundeliegenden Prozesse der axonalen Tau-Sortierung würde dazu beitragen, Behandlungen zu entwickeln, die die physiologische Sortierung unter Krankheitsbedingungen wiederherstellen und damit die neurotoxischen Auswirkungen der Tau-Fehlsortierung abschwächen können.

Interessanterweise unterscheiden sich die sechs menschlichen Tau-Isoformen in ihrem intrazellulären Sortierverhalten. Diese unterschiedliche Lokalisierung deutet auf unterschiedliche Rollen dieser Isoformen bei der Funktion von Tau in Gesundheit und Krankheit hin. Die genaue Funktion der sechs menschlichen Tau-Isoformen ist nach wie vor unbekannt, aber frühere Studien deuten auf eine isoform-spezifische Rolle bei der Tau-vermittelten neuronalen Toxizität hin. Darüber hinaus können Tauopathien bereits durch Veränderungen im Spleißen des Tau-kodierenden MAPT-Gens verursacht werden, was zu einer abweichenden Expression der Tau-Isoform führt. Daher sind wir der festen Überzeugung, dass mehr Erkenntnisse über die isoform-spezifischen Funktionen in Gesundheit und Krankheit neue therapeutische Möglichkeiten eröffnen könnten.

In dieser Studie wollten wir i) die Bedeutung des anterograden Transports für eine effiziente axonale Tau-Sortierung bestimmen, ii) Domänen, Motive und Bindungspartner von Tau entschlüsseln, die am axonalen Tau-Sortierungsprozess beteiligt sind, und iii) sortierungsspezifische Bindungspartner zwischen einzelnen Tau-Isoformen identifizieren, die auf unterschiedliche Rollen der Tau-Isoformen hinweisen könnten.

Um diese Fragen zu beantworten, haben wir zwei menschliche neuronale Modellsysteme entwickelt: Aus der menschlichen Neuroblastom-Zelllinie SH-SY5Y abgeleitete Neuronen und

aus menschlichen induzierten pluripotenten Stammzellen (iPSC) abgeleitete glutamaterge Neuronen mit einem Neurogenin 2 (*Ngn2*)-Transgen.

Zunächst haben wir die Rolle des anterograden Transports von somatischem Tau bei der axonalen Sortierung untersucht. Zu diesem Zweck schnitten wir das proximale Axon einzelner iPSC-abgeleiteter und primärer Mausneuronen mit einem hochenergetischen UV-Laser durch und quantifizierten das Ausmaß der somatischen Tau-Akkumulation und AT8-Phosphorylierung bei Axonverlust. In beiden Zellmodellen fanden wir nach der Axotomie keine Anzeichen für eine akute Tau-Pathologie, sondern eher eine leichte Verringerung der Tau-Spiegel und der AT8-Phosphorylierung zu einigen der getesteten Zeitpunkte. Diese Ergebnisse deuten darauf hin, dass die Beeinträchtigung des anterograden Transports aufgrund des Axonverlusts nicht zu einer offensichtlichen Tau-Pathologie führt. Wir argumentieren, dass diese Ergebnisse entweder auf eine untergeordnete Rolle des anterograden Transports von somatisch synthetisiertem Tau hinweisen oder die Fähigkeit der geschädigten Zelle unterstreichen, die Tau-Akkumulation zu verhindern.

Als Nächstes wollten wir die Domänen und Motive des Tau-Proteins entschlüsseln, die für eine erfolgreiche Tau-Sortierung erforderlich sind. Wir erstellten eine Bibliothek mutierter HA-markierter Tau-Konstrukte und wollten ihr Sortierverhalten analysieren. Zunächst mussten wir die Voraussetzungen für eine effiziente axonale Sortierung von exogenem Tau schaffen, ein Problem, das in früheren Studien häufig auftrat. Wir verwendeten einen kürzlich hergestellten *MAPT*-Knockout (*MAPT*-KO) Klon unserer iPSC-Linie und erreichten eine endogen-ähnliche Sortierung von exogenem 0N3R-Tau durch lentiviralen Gentransfer und eine Doxycyclin-induzierbare Promotorexpression. So konnten wir das Sortierverhalten unserer mutierten Tau-Konstrukte in *MAPT*-KO-iPSC-Neuronen analysieren. Wir beobachteten, dass große Trunkierungen die axonale Sortierung beeinträchtigen, z. B. akkumulierte die N-terminale Hälfte von Tau im Soma und bildete punktförmige Einschlüsse, die bei allen anderen Konstrukten nicht vorhanden waren.

Unsere Ergebnisse zeigten, dass die prolinreiche Region 2 (PRR2) von Tau für eine effiziente Tau-Sortierung erforderlich ist. Im Gegensatz zu früheren Studien zeigten wir, dass die effiziente axonale Sortierung unabhängig vom N-terminalen Schwanz von Tau und der allgemeinen Mikrotubuli-Affinität von Tau ist. Wir stellen die Hypothese auf, dass die PRR2-Domäne entweder für die Vermittlung von sortierungsbezogenen Tau-Interaktionen entscheidend ist oder dass sie für die Sortierung von Tau verantwortlich ist.

Wir haben die TurboID-basierte Proximity-Markierung verwendet, um das Interaktom von axonal sortiertem Tau und der nicht sortierten N-terminalen Hälfte zu vergleichen. Während die Interaktionen der N-terminalen Hälfte auf eine Akkumulation im peroxisomalen Lumen hindeuten, fanden wir nur einige spezifische Bindungspartner für die sortierenden Tau-Isoformen. Das Chaperon HSP110 aktiviert die wichtigste Tau-Phosphatase PP2A und könnte

die axonale Anreicherung durch PP2A-vermittelte Dephosphorylierung von Tau fördern, obwohl unsere vorherige Sortieranalyse gezeigt hat, dass die Tau-Mobilität keinen nachweisbaren Einfluss auf die axonale Anreicherung hat.

Als wir die Interaktome der Tau-Isoformen 0N3R und 0N4R analysierten, identifizierten wir 0N4R-spezifische Interaktoren, die an der Regulierung des präsynaptischen Vesikeltransports und der postsynaptischen Plastizität beteiligt sind. Zwei 0N4R-assoziierte Signalwege, die CDC42-Kaskade und die RAB11-Proteinsignalisierung, sind an der AD-Pathogenese beteiligt. Wir haben also Hinweise darauf gefunden, dass 0N4R- oder 4R-Isoformen im Allgemeinen an der synaptischen Funktion bei Gesundheit und Krankheit beteiligt sein könnten.

Zusammenfassend lässt sich sagen, dass unsere Studie Beweise für eine PRR2-abhängige axonale Tau-Sortierung unabhängig vom N-terminalen Schwanz und der Mikrotubuli-Affinität von Tau liefert. Wir behaupten, dass der anterograde Tau-Transport für eine effiziente axonale Sortierung relevanter ist als die axonale Retention. Unsere isoform-spezifische Interaktomanalyse ergab, dass 0N4R-Tau mit Regulatoren der prä- und postsynaptischen Aktivität assoziiert ist, die teilweise an der AD-Pathologie beteiligt sind. Unsere Arbeit deutet somit auf eine isoform-spezifische Rolle von Tau bei der synaptischen Funktion in Gesundheit und Krankheit hin. Unsere Ergebnisse ebnen den Weg für die Entwicklung maßgeschneiderter Behandlungsansätze, die darauf abzielen, i) die Tau-Fehlsortierung und ihre schädlichen Auswirkungen zu verhindern oder zu verbessern und ii) die Tau-Isoformen ins Visier zu nehmen, die für Tau-induzierte synaptische und neuronale Dysfunktion verantwortlich sind.

Summary

Alzheimer's disease (AD) constitutes a major burden to modern health care systems. Pathological alterations of the microtubule-binding protein Tau are a major hallmark of AD and many other disorders, summarized as tauopathies. Under healthy conditions, Tau regulates the dynamic assembly of axonal microtubule filaments depending on its phosphorylation state, and thus affects essential neuronal functions. Somatodendritic missorting of Tau is an early sign of Tau pathology in AD. Despite the intricate role of intracellular Tau mislocalization under pathological conditions, the mechanisms of axonal Tau sorting and somatodendritic missorting are still poorly understood. Several processes were proposed to affect efficient axonal Tau sorting, including anterograde protein transport or retrograde axonal retention, but their contribution remains elusive. There is also little known about Tau domains, motifs or cellular interaction partners that are required for successful Tau sorting. More knowledge about the underlying processes of axonal Tau sorting would help to develop treatments that can restore physiological sorting in disease conditions and thus attenuate the neurotoxic effects of Tau missorting.

Interestingly, the six human Tau isoforms differ in their intracellular sorting behaviour. This distinct localization hint at differential roles of these isoforms in Tau function under health and disease. The exact function of six human Tau isoforms remains still unknown, but previous studies suggested isoform-specific roles in Tau-mediated neuronal toxicity. In addition, tauopathies can be caused already by changes in splicing of the Tau-encoding *MAPT* gene, resulting in aberrant Tau isoform expression. Thus, we strongly believe that more insights into isoform-specific functions in health and disease could open up new therapeutic avenues.

In this study, we aimed i) to determine the relevance of anterograde transport for efficient axonal Tau sorting, ii) to unravel domains, motifs, and binding partners of Tau that are involved in the axonal Tau sorting process, and iii) to identify sorting-specific binding partners between individual Tau isoforms that may hint at distinct roles of Tau isoforms.

To address these questions, we established two human neuronal model systems: Human neuroblastoma SH-SY5Y cell line-derived neurons and neurogenin 2 (*Ngn2*)-transgenic human induced pluripotent stem cell (iPSC)-derived glutamatergic neurons.

First, we assessed the role of anterograde transport of somatic Tau in axonal sorting. To this end, we cut the proximal axon of single iPSC-derived and mouse primary neurons with a high-energy UV laser and quantified the levels of somatic Tau accumulation and AT8 phosphorylation upon axon loss. Consistent for both cell models, we found no signs for acute Tau pathology upon axotomy but rather mild reductions of both Tau levels and AT8 phosphorylation at some of the tested time points. These results indicate that impaired anterograde transit due to axon loss does not lead to overt Tau pathology. We argue that these

findings either indicate a minor role for anterograde transport of somatically synthesized Tau or underline the capacity of the damaged cell to prevent Tau accumulation.

Next, we aimed to unravel domains and motifs of the Tau protein that are required for successful Tau sorting. We generated a library of mutant HA-tagged Tau constructs and aimed to analyse their sorting behaviour. First, we had to establish a setup for efficient axonal sorting of exogenous Tau, an often-faced problem in previous studies. We used a recently engineered *MAPT*-knock out (*MAPT*-KO) clone of our iPSC line, and achieved endogenous-like sorting of exogenous 0N3R-Tau with lentiviral gene delivery and a doxycycline-inducible promoter expression. Thus, we were able to analyse the sorting behaviour of our mutant Tau constructs in *MAPT*-KO iPSC-derived neurons. We observed that large truncations compromise axonal sorting, for instance the Tau N-terminal half accumulated in the soma and formed puncta-like inclusions that were absent for all other constructs.

Our results showed that the proline-rich region 2 (PRR2) of Tau is required for efficient Tau sorting. In contrast to previous studies, we showed that efficient axonal sorting is independent of the Tau N-terminal tail and the general microtubule affinity of Tau. We hypothesize that the PRR2 domain is either crucial for mediating sorting-related Tau interactions or

We used TurboID-based proximity labelling to compare the interactome of axonally sorted Tau and the non-sorting N-terminal half. While the interactions of the N-terminal half suggested accumulation inside the peroxisomal lumen, we found only several binding partners specific for the sorting Tau isoforms. The chaperone HSP110 activates the major Tau phosphatase PP2A and could promote axonal retention by PP2A-mediated dephosphorylation of Tau, even though our previous sorting analysis showed that Tau mobility had no detectable effect on axonal enrichment.

When we analysed the interactomes of the Tau isoforms 0N3R and 0N4R, we identified 0N4R-specific interactors that are involved in the regulation of presynaptic vesicle trafficking and postsynaptic plasticity. Two 0N4R-associated pathways, the CDC42 cascade and RAB11 protein signalling are implicated in AD pathogenesis. Thus, we found evidence that 0N4R or 4R isoforms in general may be involved in synaptic function in health and disease.

In conclusion, our study provides evidence for PRR2-dependent axonal Tau sorting independent of the N-terminal tail and the microtubule affinity of Tau. We claim that anterograde Tau transport is more relevant for efficient axonal sorting than axonal retention. Our isoform-specific interactome analysis revealed that 0N4R-Tau is associated with regulators of pre- and postsynaptic activity partially involved in AD pathology. Thus, our work hints at an isoform-specific role of Tau in synaptic function in health and disease. Our findings pave the path for developing tailored treatment approaches that aim to i) prevent or ameliorate Tau missorting and its detrimental effects, and ii) target Tau isoforms responsible for Tau-induced synaptic and neuronal dysfunction.

1 Introduction

Neurodegenerative diseases such as dementia represent a major burden of modern societies and their health care systems [1]. Accompanied by an ageing population, the number of patients suffering from dementia-related syndromes increases constantly and will reach the tremendous number of over 100 million affected worldwide in 2050, according to recent projections [2]. The risk of developing dementia or other forms of neurodegenerative diseases strongly correlates with age. In Germany, 1 to 2 out of 100 individuals are affected by dementia at the age of 65 years, but more than 15 out of 100 individuals at an age of 80 years [3]. With more than 70 % of total cases, Alzheimer's disease (AD) is the most common type of dementia [4] Huge financial and research efforts are undertaken to unravel the complex AD pathomechanisms and to design effective therapeutics that can ameliorate AD-associated symptoms like cognitive decline, memory loss, or behavioural changes [4].

More than 100 years ago, insoluble aggregates called neurofibrillary tangles (NFT) were identified as one major histopathological hallmark of AD patient brains [5-7]. In the late eighties and early nineties of the 20th century, multiple studies identified the microtubule-associated protein Tau as the major component of NFTs [8-21]. The formation of Tau-containing NFTs follows a cascade of pathological Tau alterations, including hyperphosphorylation and intracellular mislocalization of axonal Tau [22, 23]. This Tau alterations are not only a secondary effect in AD but constitutes an integral driver of AD-associated pathology [22-24].

1.1 Tauopathies

AD shares Tau pathology as a feature with more than 20 neurodegenerative diseases that are summarized as ‘tauopathies’ due to their common pathological hallmark [25].
The group of tauopathies comprises a heterogeneous set of neurodegenerative diseases with diverse aetiological and pathophysiological modalities [23, 26]. One way of classifying them is by the role of Tau within the disease cascade [27-29]. In primary tauopathies, such as progressive supranuclear palsy (PSP) or Pick’s disease (PD), detrimental changes of Tau are the predominant neuropathological feature [28]. The majority of Tau-related diseases, however, belongs to the group of secondary tauopathies, in which Tau pathology is accompanied by other pathological features, often involving other aggregate-forming proteins like amyloid-beta (Aβ) in AD cases, or α-synuclein in Parkinson’s disease (PD), respectively [23, 29-31].
Another way to categorize tauopathies refers to the underlying aetiology, so whether the disease occurs after external mechanical trauma, due to genetic mutations, or sporadically [26, 32].

1.1.1 Trauma-caused tauopathies

Mechanical head trauma can cause severe Tau pathology with neuropathological features that are distinct from other tauopathies [33]. A single traumatic event can lead to the development of traumatic brain injury (TBI), characterized by severe axonal injury at the impact site [34, 35]. Accumulation of abnormally phosphorylated Tau protein becomes visible within days after the insult, and widespread NFT formation occurs within several years after the trauma [36].
Different from TBI, chronic traumatic encephalopathy (CTE) is associated with repetitive mechanical head trauma like concussions or blasts. The patients suffering from CTE, such as professional boxers [37], football players [38], or soldiers [39], exhibit widespread Tau pathology throughout the brain [33, 37, 39-46]
The nature of Tau pathology in trauma-based tauopathies is quite particular. Prominent NFT formation in CTE patients occurs in neurons of perivascular and periventricular brain regions, or within astrocytes as astrocytic tangles [33, 43]. Further, CTE-associated Tau fibrils have a unique molecular configuration that differs from NFTs found in AD or other tauopathies [47, 48]. Data from TBI mouse models revealed Tau aggregation not only in the somatodendritic part of affected neurons, as typically seen in tauopathies, but also in the axons [49].
Although patients with trauma-caused tauopathies comprise only a small subset of all tauopathy cases, the need for more suitable model systems is high. The rising awareness for

this form of sports- and profession-related neurodegeneration, and the unique Tau pathology make more tailored treatment approaches desirable.

1.1.2 Genetic tauopathies

While the majority of tauopathy cases occur sporadically, there is a considerable subset of tauopathies that can be caused by genetic variants [23, 26, 50].
More than 20 genes were reported to be associated with various tauopathies [51], and many more genes are known to correlate with the disease risk [52-57]. For the Tau-encoding *MAPT* gene, at least 50 pathogenic variants are described [58], These variants usually manifest in a form of frontotemporal dementia (FTD), the FTD with parkinsonism linked to chromosome 17 (FTDP-17) [59, 60] or, less frequently, cause the development of other primary tauopathies such as PD or PSP [61-65]. These *MAPT* mutations typically constitute missense variants or deletions that cause perturbed Tau protein function [66-69]. Studies in transgenic mice and human-derived neurons demonstrated that FTDP-17-associated mutant Tau shows aberrant cellular distribution and impairs neuronal plasticity [70]. Many *MAPT* mutations have no direct impact on protein function but disrupt the pre-mRNA splicing and thereby unbalance the Tau isoform expression pattern [71-79] or elevate the aggregation propensity of Tau [80-83].
In the case of AD, up to 95 % of cases occur sporadically without a causal gene variant [84, 85]. The small fraction of familial AD cases are not caused by mutations in the MAPT gene [23] but by pathogenic variants in the amyloid precursor protein (*APP*) gene or the APP-processing related genes presenilin (*PSEN*) 1 and 2 [31, 50, 86, 87].
In sum, genetic tauopathies appear diverse in both the underlying genetic cause and the resulting disease phenotype. Patients with pathogenic *MAPT* mutations develop primary tauopathies, mainly FTDP-17, while familial forms of AD are caused by variants in APP and APP-related genes *PSEN1* and *PSEN2*.

1.1.3 Sporadic tauopathies/AD

In numbers of patients, sporadic tauopathies are by far the most prevalent form [26]. Most primary tauopathies such as PSP, PD or argyrophilic grain disease are dominated by sporadic cases but the vast majority of sporadic tauopathy patients suffer from AD [23, 88-90].
The prevalence of sporadic AD strongly correlates with increasing age [91, 92]. Besides age, there are numerous other risk factors that are associated with AD including genetic variants like the apolipoprotein E (*APOE)* allele ε4 [93-97], co-morbidities like obesity, diabetes or viral infections, and behavioural factors like smoking or physical inactivity [30, 31, 98].
Although clinical AD symptoms typically develop in advanced age, molecular alterations can be detected many years before disease onset [30, 31]. These preclinical changes are manifold

and comprise overactivation of brain astrocytes and microglial cells leading to increased inflammatory signalling, and impairments of blood vessel and blood-brain barrier function [31, 99-103]. Abnormal levels of Aβ peptides are found years before disease onset [104, 105].
Two large cohort studies on non-selective autopsy cases revealed that initial signs of Tau pathology are visible in up to 25 % of individuals under 30 years [106, 107]. These so-called pre-tangles consist of AD-like hyperphosphorylated and missorted Tau, and they have already been found even in children before puberty [106]. Although it is a matter of debate whether these pre-tangles necessarily lead to Tau aggregation and NFT formation [108, 109], their tremendous prevalence highlights the early-onset Tau pathology within a decade-long clinically silent phase of AD. This is confirmed by cerebrospinal fluid (CSF) analysis that showed enhanced levels of hyperphosphorylated Tau species a decade prior the onset of clinical symptoms [110, 111].
Although major symptoms of AD like memory loss or cognitive impairments arise from hippocampal and cortical neurodegeneration [112], other brain areas are earlier affected by Tau pathology. Especially subcortical nuclei with projection neurons spanning throughout large parts of the brain show initial pre-tangle and tangle formation [106, 108, 112-117]. It is unclear why these neuronal subtypes are more vulnerable to Tau pathology but their high energy demand and enhanced exposure to humoral toxins and pathogens are risk factors that are being discussed [118]. The subcortical origin of pathology attracts growing interest in the field due to its potential role in a recently discovered phenomenon: spreading of pathogenic Tau between brain areas [119-125].
The chronology of Tau pathology affecting certain brain regions follows a well-characterized stereotypical pattern that, in contrast to Aβ pathology, correlates with the symptom severity [126-129]. The significant role of Tau in AD progression is underlined by studies with *Mapt* knockout mouse models, in which Tau depletion had protective effects as it prevented or ameliorated Aβ-induced neurodegeneration and memory impairments [130-136]. Tau-deficient primary rodent cultures were resistant against Aβ-induced synapse loss and axonal transport deficits [136-138]. Recent data from *MAPT*-KO human induced pluripotent stem cell (iPSC)-derived neurons corroborate the notion that Tau is a key driver of AD-related pathogenesis [139, 140].

Taken together, Tau-related neurodegenerative disease can be caused by external mechanical trauma or pathogenic variants in the Tau-encoding *MAPT* gene or many other genes. The vast majority of tauopathy patients suffer from sporadic cases, with age-related sporadic AD as the prototypic tauopathy. Although AD belongs to the secondary tauopathies, Tau pathology constitutes a key driver of AD development and the burden of Tau pathology correlates well with symptom severity in AD. Somatodendritic missorting and

hyperphosphorylation of Tau are key hallmarks of Tau pathology on the molecular level and occur already in preclinical disease stages. Thus, ameliorating these early signs of Tau pathology prior to widespread neuron loss is a promising target for therapeutic approaches. However, in order to prevent intracellular Tau missorting *in vivo*, it is crucial to first unravel the mechanisms that ensure efficient Tau sorting under physiological conditions.

1.2 Physiological role of Tau

1.2.1 Genetic background and Tau isoforms

The Tau protein is encoded by the *MAPT* gene located on chromosome 17 [141]. Two haplotypes of the *MAPT* gene locus are present with different frequencies among populations. The haplotype H2 is less common in the European population with ~20 % frequency, and it differs from the more frequent H1 haplotype by several SNPs, small deletions, and most significantly the complete inversion of the gene locus [142-147]. Notably, the H1 haplotype was reported to be a risk factor for the development of PSP and other tauopathies [142, 148].
The expression of *MAPT* is heavily regulated by alternative splicing mechanisms [11, 149-154]. While various sets of Tau isoforms are expressed in neurons of the peripheral nervous system [155-157], in glial cells [158-162], and other non-neuronal tissues like heart, pancreas, kidney or skeletal muscles [153, 163-175], six major isoforms are expressed in adult brain neurons [11, 176, 177]. These six brain isoforms derive from alternative exon skipping of the exons 2 and 3, affecting the inclusion of the N-terminal inserts N1 and N2, and of exon 10, controlling the inclusion of the C-terminal repeat domain R2. The resulting isoforms are defined by the number of inserts (0N, 1N, or 2N) and the repeat domains (3R or 4R) (Figure 1) [11].
The relative abundance of the isoforms varies in different brain regions, but 1N isoforms are usually more abundant than 0N and 2N isoforms [178-180]. The ratio of 3R and 4R isoforms is roughly equal among all brain regions [11, 181]. Notably, many disease-associated Tau mutations affect the alternative splicing of exon 10 and thereby disrupt the physiological 3R/4R Tau ratio, hinting at differential isoform functions [71, 182-184]. Recent data from *in vitro* studies corroborate the idea that the Tau isoforms fulfil distinct physiological functions, and also provided evidence for isoform-specific differences in susceptibility to AD and other tauopathies [185-189]. However, knowledge about isoform-specific interaction partners that could underlie functional differences, is still limited and requires further research efforts.

1.2.2 Structural-functional organization of Tau

Under healthy conditions, Tau is highly abundant in the axonal compartment [190, 191] and found in much smaller amounts in the soma, nucleus and dendrites [166, 187, 192-195]. Axonal Tau binds to microtubule filaments and thereby regulates their stability [177, 196-199]. The fast kinetics of binding and dissociation of Tau [200] modulate the dynamic assembly of axonal microtubule filaments. This is critical to ensure both mechanical stiffness and plasticity, probably via promoting the assembly of long labile domains [201]. By regulating microtubule dynamics, Tau is involved in many essential cellular processes, such as the neuronal differentiation and polarity, axon outgrowth and plasticity, and axonal transport by microtubule-associated motor proteins like dynein and kinesin [191, 202-213].

While two different microtubule binding sites were described for Tau [197, 214-216], it is known that the C-terminal repeat domains constitute the core binding domain (Figure 1) and interact with the taxol-binding site of β-tubulin [217]. Isoforms with four repeat domains exhibit (4R) more efficient microtubule binding than 3R isoforms, likely due to the additional repeat domain [218]. Notably, the binding capacity of the repeat domains alone is comparably weak. *In vitro* binding studies with recombinant Tau species showed that the presence of adjacent domains, namely the C-terminal pseudo-repeat (R') and especially the N-terminal proline-rich regions 1 and 2 (PRR1 and PRR2) ensures efficient microtubule interaction [199, 217]. Interestingly, truncated Tau without repeat domains was binding to microtubule much better than the repeat domains alone [199].

Tau undergoes a wide range of PTMs [219-224], of which phosphorylation is the most prevalent form [22, 23, 225-227]. The phosphorylation state of Tau directly affects its microtubule-binding function [176, 228, 229]. Generally, higher phosphorylation of Tau decreases its microtubule-binding affinity [221, 230-233]. Hyperphosphorylation of motifs within the PRR domain such as AT8 (S199, S202, and T205) or AT180 (T231 and S235) are associated with AD pathology (Figure 1) [234, 235], and antibodies recognizing the AT8 motif are used to identify early pre-tangle formation [236]. After somatodendritic missorting, strong phosphorylation of the 12E8 epitope (S262, S293, S324, S356) within the repeat domains is detectable (Figure 1) [237-240].

Tau phosphorylation is tightly controlled by a large group of Tau-targeting kinases and phosphatases [228, 241]. Accordingly, aberrant activity of several Tau-targeting kinases such as glycogen synthase kinase 3-beta (GSK-3β), cyclin-dependent kinase 5 (CDK5), and microtubule affinity-regulating kinases (MARKs) correlates with Tau phosphorylation, mislocalization and NFT burden [242-245]. The activity of protein phosphatase 2A (PP2A), the most important Tau phosphatase [23], and other phosphatases such as PP5 is significantly reduced in AD brains [246-248].

Besides phosphorylation, many other posttranslational modifications are found to regulate Tau function and pathology and thereby illustrate the complex interplay of posttranslational modifications [23]. For instance, recent studies showed that acetylated Tau is generally abundant in dendrites and more prone to aggregation [137, 249, 250] except for an aggregation-protective acetylation site in repeat domain 2 [251].
When Tau is bound to microtubules, the N-terminal half of the protein projects away from the microtubule filament [206, 214, 217, 252]. This so-called projection domain (Figure 1) acts as a spacer, helps to organize the spatial order of adjacent microtubule filaments [253], and links microtubule strands to the plasma membrane via direct interactions with annexin 2 and other membrane components [66, 209, 254].

In brief, Tau is a mainly axonal protein that dynamically regulates the stability of axonal microtubules and thereby contributes to major cellular functions. The C-terminal half of Tau comprises the microtubule-binding domain with the central repeat domains and the PRR and pseudo-repeat as flanking domains. The N-terminal half controls spacing of adjacent microtubules and acts as plasma membrane linker. The microtubule-binding affinity of Tau is controlled by various PTMs, especially phosphorylation. High levels of phosphorylation decrease the microtubule binding, and hyperphosphorylation within the AT8 or KXGS motifs are linked to Tau pathology in AD. This Tau hyperphosphorylation coincides with decreased axonal sorting and increased somatodendritic missorting.

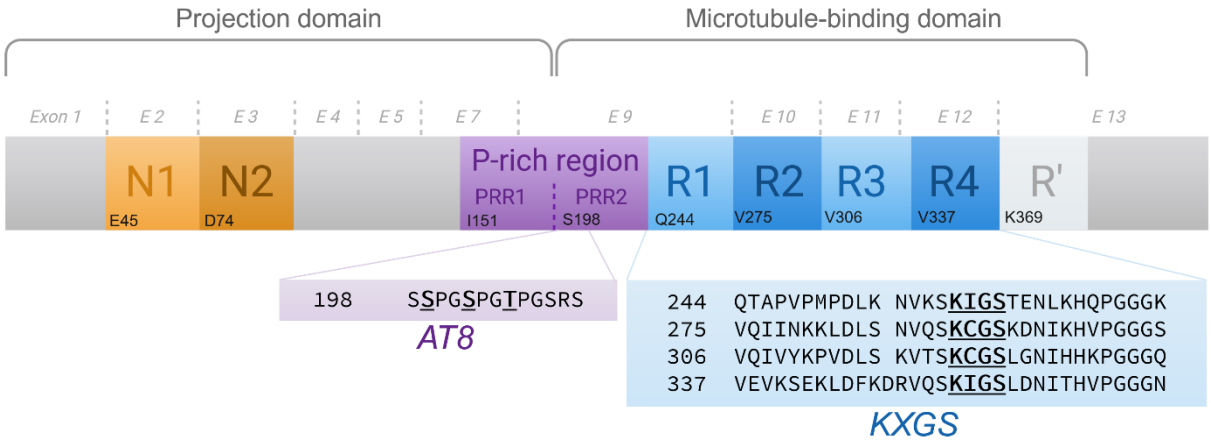

Figure 1: Domain structure and phosphorylation sites of the longest Tau isoform 2N4R.

The N-terminal half with the alternatively spliced inserts N1 (only 1N and 2N Tau) and N2 (only 2N Tau) comprises the projection domain that regulates Tau interactions and spacing of adjacent microtubule filaments. The C-terminal repeat domains R1 to R4 (for 3R Tau: no R2) constitute the core of the microtubule-binding domain. The flanking domains, namely the proline-rich region 2 (PRR2) and the pseudo-repeat domain R' contribute to the high microtubule affinity of Tau. The phosphorylation motifs AT8 (with Ser199, Ser202, Thr205, underlined in violet box) within the PRR2 and KXGS (with Ser262, Ser293, Ser324, Ser356, underlined in the blue box) within the repeat domains are associated with pathological Tau hyperphosphorylation and missorting as seen in early AD disease stages. The grey dotted lines indicate the borders of *MAPT* exons, the numbers indicate the respective exon. The first amino acid and its position in the 2N4R-Tau isoform are given by the small bottom numbers. Domain widths are proportional to the number of amino acids.

1.2.3 Mechanisms of axonal Tau sorting

Efficient axonal targeting of Tau is crucial for normal neuronal function since Tau missorting is an early sign of pathology in AD and related disease. Several mechanisms were proposed to contribute to the process of axonal Tau sorting (

Figure 2) but their relevance and underlying mechanisms are still not well understood [22, 255].

1.2.3.1 RNA-based sorting models

Findings from primary rat neurons suggest selective axonal targeting of the Tau mRNA via an axon targeting sequence in the 3'-UTR [256]. Indeed, larger amounts of Tau mRNA are visible within the proximal axon [257], and by exchanging the Tau 3'-UTR with the dendritically localizing microtubule-associated protein MAP2, Tau mRNA was erroneously redirected into the dendrite [256]. However, Tau mRNA is also found in the proximal dendrite [258, 259] and, more importantly, successful axonal sorting was seen even when Tau mRNA was lacking the 3'-UTR [185] or when Tau protein was microinjected into the cytoplasm [260], indicating a minor role for axonal targeting of Tau mRNA.

Besides axonal targeting of Tau mRNA, also preferential translation of axonal Tau mRNA was reported. Adding 5'-UTR of Tau was sufficient to induce translation of a reporter protein in the axons of young primary neurons [261]. Evidence for this mechanism in mature neurons is still missing, and the efficient sorting of recombinant Tau [185, 260] as well as local Tau translation within the dendrites [258] argues against a major role for Tau mRNA.

Taken together, there is evidence for both axonal targeting and translation of Tau mRNA, but the relevance of mRNA-based sorting for the polarization of Tau protein in mature neurons is probably minor.

1.2.3.2 Sorting by preferential degradation

There are several protein-based hypotheses describing how the Tau may be selectively enriched in the axonal compartment. Among those, the idea of enhanced dendritic degradation of Tau was supported by recent data. While pharmacological inhibition of proteasomal and autophagic degradation machineries leads to elevated levels of dendritic Tau, boosting of degradation pathways substantially reduces the amount of dendritic Tau [258]. It was hypothesized that, likely due to differential patterns of PTMs [262, 263], differential degradation of axonal and dendritic Tau could rely on compartment-specific interactions with degradation pathways [264-267]. In contrast, studies with traceable Tau species observed axonal redistribution of somatodendritic Tau instead of enhanced degradation [268].

1.2.3.3 Anterograde Tau transport

Besides protein degradation, the anterograde transport of Tau protein is another possible scenario. Free diffusion of Tau between compartments [269] and transport of Tau along microtubules at rates typical for slow motor-driven protein transport were observed [270-272]. Direct interaction of Tau with the light chains 1 and 2 of kinesin-1 was reported, and disruption of this interaction led to somatic Tau accumulation [273]. Accordingly, integrity of microtubule assembly appears critical for axonal Tau sorting as treatment with nocodazole, a drug that prevents polymerization of new microtubule filaments impaired anterograde Tau sorting in primary murine neurons [138, 268].

The measured rates of motor-driven transport are too slow to explain the efficient anterograde transit of large amounts of protein [274]. It was shown that over short distances, Tau can utilize the microtubule lattice to rapidly propagate towards the microtubule plus-end [274]. Since microtubule filaments appear highly polarized in the proximal axon [275], this unidirectional sliding would allow the transit of Tau into the axon [276].

The model of microtubule-interaction driven Tau transport has a major constraint: The high turnover rates of microtubule filaments at the axon initial segment (AIS) [185] question how efficient active transport can be achieved with a lack of stable microtubule filaments.

1.2.3.4 Axonal retention of Tau

Experiments with fixed neurons showed that Tau binds to axonal microtubules with much higher affinity than to dendritic microtubules [198]. Thus, increased axon-specific microtubule interaction of Tau could trap axonal Tau and lead to less retrograde than anterograde Tau diffusion, resulting in a net enrichment within the axon [255]. microtubule-binding affinity of Tau is negatively regulated by its phosphorylation state, which makes it less diffusible [221, 230-233]. Indeed, the phosphorylation of the KXGS motif and other sites is reduced in axonal Tau [238], resulting in stronger microtubule interaction while dendritic Tau appears highly phosphorylated and less stable in microtubule interactions [277]. The compartment-specific phosphorylation states are likely mediated by the different subcellular localizations of Tau-targeting kinases and phosphatases [23]. While PP2a is highly abundant in the axon [278], kinases like GSK-3β or different MARKs are primarily localized in the dendrites and soma [239, 279, 280].

However, recent data demonstrate that Tau shows efficient axonal Tau sorting even when the microtubule binding affinity is strongly decreased by depletion of the repeat domains [281]. Thus, axonal retention of Tau is either less relevant than previously claimed or it is mediated by domains that are not involved in microtubule binding. In fact, studies with truncated Tau suggest a key role of the interaction between the N-terminal tail of Tau and membrane-bound

annexins A2 and A6 for axonal localization as loss of this interaction caused higher somatic Tau levels [282].

1.2.3.5 Retrograde Tau diffusion barrier

Studies with traceable Tau species in primary murine cultures revealed another mechanism of axonal Tau retention that depends on the AIS rather than on interaction with axonal microtubules [185, 268].

The AIS comprises a complex network of ion channels, scaffold proteins, and cytoskeletal elements such as circular and patched filamentous actin (f-actin), and microtubule filaments [283-287], orchestrated by the master regulator Ankyrin G (ANKG) [288, 289],. The AIS-located microtubules are bundled in dense fascicles with the plus-end facing towards the axon [275, 290, 291]. This uniform orientation of microtubules depends on interactions with the AIS-specific tripartite motif-containing protein 46 (TRIM46) [275]. The AIS establishes and maintains neuronal polarity by controlling all forms of axonal transit [285-287]. Uncontrolled diffusion of lipids and membrane proteins is impeded, and erroneous transport of somatic proteins is blocked by f-actin-dependent filtering [283, 292-294]

In the above-mentioned studies, the retrograde diffusion of Tau was selectively impaired at the AIS while diffusion of other proteins was unaffected [185, 268]. When AIS microtubules were destabilized with nocodazole treatment, large amounts of axonal Tau penetrated the soma whereas stabilization of microtubules with taxol prevented Aβ-induced Tau missorting [185]. Disruption of the f-actin network with latrunculin A did not cause retrograde Tau missorting [185, 268]. Accordingly, TRIM46-depleted primary neurons lacked normal microtubule orientation at the AIS, which resulted in somatodendritic Tau missorting but not in defects of anterograde cargo transport [275]. These findings show that the retrograde Tau diffusion barrier (TDB) is independent of f-actin integrity [268], but it rather relies on polymerized microtubules.

This observation rises the questions about the underlying mechanisms of this Tau-selective barrier. Although the TDB permeability was changed for larger Tau isoforms, the protein size appears to be rather irrelevant as mutant versions of the 2N4R-Tau reverted the observed size-dependent effects [185]. It is more plausible that Tau shows specific interactions with the microtubule filaments within the AIS that trap retrogradely trafficking Tau inside the dense microtubule bundles.

In fact, the AIS-located microtubules do not only differ from the axonal microtubules in their dynamics but also the modification patterns are distinct [295-297]. This theory is missing a mechanism that prevents continuous accumulation of trapped Tau at the TDB. One possible

explanation is that the constant anterograde transport of single Tau or Tau-tubulin complexes [274, 298] clears out the trapped Tau proteins.

Alternatively, other AIS components that are functionally connected to the microtubule bundles could execute the Tau retention. Knockdown of members of the AIS architecture, such as ANKG, the neuronal cell adhesion molecule (NRCAM), or the end-binding protein 1 (EB1) [285, 286], led to increased permeability of the TDB in primary neurons. However, reduction of these proteins also compromised the general filter function of the AIS [185].

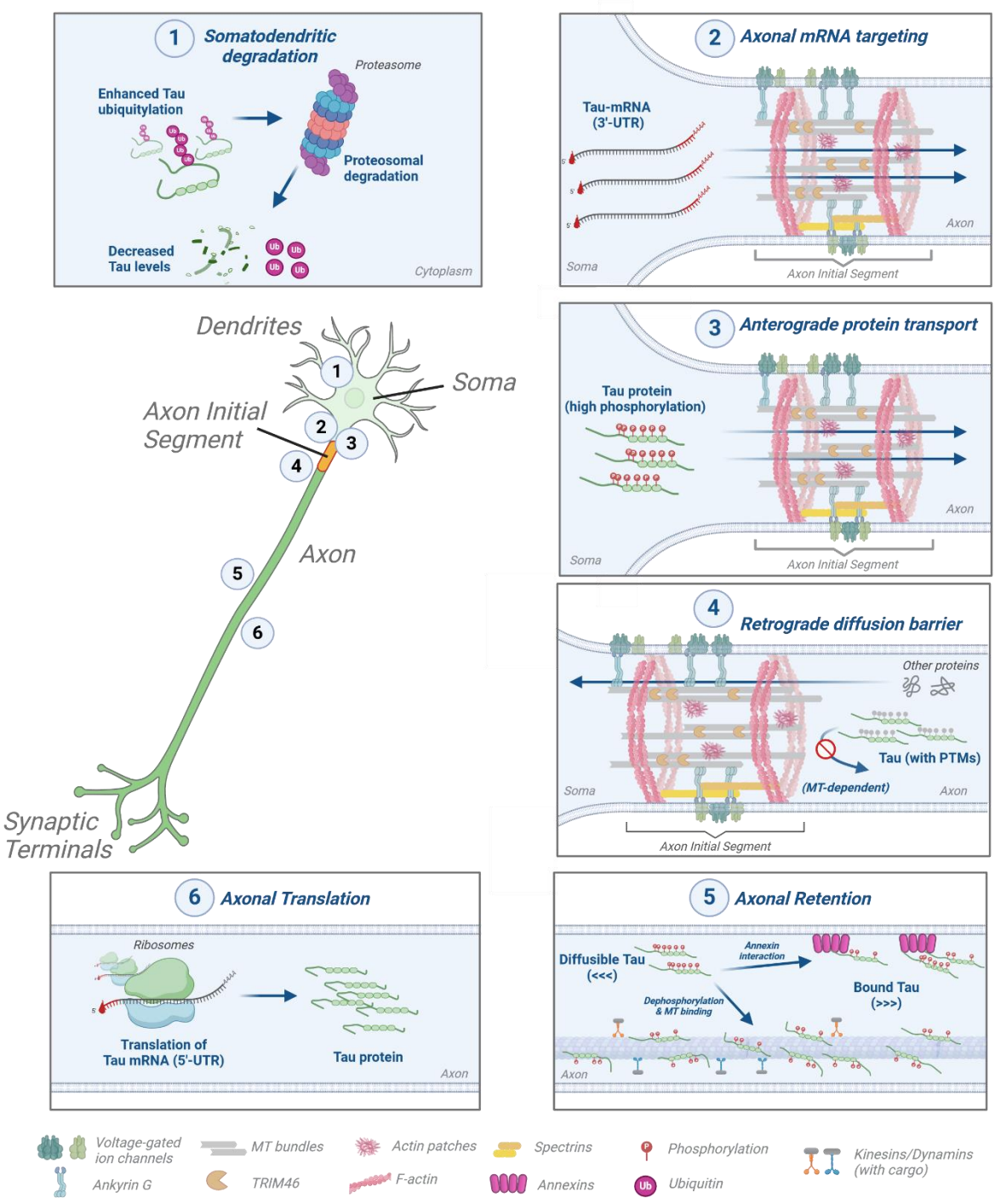

Figure 2: Mechanisms of axonal Tau sorting.

Multiple mechanisms were described to contribute to axonal sorting of Tau in mature brain neurons (middle left, dark green represents higher Tau protein levels). (1) Preferential somatodendritic degradation of Tau protein due to site-specific ubiquitylation and proteasomal degradation. (2) Preferential axonal targeting of Tau mRNA mediated by the 3'-UTR. (3) Anterograde transport of somatic Tau protein. (4) Inhibition of retrograde diffusion by a Tau-selective microtubule-dependent barrier within the axon initial segment (AIS). (5) Axonal retention of Tau due to decreased mobility caused by enhanced microtubule binding and interaction with membrane-bound annexins. (6) Preferential axonal translation of ubiquitously distributed Tau mRNA mediated by the 5'-UTR. See chapter 1.2.3 for literature.

In sum, several mechanisms were postulated to influence axonal Tau sorting (

Figure 2). While the contribution of RNA-based sorting theories seems to be minor, protein-based models appear more relevant. Preferential somatodendritic Tau degradation was postulated as one process to enrich axonal Tau but experiments with traceable showed no increased degradation of Tau in the soma. Anterograde trafficking of Tau protein could occur by free diffusion of or by microtubule-dependent motor transport, but the microtubules of the AIS are highly unstable. Another mechanism would be retention of axonal Tau, possibly by preferential interaction with microtubules or membrane-bound anchors. Axonal retention could also be mediated by the AIS-located selective Tau diffusion barrier, which depends on the phosphorylation state of axonal Tau.

The individual contribution of the described mechanisms to efficient axonal Tau sorting remains unclear. Recent studies with truncated Tau species claim that the N-terminal tail of Tau and the microtubule affinity are involved in axonal targeting [281, 282]. However, these reports lack a systematic evaluation of Tau domains or motifs and their role in Tau sorting. There are also no data available about Tau binding partners that could be crucial for the sorting process, although much effort was undertaken to unravel Tau interactions beyond microtubules and their implications for Tau function.

1.2.4 Tau interactions and non-classical functions

The cellular functions of Tau beyond microtubules are manifold [22, 23, 299, 300] and correlate to the plethora of interaction partners that were described for Tau [301]. There is strong evidence that Tau phosphorylation affects also these non-canonical Tau functions [219, 302], and that disruption of these functions is associated with the development of AD and related tauopathies.

A recent meta-analysis assessed Tau interactome data from multiple studies in humans and rodents, and counted 2081 Tau interactors in total, of which 261 proteins were found in the majority of studies [301]. The interaction data were consistent between studies using brain tissue or cell culture models. In particular, the comparison with the Tau interactome in rodent models revealed considerable differences in the number and function of interaction partners, which questions the transferability of rodent-derived data to the human context. A large subset of human Tau interaction partners comprises Tau-targeting kinases and phosphatases [22, 23]. Apart from some tyrosine kinases, the vast majority of kinases regulating Tau are serine/threonine kinases like the glycogen synthase GSK-3β [303, 304], CDK5 and MARK1-4, which are relevant for phosphorylation of the KXGS motifs [279, 305, 306], and phosphatases such as PP2A, PP5 [246, 307]. Other interactions of Tau are directly related to its main function as microtubule stabilizers, such as the N-terminal binding of membrane components like

annexin 2 or phospholipids [66, 209, 254, 300]. Interactions with other components of the cytoskeleton are reported, such as the microtubule-associated motor proteins kinesin-1 and dynein, f-actin or the light neurofilament [22, 271, 308].
In addition, Tau exhibits are large number of interactions that are not linked to microtubules but to alternative cellular functions. Especially the PRR domain is a common target for binding of various cell signaling proteins, such as Src-family kinases, members of the 14-3-3 protein family, calmodulin, growth factor receptor-bound protein 2 (GRB2), and many others [223, 224, 309-314]. The various interactions with signaling proteins led to the hypothesis that Tau might act as a scaffold for these proteins aiming for subcellular compartmentalization [309].

1.2.4.1 Nuclear Tau

Tau reaches the nuclear region, presumably by a transport mechanism that relies on phosphorylation and other modifications of Tau, possibly O-GlcNac glycosylation [166, 193, 315-317]. Inside the nucleus, Tau enter the nucleolus [318] or directly bind to chromosomal DNA via its PRR domain [315, 319, 320] and regulates genomic stability in a chaperone-like manner [315, 320-324]. Multiple studies showed that Tau is heavily involved in RNA processing, indicated by the fact that heterogeneous nuclear ribonucleoproteins (HNRNPs) and other RNA-binding proteins make up the largest subset of Tau interactors in terms of protein numbers [301, 325-328].

1.2.4.2 Synaptic Tau

Dendritic missorting of hyperphosphorylated Tau is a hallmark of AD-related Tau pathology [137, 329]. However, Tau was shown to be present at synaptic sites also under healthy conditions [302, 309, 329].
It is not fully understood how Tau is guided to the postsynapse, but there is evidence for local translation of dendritic Tau mRNA [329]. Further, the modification profile might play a role in dendritic recruitment [159, 187, 192, 330-332]. There is evidence that synaptic activity triggers the phosphorylation of Tau residues and thereby redirects Tau to the synapse, but the phosphorylated residues are controversial and may depend on the neuron type [219, 302, 332]. When Tau reaches the synapse, it shuttles the Src-family kinase Fyn to NMDA receptors enabling signal transduction. NMDA receptor activation in turn increases the phosphorylation state of Tau, which attenuates the Fyn-Tau binding to the NDMA receptor and thus prevents overactivation [137, 302, 333]. In addition, Tau was shown to be involved in AMPA receptor endocytosis to prevent excitotoxicity after site-specific phosphorylation by GSK3β [302, 334, 335]. Besides its role in modulation of synaptic activity, Tau might also directly influence

synapse plasticity via its interaction with f-actin [336-340], the major cytoskeletal component of postsynaptic spines [341, 342].
The Tau levels at the presynaptic site are rather low under normal conditions [329], despite its high axonal abundance [190, 191]. Presynaptic Tau showed neuronal activity-dependent association with proteins of the SNARE complex including synaptotagmin-1 and MINT1, and with vesicular membrane proteins like synapsin 1 and synaptogyrin-1 [343].

Overall, the Tau interactome is highly diverse and gives rise to multiple cellular functions beyond regulation of microtubule dynamics. Besides binding to many proteins involved in cellular signaling, Tau localizes to the nucleus where it binds DNA, but mainly interacts with members of RNA-binding complexes and thereby influences RNA processing. At the synapse, Tau is thought to modulate synaptic activity of glutamatergic NMDA and AMPA receptors in order to prevent excitotoxicity.

1.3 Tau missorting and pathology in AD

Under healthy conditions, Tau is enriched in the axon where it regulates the dynamic assembly of microtubules. Pathological alterations of the normal Tau function are a key event in AD development [22, 23]. In AD, pathogenic extracellular Aβ peptides induce hyperphosphorylation and somatodendritic missorting of Tau [238, 344]. Aβ-induced synaptic hyperexcitability [131, 135-137]., a key driver of Aβ neurotoxicity [345-348], depends on the presence of postsynaptic Tau [329] Although some studies describe reciprocal effects of Tau and Aβ pathology [349, 350], these findings suggest that Tau acts downstream of Aβ in the pathological cascade of AD.

This observation leads to the question how the Aβ-Tau interplay mediates neuron loss under disease conditions. Extracellular Aβ oligomers and other external stressors disturb the intracellular calcium signaling pathways [237, 238], and thereby lead to abnormal activity of Tau-targeting kinases and phosphatases [237]. As a consequence, Tau gets hyperphosphorylated, primarily at the KXGS epitopes within the repeat domains or at the AT8 motif within the PRR2 domain [238-240], and becomes enriched in the somatodendritic compartment. In the dendrites, hyperphosphorylated Tau is recruits the tyrosine kinase Fyn to the postsynaptic PSD95 complex [137], where Fyn modulates Aβ-induced excitotoxicity by phosphorylating the NMDA receptor subunit 2B [329, 351]. Tau controls NMDA receptor-mediated signaling cascades and inhibits activation-reducing pathways. Genetic or pharmacological reduction of synaptic Tau attenuated Aβ-induced excitotoxicity [130, 136, 137, 352].

In addition, dendritic Tau was shown to exhibit another toxic gain-of-function: Missorted Tau induces the breakdown of dendritic microtubule filaments as it recruits the tubulin tyrosine-like ligase 6 (TTLL6) to the subsynaptic area. TTLL6 polyglutamylates the microtubule strands and thereby triggers microtubule severing [138, 353] leading to spine destabilization and loss [138, 237, 344, 354].

Besides the toxic effects at the dendrites, axonal transport is severely disrupted under Tau pathological conditions [272, 355, 356]. It has been suggested that abnormally modified Tau either indirectly or directly interferes with motor proteins that drive axonal cargo transport [219, 274, 357]. Further, altered stability of axonal microtubules by decreased binding of hyperphosphorylated Tau is thought to impair cargo transport [201, 274]. Accordingly, microtubule-stabilizing drugs such as epothilone D restores axonal function and ameliorates cognitive deficits in AD models [358-361].

Pathological Tau also affects the nuclear functions of Tau. Altered interaction of nuclear Tau with HNRNPs or other RNA-binding proteins is observed under pathological Tau conditions [301], and several HNRNPs were found to aggregate with Tau inside NFTs of AD patient brains

[301]. Other studies observed DNA damage, cell cycle re-entry [362], critical mechanisms for neuron death in AD [23], in human Tau-expressing *Drosophila* models. Loss of heterochromatin and aberrantly increased gene expression were observed in *Drosophila*, transgenic mice and AD brains [363]
Besides the induction of synaptic and axonal instability and the effects on nuclear function, AD-related hyperphosphorylation increases the aggregation propensity of Tau and promotes assembly of oligomers and eventually insoluble filaments that form Tau neurofibrillary tangles (Tau-NFTs) [22, 23].

Taken together, somatodendritic missorting and abnormal hyperphosphorylation of Tau, especially at the KXGS and AT8 motifs, are early events in AD-related Tau pathology. These Tau changes are induced by extracellular Aβ peptides. The interplay between Tau and Aβ pathology appears reciprocal since pathological Tau is required for mediating Aβ-induced synaptic excitotoxicity via Fyn kinase recruitment. In addition, dendritic Tau induces TTLL6-mediated microtubule breakdown leading to synaptic loss. In the nucleus, pathological Tau alters RNA processing and induced chromosomal instability. Eventually, the increased aggregation propensity of hyperphosphorylated Tau results in the formation of Tau oligomers and insoluble Tau-NFTs.

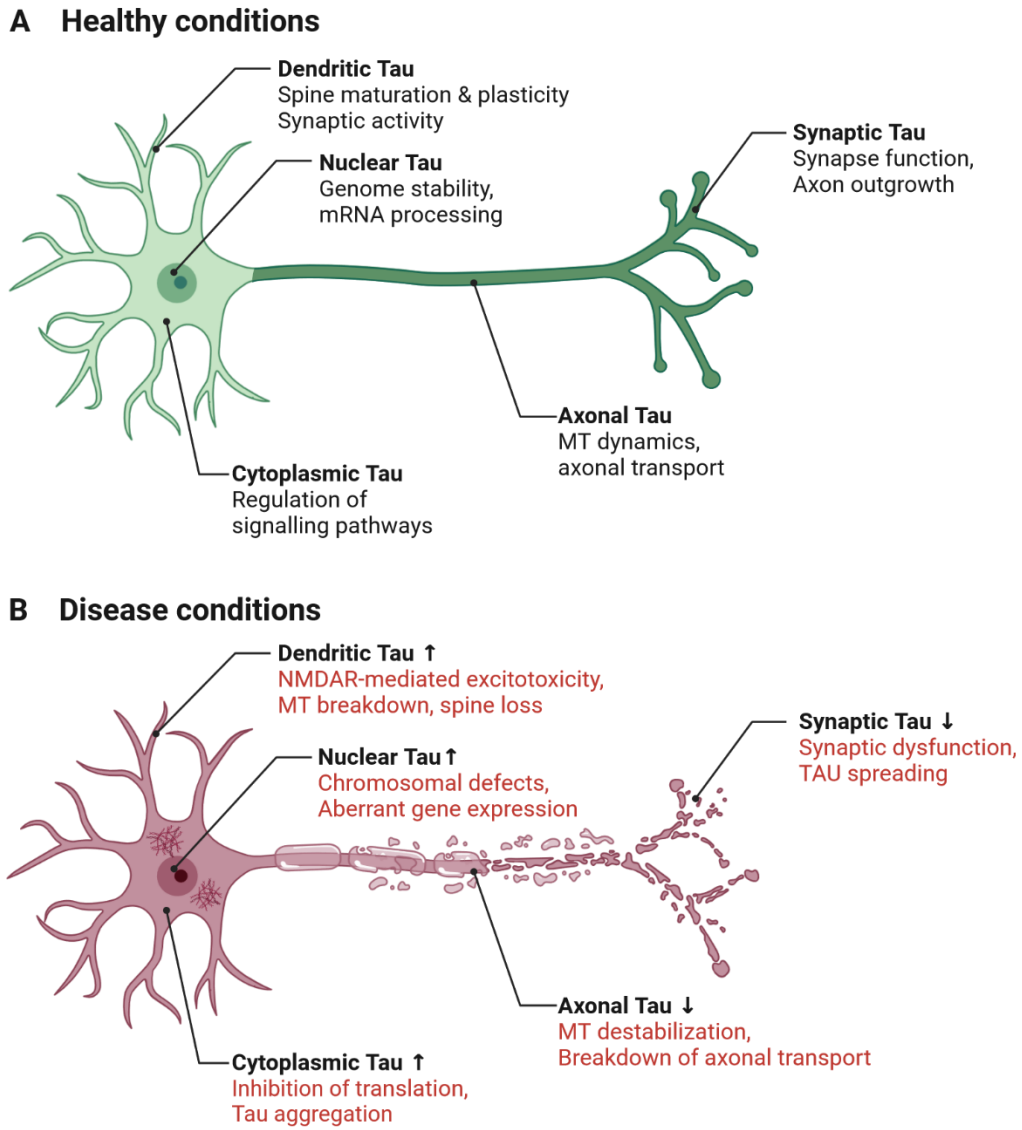

Figure 3: Roles of Tau in healthy and disease conditions.

A: Under normal conditions, Tau is mainly localized to the axon where it controls dynamic microtubule assembly and thus regulates axonal outgrowth, plasticity and cargo transport. Besides its canonical function, Tau is involved in multiple other cellular processes. Presynaptic Tau acts on synapse function and axon outgrowth, while dendritic Tau supports maturation and plasticity of postsynaptic spines and modulates synaptic activity. In the nucleus, Tau supports genome and heterochromatin integrity and processing of mRNA while cytoplasmic Tau interacts with signalling proteins and potentially enables sub-compartmentalization. B: Under disease conditions, hyperphosphorylated loses microtubule affinity and gets missorted to the somatodendritic compartment. This causes abnormal dynamics of axonal microtubules and impaired cargo transport, which leads to axon destabilization and synaptic dysfunction. Enhanced levels of dendritic Tau induce a toxic gain-of-function at the synapse. Recruitment of Fyn kinase mediates Aβ-induced NMDAR-dependent excitotoxicity while recruitment of microtubule-severing enzymes induces subsynaptic microtubule breakdown and spine destabilization. In the nucleus, pathogenic Tau causes chromosomal defects and abnormal gene expression. Tau aggregation and inhibited translation are observed in the cytoplasm under disease conditions. MT = microtubules, NMDAR = NMDA receptor. Illustration adapted and modified with permission from [180].

1.4 Models for Tau sorting research

Experimental models are crucial for investigating the mechanisms of axonal Tau sorting and pathological missorting since directed data from human patients are limited to post-mortem brain tissue.

Primary cortical or hippocampal neuron cultures from rodents were the most popular model system over the past decades. Their use requires the killing of animals, but the resulting cultures exhibit the neuronal subtype of affected brain regions and show fast maturation including extensive neurite outgrowth and spine formation within several days to few weeks [364]. Most previous studies on Tau sorting were performed in rat or mouse primary cultures, including work on the axonal transport and translation of Tau mRNA [256, 257, 261], anterograde Tau transport [138, 268, 269], retrograde Tau retention at the AIS [185, 268, 275], and more recently on sorting behavior of truncated Tau constructs [281, 282]. Despite their popularity in the field, primary rodent neurons have multiple limitations beyond the use of animals. the rodent Tau differs from the human Tau in major characteristics such as the isoform expression pattern [365], the Tau interactome and thereby also possible interactions the influence Tau (mis)sorting deviate between rodents and humans [301]. Lastly, the sorting capacity of exogenous Tau is poor, which complicates approaches with Tau overexpression [366].

Human-derived neuronal models can overcome these limitations. The human neuroblastoma cell line SH-SY5Y has been widely used in Tau and AD studies since the neuronal differentiation is comparably easy and fast [367]. However, the maturity of SH-SY5Y-derived neurons varies between different treatments, and the suitability of these neurons for Tau sorting studies has not been previously assessed.

The rise of induced pluripotent stem cells (iPSCs) paved new avenues for Tau studies in the human context. Human iPSCs can be differentiated in various neuronal subtypes and even glial cell types with high maturity, and by reprogramming patient-derived fibroblasts, the resulting iPSC-derived neurons carry the genetic background of familial and sporadic AD patients [368-370]. The direct conversion of patient cells into neurons without the functional and epigenetic reset of the stem cell stage allows to study age-associated features such as methylation or DNA damage [371-373].

However, most available differentiation protocols for iPSC-derived neurons are resource-consuming and lengthy, and neuronal cultures often suffer from unintended impurity [374-376]. Human iPSC-derived neurons also often lack proper spine formation and expression of all six major brain Tau isoforms. The development of a Neurogenin 2 (*Ngn2*)-transgenic iPSC line allowed for rapid differentiation into homogeneous glutamatergic neurons without embryoid body formation and daily medium changes [377, 378]. The Ngn2-transgenic iPSC-derived

glutamatergic neurons were used to study the roles and interactions of FTD-related mutant Tau compared to unaffected Tau isoforms [70, 343].

In sum, rodent primary neurons constitute the classical model for Tau sorting research since they show fast differentiation into mature neuronal cultures without further treatment. However, mouse and rat neurons suffer from differences in Tau physiology between rodents and humans. Human-derived neuronal models such as SH-SY5Y-derived and iPSC-derived neurons overcome these limitations and constitute promising tools for Tau sorting research in the human context.

2 Aims of the Study

The microtubule-associated protein Tau is highly enriched in the axonal compartment of mature brain neurons, where it regulates the stability of microtubules. Pathological alterations of Tau physiology play a key role in AD and related neurodegenerative diseases, so-called tauopathies. Strikingly, somatodendritic missorting of hyperphosphorylated Tau constitutes an early pathological hallmark in AD and other tauopathies. Pathological Tau induces postsynaptic destabilization and disruption of microtubule-based axonal transport, both leading to neuronal dysfunction and cell death.

Given this pathological cascade, effective prevention of Tau missorting could ameliorate disease progression in AD and tauopathy patients. In order to understand the failure of axonal Tau sorting, more profound knowledge about the mechanisms of physiological Tau sorting is required. Multiple mechanisms have been postulated to ensure efficient axonal sorting, including mRNA-based sorting, preferential degradation, anterograde Tau protein transport or axonal retention of Tau, but their individual impact remains unclear.

Further, the question arises which protein domains, motifs or modifications of Tau are required for efficient axonal sorting. Existing reports are sparse, were performed in non-human cells, and mostly focused on single motifs.

The interactome of Tau is well-studied in both rodent and human tissues and cell models and comprises proteins of manifold cellular functions, indicating the diversity of Tau function beyond microtubule binding. None of these studies compared the binding partners of sorting and non-sorting Tau constructs, which could give valuable hints to the underlying cellular sorting mechanisms. The isoform-specific interactome of human Tau is also unknown although there is strong evidence for distinct functions of Tau isoforms in health and disease [180]. Data about different interactors could help to find more tailored treatment approaches in AD and other tauopathies.

In this work, we aimed to elucidate major/open questions of axonal Tau sorting and the Tau interactome by using suitable *in vitro* model systems. This is not trivial since current Tau

research i) relies on animal models and derived primary neuronal cultures despite inter-species differences in Tau physiology such as distinct isoform expression pattern [259] and deviating interaction partners [301], and ii) suffers from poor axonal sorting of overexpressed Tau constructs [366]. Thus, we strived to:

AIM 1: Establish human-derived neuronal cell models that allow studying Tau intracellular distribution and interactomes.

Using these human neurons, we aimed to:

AIM 2: Investigate the relevance of anterograde transport for axonal enrichment of Tau using an axon depletion model that resembles axon injury observed in trauma-caused tauopathies such as TBI and CTE.

AIM 3: Unravel intrinsic features of Tau, such as certain domains or phosphorylation motifs, and cellular interaction partners that are required for efficient axonal Tau sorting.

AIM 4: Compare the interactome of sorting and non-sorting Tau to identify underlying sorting mechanisms, and reveal distinct binding partners of individual human Tau isoforms that help to explain isoform-specific roles in health and disease.

By addressing these questions, we strived to extend the understanding of physiological Tau sorting mechanisms and their internal and external mediators. This knowledge is crucial for the development of therapeutic approaches that ultimately prevent somatodendritic missorting and its detrimental consequences in AD and related tauopathies.
Further, we pave the path for isoform-specific treatment avenues that arise from distinct cellular functions and their roles in development of AD and other neurodegenerative diseases.

3 Publications and preprints

3.1 Overview

The results of this doctoral thesis have been summarized in three independent research articles that were accepted and published in peer-reviewed journals. One more article with original research data from this thesis was uploaded to the publicly available preprint server BioRxiv. Additionally, two project-related review articles were published in peer-reviewed journals.

In this chapter, I will describe the main findings from these articles, their contribution to the thesis aims, and my individual merits in conceiving, data collecting, and writing the articles.

Table 1: Overview of manuscripts relevant to this thesis.

Publication	Year	Type	Status	Contribution to Aim…			
				1	2	3	4
Article #1	2021	Research article	Published	X	X		
Article #2	2021	Perspective (with original data)	Published	X			
Article #3	2021	Review	Published	-			
Article #4	2024	Research article	Published	X			
Article #5	2023	Research article	Published		X		
Article #6	2024	Research article	Preprint/Submitted			X	X

3.2 Article #1

Title	Axonal TAU Sorting Requires the C-terminus of TAU but is Independent of ANKG and TRIM46 Enrichment at the AIS
Authors	Michael Bell, Sarah Bachmann, Jennifer Klimek, Felix Langerscheidt, Hans Zempel
Status	Published
Year of publication	2021
Journal	Neuroscience
Publisher	Elsevier
DOI	10.1016/j.neuroscience.2021.01.041

3.2.1 Key findings relevant to the thesis

In this study, we optimized the neuronal differentiation of the human neuroblastoma cell line SH-SY5Y, a commonly used model for in vitro studies on Tau [367, 379]. Four different protocols were evaluated and neuronal maturity was assessed with different morphological and biochemical readouts. We could demonstrate that combinatorial treatment with retinoic acid (RA) and the brain-derived neurotrophic factor (BDNF) in different serum conditions results in neuronal cells with profound axonal Tau sorting and somatodendritic MAP2 retention. We demonstrated that the N-terminal half of Tau is not sufficient for axonal targeting of the protein in our SH-SY5Y-derived neurons and in mouse primary neurons using expression of recombinant Tau constructs. Tau lacking the C-terminal microtubule-binding domain was enriched in the somatic compartment of both neuron types.

Moreover, the absence or lack of enrichment of two crucial mediators of axon initial segment (AIS) formation at the proximal axon in SH-SY5Y-derived neurons, namely ANKG and TRIM46, suggested that successful axonal Tau sorting does not only depend on classical AIS formation. This finding is in contrast to recent studies in murine neuronal models, and requires more detailed studies with protein-specific deficiency models.

In sum, our study provides a comparative analysis of differentiation protocols for the generation of SH-SY5Y-derived neurons, it demonstrates the necessity of the C-terminal half for axonal Tau targeting with consistent data in two neuronal models, and it reveals the capacity of human neuronal cells to enrich axonal Tau without the detectable formation of an AIS at the proximal axon.

3.2.2 Individual contribution

As the first author of the study, I conceived, conducted, and analysed the large majority of experiments. Data interpretation, visualization and the manuscript drafting were also performed by me, while it was reviewed and edited by all co-authors, especially Hans Zempel.

3.3 Article #2

Title	A simple human cell model for TAU trafficking and tauopathy-related TAU pathology
Authors	Michael Bell & Hans Zempel
Status	Published
Year of publication	2021
Journal (IF)	Neural Regeneration Research
Publisher	Publishing House of Neural Regeneration Research
DOI	10.4103/1673-5374.322450

3.3.1 Key findings relevant to the thesis

In this perspective, we discussed the suitability of SH-SY5Y-derived neurons for Tau sorting research with a special focus on Tau missorting-induced spine loss, Tau aggregate formation, Tau pathology observed in patients with traumatic axon injury (TAI), and neuron-subtype specific effects. Besides description of relevant literature, we also provide original research data from experiments that we performed to assess the neuronal identity of our cultivated SH-SY5Y-derived neurons.

3.3.2 Individual contribution

For this perspective article, I was responsible for the article design, literature review and visualization, drafting of the manuscript, which was reviewed and edited by the co-author.

3.4 Article #3

Title	SH-SY5Y-derived neurons: a human neuronal model system for investigating TAU sorting and neuronal subtype-specific TAU vulnerability
Authors	Michael Bell & Hans Zempel
Status	Published
Year of publication	2021
Journal (IF)	Reviews in the Neurosciences
Publisher	DeGruyter
DOI	10.1515/revneuro-2020-0152

3.4.1 Key findings relevant to the thesis

The human neuroblastoma cell line SH-SY5Y is a commonly used cell model in neuroscience research as it combines all advantages of an immortal cell line with the ability to differentiate into cells exhibiting many neuron-typical features. Moreover, SH-SY5Y-derived neurons are popular for studies on Tau physiology and Tau-related pathology since the neurons are reported to mimic neuronal subtypes that are early affected in brains of AD patients.
In our review, we critically evaluate the suitability of SH-SY5Y-derived neurons for Tau and Tau-sorting related research, and we provide a comparative analysis with other commonly used models for Tau sorting studies. In the second part, we focus on the reports about neuronal identity of SH-SY5Y-derived neurons and discuss benefits and limitations of these cells when studying neuronal subtype-specific vulnerability for AD and other tauopathies.

3.4.2 Individual contribution

For this review article, I was responsible for the article design, literature review and visualization, drafting of the manuscript, which was reviewed and edited by the co-author.

3.5 Article #4

Title	Cultivation, differentiation, and lentiviral transduction of human induced pluripotent stem cell (hiPSC)-derived glutamatergic neurons for studying human TAU
Authors	Sarah Buchholz #, Michael Bell-Simons #, Çağla Cakmak, Jennifer Klimek, Hans Zempel (# equal contribution)
Status	Published
Year	2024
Journal	Methods in Molecular Biology: Tau series
Publisher	SpringerNature
DOI	10.1007/978-1-0716-3629-9_31

3.5.1 Key findings relevant to the thesis

Pathological alterations of the Tau protein are major hallmarks of Alzheimer's disease and other tauopathies, but they still remain poorly understood. While primary rodent cultures are commonly used to study Tau sorting, hyperphosphorylation, or downstream pathological cascades, these non-human models have substantial drawbacks, e.g. different isoform expression and interaction profiles. Neuron cultures derived from human induced pluripotent stem cells (iPSCs) are emerging as the model of choice. However, most differentiation protocols are not only complicated and resource-intensive but also fail to generate reproducible and homogeneous neuron cultures [374-376].

In this method paper, we describe a rapid and reproducible strategy to obtain glutamatergic cortical neuron cultures from WTC11 human iPSCs. The presence of an inducible Neurogenin 2 (Ngn2) transgene in the safe harbour gene locus AAVS1 allows for precise steering of neuronal maturation [94, 378]. We also demonstrate how co-cultured murine astrocytes can be used to promote neuronal maturity such as electrical activity in iPSC-derived glutamatergic neurons.

In the second part of the article, we provide guidelines for efficient lentiviral transduction of the iPSC-derived neurons with various constitutive and inducible lentiviral expression vectors. The high transduction rates make lentiviruses superior to non-viral transfection and thus the method of choice for studies with exogenous protein expression in neurons.

Taken together, we describe a powerful human iPSC-derived neuronal model for investigating molecular mechanisms of Tau-related pathology in AD and related tauopathies, and we provide protocols for glial co-cultivation and high-efficiency lentiviral transduction. Major parts of the work for this thesis have been conducted in differentiated Ngn2-transgenic iPSC-derived neurons using lentiviral transduction strategies.

3.5.2 Individual contribution

The establishment and optimization of the differentiation protocols was done collaboratively by me and the other first co-author, Sarah Buchholz. I conceived and drafted the introduction, parts of the general protocol and specifically the lentiviral transduction chapter including the visualization. Final revision was done by me and all other co-authors.

3.6 Article #5

Title	Laser-induced axotomy of human iPSC-derived and murine primary neurons result in decreased somatic Tau levels and phosphorylation but no overt Tau pathology
Authors	Michael Bell-Simons, Sarah Buchholz, Jennifer Klimek, Hans Zempel
Status	Published
Year of publication	2023
Journal	Cellular and Molecular Neurobiology
Publisher	SpringerNature
DOI	10.1007/s10571-023-01359-z

3.6.1 Key findings relevant to the thesis

Several mechanisms are thought to mediate the axonal Tau enrichment in healthy neurons but the contribution of these mechanisms is still poorly understood. In this article, we developed an experimental approach to assess the impact of anterograde Tau protein transport. We established a protocol that allows precise and highly-controlled UV laser-based axotomy in mouse primary neurons and human iPSC-derived glutamatergic neurons with live-cell monitoring and post-fixation identification of axotomized neurons for Tau analysis.

We used this method to quantify the somatic levels of Tau and AT8-motif phosphorylation after acute axonal damage. Notably, we could not observe any pathological alterations of Tau in both neuronal models. These findings provide evidence that mechanical axon depletion and impaired soma-to-axon transport of Tau may not be crucial for somatic Tau pathology.

In brief, we report a laser-induced axotomy model with single-cell resolution suitable for live-cell and post-fixation analysis, and by using this technique we demonstrated that acute axon loss does not lead to somatic Tau accumulation and pathological phosphorylation.

3.6.2 Individual contribution

As the first author of the study, I conceived, conducted and analysed the large majority of experiments. Data interpretation, visualization and the manuscript drafting were also performed by me, while it was reviewed and edited by all co-authors, especially Hans Zempel.

3.7 Article #6

Title	Axonal Tau sorting depends on the PRR2 domain and 0N4R-specific interactions hint at distinct roles of Tau isoforms in synaptic plasticity
Authors	Michael Bell-Simons, Sarah Buchholz, Jennifer Klimek, Hans Zempel
Status	Preprint
Date of upload	2024
Preprint server	BioRxiv
DOI	10.1101/2024.06.28.601286

3.7.1 Key findings relevant to the thesis

Despite the major role for Tau localization under health and disease, many aspects of the Tau sorting process are still unresolved. The data about domains and motifs of Tau and about cellular interaction partners that are required for successful sorting are sparse. Further, the different Tau isoforms show different cellular distribution, suggesting distinct roles in Tau function in healthy neurons and under pathological conditions.

In this study, we used human *MAPT*-KO iPSC-derived neurons to unravel basic factors of axonal Tau sorting. We analysed the sorting behaviour of truncated and mutated Tau constructs after delivery with inducible lentiviral expression vectors. We found evidence that, in contrast to previous findings, the N-terminal tail and its interaction with axonal annexins are not needed for axonal enrichment. Further, the sorting efficiency was independent of the C-terminal repeat domains and of the general microtubule affinity of the protein. In turn, we observed that the proline-rich region 2 (PRR2), which is part of the microtubule-binding domain and mediator of multiple Tau interactions, is critical for efficient Tau sorting. The underlying mechanism is independent of phosphorylation of the AT8 motif. When we compared the interactome of non-sorting and sorting Tau species, we did not identify sorting-specific Tau interactors that could directly be linked to the PRR2 domain. Thus, further effort is needed to shed light on the details of PRR2-mediated axonal sorting.

We further used the TurboID proximity labelling technique to unravel Tau interactors that are specific for 0N3R or 0N4R isoforms in human *MAPT*-KO iPSC-derived neurons. Our results suggest specific binding of 0N4R to proteins that play important roles in pre- and postsynaptic plasticity. This includes regulators of the postsynaptic CDC42 pathway involved in spine formation and of exocyst-mediated exocytosis. Strikingly, 0N4R could affect CDC42-associated synapse pathology as observed in AD, since it interacts with a key regulator of CDC42, the protein MARCKS, and the downstream effector GSK-3β. Another 0N4R-interactor, RAB11, has a functional connection to AD-related Aβ pathology.

Taken together, our findings from human *MAPT*-KO iPSC-derived neurons unravelled how domains and other features of Tau influence efficient axonal Tau sorting. The isoform-specific interactome study provided evidence for distinct roles of 0N4R or generally 4R isoforms in synaptic function, with potential implications for isoform-specific roles in pre- and postsynaptic dysfunction.

3.7.2 Individual contribution

As the first author of the preprint, I conceived, conducted and analysed the large majority of experiments. Data interpretation, visualization and the manuscript drafting were also performed by me, while it was reviewed and edited by all co-authors, especially Hans Zempel.

4 Discussion

Tau pathology is a key hallmark of Alzheimer's disease (AD) and related tauopathies [22, 23, 357], but no curative treatments targeting pathological Tau alterations are available to date [4]. Under disease conditions, hyperphosphorylated Tau gets missorted into the somatodendritic compartment where it mediates microtubule breakdown and synapse destabilization [137, 138, 237, 238, 329, 344, 349].Despite the role of Tau missorting, the knowledge about the mechanisms underlying both axonal Tau sorting and pathological missorting is sparse. Several sorting models were proposed for Tau, including anterograde Tau transport or retrograde axonal retention, but their contributions remain unclear [255]. Little is also known about functional domains, motifs or cellular interaction partners of Tau that might be required for successful axonal targeting. The manifold interactome of Tau is well-studied and represents the enormous diversity of Tau function beyond microtubule binding [301]. Comparative studies of interactors from axonally sorted and non-sorting Tau species are still lacking. Novel insights into the process of axonal Tau sorting could help to develop effective therapeutic approaches for prevention of Tau pathology in AD and other diseases.

The six Tau isoforms, that are expressed in the human brain, show distinct intracellular sorting [185, 186], and there is strong evidence for isoform-specific roles in disease development as some tauopathies are caused by an unbalanced isoform expression pattern [72-74, 183]. Knowledge about isoform-specific interactions would help to unravel distinct roles of Tau isoforms in health and disease conditions. This would allow the design of tailored treatment strategies targeting specific Tau isoforms.

In this work, we aimed to expand the current knowledge about the mechanisms of axonal Tau sorting. We established and characterized suitable human-derived neuronal cell models, SH-SY5Y-derived neurons and iPSC-derived glutamatergic neurons, for studying our major research questions (AIM 1). Using these models, we investigated the role of anterograde Tau transport using an axon depletion model that could serve as an *in vitro* model for trauma-caused tauopathies (AIM 2). We generated a comprehensive library of truncated and phosphorylation-mutant Tau species to elucidate intrinsic features of Tau that are required for efficient axonal Tau sorting (AIM 3). By using proximity labelling strategies and subsequent interactome analysis, we identified interaction partners specific for sorting and non-sorting Tau species. We used the same approach to unravel interactors specific for individual Tau isoforms to expand the knowledge about isoform-specific cellular function that may be relevant for disease susceptibility.

4.1 AIM 1) Establish human neuronal cell models for Tau sorting research

4.1.1 SH-SY5Y-derived neurons are a simple model system for basic Tau sorting research with considerable limitations

The human neuroblastoma cell line SH-SY5Y [380] is commonly used in neuroscience research due to its robustness, fast proliferation, and the abundance of differentiation protocols [367, 381, 382]. Previous studies reported homogeneous cultures of SH-SY5Y-derived neurons that exhibit neurite outgrowth [367, 383, 384], electrical excitability [385-389], and the strong expression of Tau, MAP2, and other maturation markers [383, 384, 390-393], after one to two weeks of differentiation. Depending on the treatment, SH-SY5Y-derived neurons were reported to resemble either dopaminergic [390, 394, 395], noradrenergic [383, 390, 392, 395] or cholinergic neurons [396], all of which are early affected in AD patient brains [23]. We therefore decided to systematically test four common differentiation protocols and evaluate the suitability of the resulting SH-SY5Y-derived neurons for our studies on axonal Tau sorting (Article #1).

When we differentiated SH-SY5Y cells with the vitamin A derivative retinoic acid (RA), with 12-O-Tetradecanoylphorbol-13-acetate (TPA) or a sequential treatment of RA and TPA [392, 393, 397-400], the differentiation efficiency was low, and the neuronal cells showed weak neurite outgrowth, little expression of Tau and MAP2 and poor axonal Tau sorting (Bell 2021). In contrast, the sequential differentiation with RA and the brain-derived neurotrophic factor (BDNF) resulted in homogeneous neuronal cultures with extensive axon outgrowth and profound Tau and MAP2 expression after two weeks, which is in line with studies describing the beneficial effects of BDNF in SH-SY5Y cell differentiation [401]. The axonal enrichment of Tau in RA/BDNF-treated SH-SY5Y-derived neurons was ~2.5-fold higher but still significantly less than in primary mouse neurons. However, the endogenous-like axonal sorting efficiency of overexpressed Tau in SH-SY5Y-derived neurons made them suitable for sorting studies with recombinant Tau species. Using RA/BDNF-treated SH-SY5Y-derived neurons, we could analyse the sorting behaviour of N-terminal Tau, and the results were consistent with findings from primary mouse neurons, namely a lack of axonal targeting and accumulation in puncta-like inclusions (Article #1).

Despite the simple and fast differentiation with RA/BDNF and the strengths of the resulting neuron cultures, we also observed major drawbacks of this cell model for more advanced studies. First, the neuronal maturity appears questionable due to the reduced axonal and dendritic outgrowth, and the lack of a proper AIS formation compared to primary rodent or human iPSC-derived neurons (Article #1). Consistently, reports about expression of synaptic or other maturity markers lack convincing evidence for profound maturity [385, 387, 391, 393,

402, 403] (reviewed in Article #3). Further, the cultures are hard to transfect and show considerable neuron loss after transfection (Article #1). Using neuronal subtype-specific marker, we could demonstrate that the neuronal identity of these neurons does not resemble brain cell populations early affected in AD (Article #2), and that the expression profiles are inconsistent and rather hint at gradual variations than distinct cell types (reviewed in Article #3). More profound analyses, such as single-cell transcriptomics of differently treated SH-SY5Y cultures could clarify the neuronal identity. However, further efforts on disentangling the neuronal identity appear inexpedient considering the limitations of this cell model.

Taken together, SH-SY5Y-derived neurons combine the advantages of a proliferative and robust cell line with simple differentiation procedures that result in neuron cultures with axonal outgrowth and profound Tau expression and axonal sorting. With their endogenous-like sorting of overexpressed Tau, these cells outcompete commonly used neuron models. However, the apparent lack of neuronal maturity and sensitivity to transfection methods constitute major limitations for advanced Tau sorting studies, especially when using exogenous protein expression.

4.1.2 Human WTC11 iPSC-derived glutamatergic neurons are suitable for studying intracellular sorting

Human induced pluripotent stem cells (iPSCs) are a powerful tool for studying AD pathology *in vitro* research with increasing popularity [368-370, 404, 405]. The differentiation of human iPSCs into functional neuron usually suffers from laborious differentiation protocols that are time-consuming and often lead to highly variable cultures with a substantial proportion of non-neuronal cells [374, 375, 406]. To overcome these constraints, we used a derivative of the WTC11 iPSC line harbouring an doxycycline-inducible transgene of the transcription factor Neurogenin 2 (Ngn2) [377, 378, 407]. Induced expression of Ngn2 guides differentiation into glutamatergic neuronal cultures that are stable/viable in culture for months [378, 407]. Already after two weeks, these hiPSC-derived neurons show signs of maturity, i.e., extensive neurite outgrowth and branching, AIS formation and electrical activity, expression of Tau, MAP2, the neuronal marker neuronal nuclei (NeuN), and the synaptic markers vesicular glutamate transporter 1 (VGLUT1), synaptophysin, and postsynaptic density 95 (PSD95) [378, 407, 408]. We further demonstrated that co-cultivation with primary mouse astrocytes resulted in increased synapse formation and neuronal activity (Article #4).

The axonal enrichment of endogenous Tau in our iPSC-derived neurons raises rapidly after inducing differentiation in parallel with formation of the AIS (unpublished data, Breuer et al.), and is comparable with the sorting efficiency in primary mouse neurons from week 3 on (Preprint #1). Since expression and axonal sorting of different recombinant/exogenous Tau

species was an integral part of our studies, we had to optimize the delivery and localization of exogenous Tau in our iPSC-derived neurons. Previous studies with overexpressed Tau only achieved modest axonal sorting [186, 366]. To boost the sorting efficiency of exogenous Tau, we used a recently engineered Tau-depleted *MAPT*-KO derivative of the Ngn2-transgenic WTC11 iPSC line (Preprint, Buchholz 23?) that showed unaffected differentiation efficiency despite modest effects on neurite outgrowth and neuronal activity.

For young iPSC-derived neurons, we achieved mosaic transfection with polymer-based transfection reagents that allowed for conducting live-cell axon lesion studies. However, this technique was not suitable for older neurons (>2 weeks) due to low transfection efficiency. We thus developed a protocol for lentiviral transduction of iPSC-derived neurons that allowed for transduction up to 6 weeks without a decrease in efficiency and tuneable transduction rates from single neurons to complete coverage (>99 % transduced neurons). A similar approach was successfully used for efficient Tau sorting before. However, the authors worked with primary rodent neurons and used a fusion protein of Tau and GFP, which might alter the intracellular sorting and interaction profile [281].

For our Tau sorting and interactome studies, we combined lentiviral transfer with a specialized expression vector bearing two major advantages: I) The sequences of the protein of interest and the reporter protein dTomato were linked via a 2A peptide, which allows equimolar expression of two separated proteins through co-translational site-specific cleavage [409]. II) The doxycycline-inducible promoter enabled fine-tuning of the expression levels and duration for efficient axonal sorting. Strikingly, we observed endogenous-like sorting of overexpressed 0N3R-Tau^{HA}, the control construct of our sorting study, and almost endogenous-like sorting of our BirA-Tau^{HA} fusion constructs used in our TurboID-based Tau interactome studies.

While we could show that our approach in iPSC-derived neurons was suitable for the needs of this study, they also have limitations that have to considered for future approaches. We did not observe the formation of dendritic spines in our cultures, regardless of single cultivation or co-cultivation with glial cells (Article #4). This synaptic immaturity is typical for iPSC-derived neurons and makes it puzzling to determine the postsynaptic toxicity of missorted Tau. Studying the effects of postsynaptic Tau levels is further impaired by rather basic dendritic levels of exogenous Tau, which we observe for most constructs.

In sum, we established homogeneous cultures of human iPSC-derived neurons with signs of neuronal maturity from transgenic WTC11 iPSCs, and thereby we overcome several limitations of non-human models like primary rodent neurons or cell line-based models such as SH-SY5Y-derived neurons. The use of Tau-depleted iPSC-derived neurons enabled us to analyse Tau sorting and identify sorting-related interaction partners without interference with endogenous Tau. For precise and efficient expression and analysis of our mutant Tau species in matured

iPSC-derived neurons, we developed a workflow for inducible lentiviral expression of our target genes with equimolar reporter gene expression.

4.2 AIM 2) Investigate the relevance of anterograde Tau transport

Altered intracellular Tau localization is an early pathological hallmark in AD patients [106]. Thus, one possible strategy to counteract early AD pathology is to restore efficient axonal targeting of Tau and thereby prevent the toxic effects of somatodendritic Tau accumulation. The mechanisms underlying physiological Tau sorting are still not fully understood. There is, however, growing evidence that active anterograde transport (and axonal retention) of Tau protein determine Tau localization rather than mRNA-based sorting processes [255].

4.2.1 Studying Tau sorting and trauma-related tauopathies with laser-induced axotomy

We developed an experimental setup that combines a UV laser ablation unit with a high-speed spinning disc microscopy that enables multiplexed live-cell monitoring of Tau to study possible somatic Tau pathology after physical axon disruption (Article #5). Our approach has major benefits for Tau sorting research: We successfully applied our setup to primary mouse neurons, a common Tau research model system [138, 185, 238, 268, 281, 282], and human iPSC-derived glutamatergic neurons, an *in vitro* model with great potential for studying Tau [70, 368-370, 406, 407, 410, 411]. Further, our approach allowed for post-fixation identification and analysis of pathological Tau features such as localization or site-specific phosphorylation due to cultivation chambers with spatial orientation. The precise lesion induction of the ablation laser enabled investigation of potential Tau pathology on a single-cell level.
In addition, our system could be also useful to mimic tauopathies that are caused by external trauma such as CTE or TBI [33]. In these diseases, either repetitive or singular physical impact results in the hyperphosphorylation and missorting of Tau, and eventually formation of neurofibrillary tangles (NFT) in both the axonal and somatodendritic compartments [33, 43, 47-49]. Available animal models for CTE or TBI work mimic the traumatic axon injury that underlies the detrimental cascade of Tau pathology, are based on diffuse external harm by poking the cortical surface with a metal tip after craniotomy [49, 412, 413]. Our single-cell approach bears great potential to complement current techniques with insights into pathological alterations on the molecular level.

4.2.2 Laser-mediated axon severing does not cause somatic Tau accumulation or AT8-associated hyperphosphorylation

We induced acute axon depletion and determined Tau pathology in iPSC-derived and mouse primary neurons. Our experiments revealed no acute increase of somatic Tau levels or

phosphorylation at the tauopathy-associated AT8 motif as signs of Tau pathology after laser-based axon ablation. This was true for both model systems studied primary mouse and human iPSC-derived neurons at multiple time points, i.e. 0.5, 1 and 3 hours after axotomy. These observations are surprising as we expected somatic Tau accumulation of newly synthesized somatic Tau that cannot transit anterogradely, and possible reflux of Tau from the axon stump, due to known models of anterograde and retrograde Tau transit [255].

These findings have two possible implications: The lack of Tau pathology could suggest a more prominent role of axonal Tau protein synthesis and a minor role of somatically synthesized protein. This would be in contrast to former studies claiming a major role for protein-based Tau sorting [185, 260]. On the other hand, pathological changes of Tau could occur beyond our observation time of 3 hours. However, in studies with application of stressors like Aβ oligomers, excitotoxic agents or reactive oxygen species (ROS), prominent somatic Tau accumulation occurred within minutes to several hours with the peak around 3 hours after application [138, 238, 414, 415]. An alternative explanation are regulatory effects of a post-lesion signalling cascade that influence transcription or translation, degradation of Tau mRNA and protein, and potentially other axon-targeted proteins.

Our setup provides the basis to disentangle these potential factors experimentally. The multi-cell live imaging allows to combine axotomy with compartment-specific photoconversion of traceable Tau and imaging for several hours. This allows to monitor the synthesis and degradation rates of somatic Tau as well as possible impairments of the retrograde diffusion barrier after the axon injury. Further, pharmacological interventions with drugs inhibiting protein synthesis or degradation are feasible in our system.

What are the implications of our results for its suitability in trauma-caused tauopathy modelling? Our approach proved its benefit for monitoring and assessing acute molecular changes of Tau on single-cell resolution. However, first signs of Tau pathology after traumatic head injuries get visible after several days to weeks [34, 416]. Therefore, further optimization of our approach is necessary to enable long-term-monitoring (>24 hours) that mimics the chronology of TAI-induced pathology more accurately. In case of success, our system bears great potential to complement existing models of trauma-based tauopathies, which use diffuse injury induction to the cortical surface [49, 412, 413].

All in all, we established a laser-based axon lesion model for investigating acute axon depletion and its effects on intracellular Tau pathology by multi-cell live imaging and post-fixation analysis. We used this model to study the role of protein-based Tau sorting in two common in vitro models, primary mouse neurons and human iPSC-derived neurons. Our findings provide evidence that acute axon depletion does not result in tauopathy-related somatic Tau alterations, hinting towards a minor role of anterograde Tau transport or acute cellular

adaptations to prevent somatic accumulation of axonal proteins. Further experiments are necessary to unravel the reasons for this lack of Tau pathology after axotomy.

4.3 AIM 3) Role of individual Tau domains and phosphorylation sites in axonal Tau sorting

Under healthy conditions, Tau shows pronounced axonal enrichment in mature brain neurons [190, 191]. This efficient polarization of Tau gets compromised in early stages of AD, in which hyperphosphorylated Tau accumulates in the somatodendritic compartment [106, 107]. In the dendrites, pathological Tau induces microtubule breakdown and synaptic dysfunction [138, 237, 329]. Despite the detrimental effects of Tau missorting, the mechanisms of successful axonal Tau sorting and pathological missorting are still poorly understood. The studies about Tau domains and motifs that are required for axonal Tau sorting are sparse [185, 281, 282]. More knowledge about the molecular processes of Tau sorting would help to develop novel approaches for ameliorating Tau missorting under disease conditions.

4.3.1 Sorting analysis of the Tau construct library in *MAPT*-KO human iPSC-derived neurons

In our work, we aimed to systematically assess domains, motifs and posttranslational modifications of Tau that are necessary or sufficient for successful axonal Tau sorting. We generated a comprehensive library of HA-tagged Tau constructs that were either lacking specific protein domains or mutated the phosphorylation sites of the KXGS and AT8 motifs, which are hyperphosphorylated under disease conditions and may be involved in the Tau missorting process [238-240] to mimic permanent or absent phosphorylation [185, 331].
One major constraint of Tau sorting studies is the poor sorting efficiency of overexpressed exogenous Tau [281, 366]. We used lentiviral gene delivery of our Tau^{HA} constructs with a 2A-coupled reporter dTomato under a doxycycline-inducible promoter, similar to previous studies with GFP-tagged Tau in primary rodent neurons [281].
With our approach, we achieved endogenous-like axonal sorting of exogenous 0N3R-Tau^{HA} in 8-weeks old *Ngn2*-transgenic human iPSC-derived glutamatergic neurons (Article #4).
Prior to the sorting analysis, we validated the correct expression of all Tau^{HA} constructs and their relative protein levels to determine both the correct biosynthesis and possible cellular degradation. Most constructs were correctly sized and showed no signs of strong protein degradation after two weeks of expression, except for the repeat domain constructs only3R-Tau and only4R-Tau. These fragments were not detectable, possibly due to nonsense

mediated decay, enhanced proteasomal targeting or other pathways of protein quality control [417-419].

The KXGS-mutated Tau constructs exhibited slightly smaller extra band that was stronger with more mutated residues. This effect was visible for both pseudophosphorylated and dephosphorylated mutants. One explanation could be a disturbed interaction with protein-modifying enzymes that target KXGS or adjacent residues, leading to altered modifications of other protein domains. Tau is known to undergo proteolytic cleavage under pathological conditions [22]. The KXGS mutations could induce protein truncation. In AD-related synaptosomes, several Tau fragments with truncations at the Tau C-terminal tail were detected [420], and the size range of 40 to 53 kDa would match our band pattern [22].

Notably, we did not observe any changes for nuclear Tau levels in our analysis. The details of nuclear Tau import are still unclear but several studies suggest a role of Tau phosphorylation, probably in balance with the abundance of another modification, Tau O-GlcNAc glycosylation [194, 316, 317]. According to this, higher levels of phosphorylation decrease the degree of O-GlcNAc glycosylation and impair successful nuclear targeting [317]. However, also our pseudophosphorylated AT8 and KXGS constructs showed no changes for nuclear levels. Whether certain residues are more critical for the reciprocal regulation of O-GlcNAc glycosylation, or whether other factors influenced nuclear import in our neurons, remains elusive.

The sorting analysis of our constructs revealed that large truncations generally prevent proper axonal Tau sorting. Neither the C-terminal half nor the N-terminal half alone were enriched in the axonal compartment. The insufficiency of N-terminal Tau for axonal sorting was confirmed in SH-SY5Y-derived and primary rodent neurons in our earlier study (Article #1). So, either domains of both the N-terminal and C-terminal half are required for axonal sorting, or the intracellular filtering, e.g. the AIS-located transit filter [285-287], prevents undisturbed localization of these artificial fragments. Notably, across all neuronal models, we saw puncta-like inclusions for $Nterm^{HA}$ in the soma. These characteristic inclusions were absent for all other fragments and might hint at specific targeting of Nterm-Tau to a distinct cellular compartment. For Tau fragments of similar amino acid sequence, that occur due to proteolytic cleavage in disease, no such puncta-like distribution was described [421-423], Our interactome analysis using TurboID proximity labelling suggests that Nterm-Tau is recognized by the peroxisomal import machinery and localizes inside the peroxisomal lumen (see chapter 4.4.3).

The N-terminal tail of Tau domain was previously claimed to be critical for axonal Tau sorting. Absence of the N-terminal tail caused enhanced retrograde flux of axonal Tau coupled to photoactivatable GFP in primary rodent neurons, which was explained by the missing connection with membrane-bound annexins in the axon [282]. However, we did not see any

sorting deficits for N-terminal-lacking construct noNTail-Tau in our sorting analysis. One explanation for this discrepancy is that we measured not only retrograde protein flux but total sorting efficiency. Strong anterograde transport [255, 274] could compensate for the described lack of annexin-mediated axonal retention.

In fact, the same effect was observed in studies with traceable 2N4R-Tau with complete KXGE pseudophosphorylation in rodent neurons showed that, although highly diffusible Tau leaks through the retrograde Tau diffusion barrier, the net axonal enrichment is even higher than for unmutated 2N4R-Tau [185]. Consistently, Tau failed to enrich axonally when it exhibited strong microtubule affinity due to a dephosphorylation-mimetic proline-rich region 2 (PRR2)[281]. These findings suggest that strong microtubule binding prevents efficient Tau sorting while diffusible Tau shows proper axonal enrichment due to efficient anterograde transport. However, Tau mutant that lacked the entire PRR2 region and thus had less microtubule binding showed strongly decreased axonal sorting [281], hinting at microtubule-independent effects.

In our sorting analysis in human *MAPT*-KO iPSC-derived neurons, the phosphorylation-mimetic AT8-mutant Tau constructs showed no sorting deficits, which supports the idea of accurate sorting for diffusible Tau [185, 281]. However, the dephosphorylation-mimetic AT8 mutants with tight microtubule binding showed similar sorting efficiency, contradicting previous claims [281]. It remains questionable, whether phosphorylation-dead mutants of the AT8 motif or the entire PRR2 have such a strong impact on microtubule binding, at all. The changes in microtubule binding are surprisingly small for PRR2-mutant Tau compared to wildtype Tau [281]. When we depleted the entire PRR2 domain (noPRR2-Tau) instead of mutating the AT8 motif and thereby slightly decreased the microtubule affinity [199], we saw considerable impairment of axonal sorting. An effect, that was in fact also seen in the previous study [281].

Based on these findings, we conclude that i) diffusible Tau overcomes the retrograde retention mechanisms but still shows efficient sorting due to anterograde transport processes [185, 255, 281], ii) successful axonal Tau sorting occurs independent of the microtubule affinity of Tau, and iii) the PRR2 domain plays a key role in the sorting process. These conclusions were corroborated by the sorting behaviour of the KXGS-mutant Tau constructs. All KXGS-Tau mutants, regardless whether pseudophosphorylated or phosphorylation-dead, showed normal axonal enrichment. This observation questions the relevance of axonal Tau retention via dephosphorylation by site-specific phosphatase such as PP2A [255].

Based on these findings, the question arises on how the PRR2 domain mediates axonal Tau sorting. Since mutations of the AT8 motif did not affect axonal targeting, the sorting process seems to be independent of the AT8 phosphorylation state. However, previous data suggest that PRR2-wide dephosphorylation disrupts axonal sorting [281]. The PRR2 domain contains other phosphorylation motifs that are associated with Tau missorting, such as the AT180 motif [23]. Given the fact, that phosphorylation of two major hallmarks of pathologically missorted

Tau, AT8 and KXGS, does not affect successful sorting, an involvement of AT180 phosphorylation appears unlikely. Besides phosphorylation, raised Tau acetylation was recently found to affect Tau function in health and disease [424]. It was claimed that acetylated Tau compromises the Tau retention function of the AIS and thus gets missorted [425]. The studies acetylation residues lie all outside the PRR2 domain. Alternatively, the PRR2 domain could bind cellular interactors that are critical for efficient anterograde sorting and/or retrograde retention effects. The PRR domain interacts with various proteins including SH-containing kinases such as Src and other signalling proteins, nucleic acids, cytoskeletal networks of f-actin or kinesin motor proteins [22, 23]. The lack of interaction of Tau to a PRR2 interactor could explain the seen sorting deficits. It is also possible that the domain truncation causes structural changes of Tau which in turn impede axonal sorting, for instance by masking protein interaction residues. It remains, however, unclear whether PRR2-wide dephosphorylation [281] would induce the same protein configuration change.

Taken together, we analysed the sorting behaviour of mutant HA-tagged Tau constructs to reveal the Tau domains and motifs that are crucial for efficient axonal sorting of Tau. For the sorting analysis, we transduced our doxycycline-inducible constructs into human *MAPT*-KO iPSC-derived glutamatergic neurons and achieved endogenous-like sorting efficiency of exogenous Tau.

Our analysis showed that larger truncations of the N- or C-terminus prevent axonal sorting, and lead to the accumulation of puncta-like inclusion for the N-terminal half. We also found that efficient axonal Tau sorting was independent of the N-terminal tail and of the microtubule affinity of Tau, in contrast to the literature [281, 282]. We claim that the PRR2 domain of Tau is required for successful axonal Tau sorting. PRR2-dependent Tau sorting seems to be independent of AT8 phosphorylation. PRR2-specific Tau interactions or other posttranslational modifications could mediate the sorting process but more research is needed to decipher the underlying molecular mechanisms.

4.4 AIM 4) Role of Tau interactions in axonal sorting

The interactome of Tau is remarkably diverse and reflects the wide range of cellular functions of Tau beyond its role in axonal microtubule regulation [22, 23, 299, 301]. Tau binds other cytoskeletal components like f-actin and neurofilament [308], regulates synaptic maturation and plasticity [302, 309, 329, 426], DNA stability and RNA processing[301, 319, 427, 428], and is involved in multiple signalling pathways [301, 327, 328, 429]. There is strong evidence, that loss or malfunction of this non-classical Tau functions contributes to neurodegeneration in tauopathies [301, 329, 430]. A recent meta-analysis evaluated previous Tau interactome studies and identified cytoskeletal proteins, ribosomes, proteasomes and especially RNA binding proteins as major Tau interaction clusters [301]. Interaction with RNA binding proteins such as heterogeneous nuclear ribonucleoproteins (HNRNPs) were found to be associated with early disease progression, suggesting a causal role in AD-related Tau pathology [301].
None of the existing studies compared the interactomes of human Tau isoforms [301] although the six human Tau isoforms were shown to have distinct patterns of intracellular distribution [185, 186], and different cellular functions [180]. There is also evidence that Tau isoforms differ in their toxicity and contribution to pathology in AD and other tauopathies [180]. In this study, we aimed to identify Tau binding partners that are different between 3R and 4R isoforms and provide novel insights into the different functional role of the human Tau isoforms.
We further wanted to unravel binding partners of Tau that are required for axonal Tau sorting. Despite the key role of intracellular Tau sorting in health and disease, there is a lack of data on sorting-specific Tau interactions. We compared the interactome of the 0N3R isoform, which shows efficient axonal sorting, with the Tau N-terminal half that lacks axonal sorting but accumulates inside the soma (Articles #1, 6). A better understanding of the proteins involved in Tau sorting would open new avenues for treatments that restore Tau sorting and prevent deleterious Tau missorting.

4.4.1 Tau interactome study in *MAPT*-KO iPSC neurons with TurboID proximity labelling

In this work, we aimed to elucidate i) proteins that interact differently with sorting and non-sorting Tau constructs, and ii) isoform-specific Tau interaction partners of 0N3R and 0N4R. To this end, we combined our inducible lentiviral expression system with the recently developed proximity labelling technique TurboID, based on the promiscuous biotin ligase BirA [431, 432]. We fused the Tau isoforms 0N3R and 0N4R, and the non-sorting construct Nterm with HA-tagged BirA in order to investigate their interactomes. Before starting the interactome study, we validated our experimental approach. After testing for correct expression and efficient 2A

peptide cleavage, we ensured that the fusion of BirA did not affect the sorting behaviour of the isoforms and the somatic accumulation of Nterm-Tau. Further, we confirmed the sufficient axonal invasion of biotin during incubation time, and could show that only basal levels of unspecific biotinylation occurred during 12-13 days of construct expression prior to biotin application.

For our actual sample collection, we transduced *MAPT*-KO iPSC-derived neurons with the described constructs and two controls, only $BirA^{HA}$ and only $0N3R^{HA}$, to correct for unspecific labelling. After affinity-based isolation of biotinylated proteins, all samples were analysed with mass spectrometry and further processed with bioinformatical methods. In total, we identified 640 interactors of either BirA-$0N3R^{HA}$, BirA-$0N4R^{HA}$, or BirA-$Nterm^{HA}$ before, and 61 after background subtraction. The absolute number of identified interactors for the Tau isoforms (32 proteins) was low compared to a similar study by Tracy et al who used APEX2-tagged Tau for biotinylation in the same iPSC line (222 proteins). However, no correction for binding partners of the fusion protein APEX2 and unspecific cellular labelling was performed, which could explain the gap in interactors and making our approach more targeted. The validity of our data was corroborated by a gene ontology (GO) term analysis, which revealed classical cellular functions of Tau, such as cytoskeleton organization and nucleotide binding, as major biological processes of the interactome. Notable, more than 75 % of our identified proteins were already described in one or multiple earlier interactome studies (Kavanagh), and almost 60 % were described in Tracy et al (22), underlining the relevance of our approach.

4.4.2 Differential interactome of the Tau isoforms 0N3R and 0N4R

In brain neurons, Tau regulates axonal microtubule dynamics and affects a wide range of cellular functions beyond its classical function [22, 23, 301]. The functional differences among the six Tau isoforms, which are present in the brain [11], are still understudied [180], although there is growing evidence for isoform-specific effects on neuronal function in health and disease conditions. For instance, the expression ratio of Tau isoforms varies between different brain regions and neuronal subtypes that are differentially susceptible to Tau pathology [179, 433-435], and misbalanced splicing of 3R and 4R isoforms underlies many familial tauopathies [23, 77, 180, 183].

Notably, the effect of Tau isoforms on basic microtubule traits such as stability, number or growth rate was similar for all isoforms in human neuroblastoma cells [186]. Data from human and murine neuron cultures showed that the subcellular distribution of Tau isoforms varies in human and murine neuron cultures after overexpression, with efficient axonal sorting of 0N isoforms and higher somatodendritic levels of 1N and 2N isoforms [139, 186, 189]. The use of isoform-specific antibodies revealed more 1N4R- and 2N4R-Tau in the dendrites, and

increased nuclear localization of 1N Tau isoforms in mouse brain [187]. These findings clearly suggest isoform-specific localization mechanisms.
Besides the differential localization, there are data from murine Tau isoforms 0N4R, 1N4R and 2N4R that demonstrate major isoform differences also for the interactome [188]. While 0N4R interacted with proteins involved in cellular homeostasis and glycolysis pathways, and 1N4R bound several glycolysis-related proteins, the longest isoform 2N4R was more involved in synaptic transmission [188], which is in line with its increased dendritic levels [185, 186]. Unfortunately, these results are hard to translate to the human context, for which comparative studies are lacking, since a recent meta-analysis revealed substantial differences of the murine and human Tau interactions [301].
Thus, our goal was to elucidate isoform-specific interaction partners that may hint at distinct cellular functions of 0N3R and 0N4R in human neurons. We compared the interactome of 0N3R and 0N4R in our human *MAPT*-KO iPSC-derived neurons by using TurboID-based proximity labelling [431, 432].

Our analysis revealed 32 interaction partners for both isoforms in total, of which 9 proteins were specific interactors of 0N3R, and 10 proteins exclusively bound to 0N4R. Out of the 0N3R-specific interactors, five proteins were not described to bind Tau before, the SH3 and PX domain-containing protein 2B (*SH3PXD2B)*, dedicator of cytokinesis protein 7 (*DOCK7)*, Zinc finger CCHC domain-containing protein 3 (*ZCCHC3)*, proline-rich and coiled-coil A2 (*PRRC2A)*, and leucine zipper-containing protein 1 (*LUZP1)*. The *SH3PXD2B*-encoded protein SPD2B recruits metalloproteases to podosomes, conical actin-rich structures at the cell surface, and controls remodelling of the extracellular matrix during cellular development [436, 437]. Mutations in the *SH3PXD2B* locus are linked to developmental ophthalmic disorders [438, 439]. The DOCK7 protein is involved in establishing neuronal polarity [440]. The *ZCCHC3*-encoded ZCHC3 protein binds DNA and RNA and contributes to innate virus defence [441, 442]. The function of the *PRRC2A*-encoded PRC2A protein is largely unknown but there is evidence that it interacts with RNA and regulates splicing of pre-mRNAs [443]. Lastly, we found LUZP1 as a novel interactor of 0N3R-Tau. LUZP1 is a regulator of f-actin stability and plays a role in cilia formation [444, 445]. In the brain, LUZP1 is predominantly expressed in the cerebral cortex where it acts as f-actin crosslinking protein [446, 447]. LUZP1 deficiency is associated with neural tube closure in mice indicating a crucial role in brain development [448]. LUZP1 was not the only actin-related protein that we found to interact specifically with 0N3R-Tau. The scaffold protein ZO1, a known Tau interactor encoded by the tight junction protein 1 (*TJP1)* gene [343], links tight junction proteins to f-actin and thereby regulates paracellular diffusion and formation of the blood-brain barrier [449, 450]. Reports about ZO1 function in brain neurons, however, are sparse.

On the other hand, we detected two specific interactors of 0N4R-Tau that directly modulate f-actin function, the actin-related protein 2 (ARP2), encoded by the *ACTR2* gene, and the myristoylated alanine-rich C kinase substrate (MARCKS). ARP2 is part of the ARP2/3 nucleation complex that induces polymerization of branched f-actin networks in the cytoplasm [451] and inside the nucleus [452, 453] thereby regulating gene transcription and DNA damage repair. The signalling protein MARCKS regulates various cellular functions including cell motility, adhesion and chemotaxis by binding to the f-actin cytoskeleton [454-456]. The identification of multiple actin-related binding partners for both isoforms underline the crucial regulatory role that Tau has for the plasticity of the f-actin network [23].
Further, we found specific interactors that are associated with microtubule function. 0N3R-Tau interacts with the microtubule-associated protein 4 (MAP4), which is closely related to Tau and has a similar domain structure. Classically considered a non-neuronal member of the MAP family, MAP4 also regulates microtubule stability in neurons [457], resulting in more branched microtubules compared to Tau or MAP2 in SH-SY5Y cells [458]. In contrast, 0N4R-Tau specifically bound to α-tubulin N-acetyltransferase 1 (ATAT1), the major enzyme responsible for acetylation of long and stable microtubules [459, 460]. These interactions suggest that 0N4R-Tau might promote microtubule stability while 0N3R enhances plasticity and branching, in line with previous reports about the importance of Tau for producing labile microtubule domains [201]. But recent *in vitro* studies revealed no differences for number, stability, length or growth rate of microtubules when single isoforms were overexpressed in SH-SY5Y cells [186].

The microtubule-binding domain of Tau is known to interact with many proteins that play a role in cellular signalling pathways, including 14-3-3 proteins or calmodulin [237, 429]. The role of individual repeat domains for binding efficiency is still unclear. In our interactome study, we found several signalling proteins that specifically bound to 0N4R-Tau. One of these interactors is the actin-binding and signalling protein MARCKS, which was shown to affect endocytic and exocytic processes through lipid sequestering or direct docking of vesicle proteins [449, 450]. At the postsynapse, MARCKS modulates synaptic plasticity via activation of the CDC42 pathway [461]. The cell division control protein 42 (CDC42) is a membrane-bound Rho GTPase that, when activated, promotes dendritic growth and spine formation [462, 463]. Of note, the CDC42 signalling pathway was found to be upregulated in cortical sections of AD patients [464, 465]. Recent studies revealed that overactivation of CDC42 causes an AD-related phenotype with severe cognitive impairments in mice, accompanied by f-actin depolymerization, Tau hyperphosphorylation and subsequent synapse loss [463]. The breakdown of f-actin was likely due to the aberrant CDC42-induced activity of the actin depolymerizer cofilin [463]. Regarding the observed Tau pathology, the authors claimed that the Tau-targeting kinase glycogen-

synthase kinase 3-beta (GSK-3β), a downstream effector of CDC42, was responsible for Tau hyperphosphorylation as the GSK-3β activity was increased drastically upon CDC42 overactivation [463]. Interestingly, our interactome analysis also revealed GSK-3β as a specific interactor of 0N4R-Tau. This cannot be simply explained by the additional repeat domain since the primary targets of GSK-3β lie within the PRR2 domain and the extreme C-terminal tail present in both isoforms [237]. These observations suggest that 0N4R-Tau could be a crucial mediator of CDC42-induced pathology at two steps of the cascade, by binding the upstream activator MARCKS and the downstream effector GSK-3β.

The 0N4R-interactor MARCKS also acts at the presynaptic site, where it binds to RAB10-positive vesicles and appears crucial for axonal development [466]. Strikingly, we detected more 0N4R-specific interactors that are involved in presynaptic function. The small Ras-related GTPase RALA locates at the presynaptic membrane, where it controls vesicle trafficking and neuronal polarity, mainly via interaction with the exocyst complex [467, 468]. The exocyst is an octameric membrane complex that tethers secretory vesicles to the membrane and thereby plays a fundamental role in polarized exocytosis in eukaryotic cells [469]. In neurons, exocyst-mediated exocytosis regulates neurite outgrowth, neuronal polarity, and synaptic receptor transport [467, 468, 470-472]. RAB11A and RAB11B, also members of the Ras superfamily, are master regulators of intracellular membrane trafficking and membrane organization [473]. Studies from yeast revealed a close connection of RAB11 proteins and the exocyst [474]. RAB11 binds to multiple exocyst complex proteins and regulates the tethering of secretory vesicles to the plasma membrane, similar to RALA [475].
The interaction of Tau with key regulators of the exocyst complex, RALA and RAB11A/B, hint at a regulatory role of 0N4R-Tau in exocyst-mediated vesicle release. Direct interaction with the octameric exocyst appears unlikely since none of the complex proteins was detected in our or previous studies [301], except for one protein (SEC8) found in a single study [343]. Our results are consistent with former data showing a close association of Tau with presynaptic vesicles and proteins of the active zone [343, 430, 476]. We provide the first evidence that the interaction pattern and thus the regulatory of Tau at the synapse is isoform-specific.
The specific interaction of 0N4R with RAB11 proteins might also have implications in disease context [474]. As a major regulator of endosomal recycling pathways, RAB11 controls the recycling of membrane-bound β-secretase (BACE1) [477], and reduction of RAB11 has been shown to increase the amyloidogenic APP cleavage by BACE1 and accumulation of Aβ [474, 478]. The impact of Tau-RAB11 interaction in this process remains unclear. Disruption of RAB11-mediated endosomal recycling can also impair other signalling pathways, such as the BDNF/Trk pathway, which may lead to neurodegenerative diseases [479].

In sum, our isoform-specific interactome study revealed several 0N4R-Tau interactors that have important roles in regulating pre- and postsynaptic function and plasticity. The postsynaptic CDC42 pathway controls dendrite growth and spine formation, and is activated by the 0N4R-interactor MARCKS. Overactivation of CDC42 was found in AD and results in Tau hyperphosphorylation via GSK-3β recruitment, another 0N4R-specific interactor. In addition, 0N4R binds several regulators of the presynaptic exocyst complex, including RAB11. Reduction of the endosomal recycling protein RAB11 activity is linked to amyloidogenic APP cleavage and Aβ accumulation. Thus, our results provide evidence for isoform-specific synaptic functions with possible implications for the progression of AD pathology.

4.4.3 Differential interactome of sorting and non-sorting Tau constructs

Next, we compared the interactome of axonally sorted Tau isoforms and a somatically retained Tau construct, the Tau N-terminal half (Nterm-Tau), to reveal sorting-specific Tau interactors. The interactome analysis revealed numerous upregulated interactions of Nterm-Tau. The GO term analysis showed that the majority of these proteins are either parts of the peroxisomal structure or components of the lipid metabolism that is taking place inside the peroxisomal matrix [480, 481]. Localization of Tau within the peroxisome, an organelle with major roles in metabolizing fatty acids and other lipids and detoxification of reactive oxygen species or alcohol [480, 481], was neither described under healthy nor under disease conditions before. Thus, we can only speculate about the circumstances of peroxisomal association of Nterm-Tau.
Most proteins targeting the peroxisome are imported via recognition of a tripeptide peroxisomal targeting signal (PTS1) by the receptor peroxin 5 (PEX5), and translocation by the PEX5-associated receptor complex [482]. In our study, Nterm-Tau interacts with PEX5 and another protein of the complex, PEX14, which strongly indicates recognition of Nterm-Tau by the PTS1-binding receptor and potential translocation to the peroxisomal matrix. However, the prototypic C-terminal PTS1 sequence S-K-L [483] is not present in Nterm-Tau. Numerous non-canonical PTS1 sequences are described, and there is evidence for the residues upstream of the C-terminal tripeptide to modulate PEX5 binding [484-486]. We used two online PTS1 prediction tools to estimate the possible recognition of Nterm-Tau, and both classified the C-terminal sequence as non-targeting (PTS1 predictor: https://mendel.imp.ac.at/pts1/, PSORTII: https://psort.hgc.jp/form2.html). Not only the mechanism of peroxisomal targeting remains elusive but also the fate of peroxisomal Nterm-Tau. While Nterm-Tau alone was not detected anymore in Western blot analysis indicating low construct stability, the BirA-fused Nterm-Tau showed no signs of degradation. Since both constructs have a consistent localization pattern, the question of Nterm-Tau stability remains unresolved.

In brains of AD and other tauopathy patients, Tau fragments with similar truncations as Nterm-Tau were described to show abnormal cellular localization. So-called NH2-Tau, comprising the amino acids Q26-R230, was enriched in the synaptosomal mitochondria [422] where it impaired the ATP transport and compromised normal mitophagy function [22, 421, 422]. Another N-terminal fragment found in AD and ALS patients, Tau_{45-230} [423], had neurotoxic effects in CHO cells, hippocampal rodent neurons [423], and humanized APP/Tau mice [136, 487], but no compartment-specific distribution was mentioned. Of note, we did not observe any cytotoxic effects of expression and peroxisomal sequestration of Nterm-Tau or BirA-Nterm fusion proteins.

Seven proteins specifically interacted with the axonally sorted Tau isoforms 0N3R- and 0N4R-Tau. Of note, five of these proteins were found to specifically interact with either 0N3R (*CPS1, LUZP1*) or 0N4R (*RALA, MAPT, ACTR2*). Assuming equally efficient sorting of both isoforms [185, 186], a crucial role of these candidates in the sorting process appears unplausible.
Besides the five mentioned proteins, *CELSR3* and *HSPH1* were differential interactors between the axonally sorted (Tau isoforms 0N3R and 0N4R) and somatically enriched Tau (Nterm-Tau). The G-protein coupled receptor CELR3, encoded by the *CELSR3* gene, plays a role in the WNT/PCP signalling pathway, which controls the guidance of growing cortical and thalamic projection neurons [488, 489]. Depletion of synaptic CELR3 results in loss of corticothalamic projections and drastic reduction of glutamatergic synapses [490, 491]. However, despite the known roles of Tau at the pre- and postsynapse, there is no evidence for a functional connection of Tau and CELR3. Notably, CELR3 was not described as Tau interactor previously [301].
The heat shock protein 105 kDa (HSP110), encoded by *HSPH1*, is highly expressed in the brain and acts as functional co-factor for chaperones such as HSPA1A and HSPA1B [492]. Together with other quality control proteins, HSP110 can form a multi-protein complex with disaggregase activity that is critical for clearing protein aggregates such as α-synuclein [493, 494]. Direct interaction with Tau is well-described for HSP110 [301]. Recent data show that HSP110 binds to axonal Tau and the major axonal Tau phosphatase, PP2A, in mice. Moreover, genetic HSP110 depletion resulted in Tau hyperphosphorylation and aggregation due to disrupted PP2A activity [495]. Thus, HSP110 directly regulates the axonal Tau phosphorylation state via PP2A activity control. Since phosphorylated Tau binds less efficient to microtubules [221, 230-233] and shows enhanced retrograde diffusion [185], HSP110 could be a key factor for efficient axonal retention of Tau.

In sum, we identified several interactors of Tau that were either specific for the non-sorting Nterm-Tau or for the axonally enriched Tau isoforms 0N3R and 0N4R. Nterm-Tau was mainly

associated with import proteins and enzymes of the peroxisome, suggesting localization of Nterm-Tau in the peroxisomal lumen. The mechanism of Nterm-Tau import, and the protein fate inside the peroxisome remain elusive. The chaperone HSP110 binds specifically to sorting Tau, and it could be a mediator of axonal Tau retention via PP2A activity control. Strikingly, none of our isoform-specific binding partners could be linked to the axon initial segment (AIS), the structure at the proximal axon, which might be heavily involved in Tau sorting by both anterograde protein transit and selective retrograde retention.

5 Resumé, limitations & outlook

Somatodendritic missorting of Tau is an early hallmark of AD-related Tau pathology [106, 107]. The mechanisms that underly axonal Tau sorting under healthy conditions and cause its disruption under pathological conditions, are poorly understood. The human Tau isoforms show different intracellular distribution, and are associated with disease development [180]. However, the isoform-specific roles of Tau in health and disease are still unclear. Thus, we aimed i) to examine the importance of anterograde Tau transport for overall axonal sorting, ii) to unravel domains, motifs, and cellular interaction partners of Tau required for efficient axonal Tau sorting, and iii) identify isoform-specific interactors that provide evidence for distinct roles of Tau isoforms in health and disease. Here, we described how we addressed these questions methodologically, we explained our findings and set them in relation to recent and former studies to assess their significance and novelty. In sum, our studies provided interesting novel insights into basic aspects of Tau physiology and paved the path for future research on Tau-related neurodegeneration, but much effort is necessary to provide more detailed answers and to eventually develop Tau-targeting therapeutic approaches.

5.1 Tau analysis in an axon severing model: Implications for Tau sorting research and trauma-based tauopathies

Previous studies reported anterograde transport of somatic Tau protein but conclusive evidence for its importance is missing. Here, we developed a neuronal model of acute laser-induced axon depletion, to determine the degree of somatic Tau pathology upon axon loss in human iPSC-derived glutamatergic neurons (Article #5). We found no overt signs for Tau pathology as somatic Tau levels and AD-related AT8 phosphorylation were not elevated after severing. We conclude that either transport of somatically synthesized Tau plays no major role in axonal sorting, or that the injured neurons adapt to the rising somatic levels of axonal protein by compensatory mechanisms.

It is possible that a stop of transcription and/or translation as well as enhanced degradation of Tau and other axonal protein are induced. Pharmacological inhibition of proteasomal or lysosomal activity could reveal whether the protein degradation machinery becomes activated. By using Tau species tagged with photoconvertible proteins such as dendra2 or mEos2, one could directly track the fate of somatic Tau after axotomy. When we conducted preliminary studies with a combination of axon severing and photoconversion, we faced the problem of severing-laser induced conversion. Alternative approaches are required, such as tags with different excitation properties or different severing strategies, to study Tau trafficking in our approach.

We envisioned our axon depletion model to be suitable not only for Tau sorting research, but also to study trauma-based tauopathies such as TBI (McKee). Most current models work with diffuse lesion induction, in which outer mechanical impact causes damage of unknown extent to entire populations of neurons [412]. In contrast, our model would allow precisely steerable and thus reproducible axon injuries of individual neurons. Studies showed that one major cause of Tau pathology is abnormal spreading of Tau released by axonal injury. Due to post-fixation identification of severed neurons, we could also compare the Tau pathology burden in neurons adjacent to the lesion site. One major challenge is, however, that Tau pathology in TBI and CTE occurs months or years after the mechanical insult [33]. Further, our system lacks the cellular environment of the brain, and so the role of microglia or astrocytes or other brain cells that were shown to contribute to the pathogenesis in TBI and related tauopathies [496, 497] remain unaddressed.

5.2 The role of Tau domains and interactions in axonal Tau sorting

Few previous studies addressed the role of single Tau domains and motifs in successful axonal Tau sorting, and the results are incomplete. Thus, we aimed to address this question more systematically with a library of multiple mutant Tau constructs. We started our analysis using human SY-SY5Y neuroblastoma cells that we differentiated into neurons, and found somatic accumulation of the N-terminal half (Article #1). However, the use of SH-SY5Y-derived neurons had considerable limitations, as the cells showed signs of immaturity such as no visible formation of the axon initial segment (AIS), unclear neuronal identify (Articles #2,3), and limited long-term culture stability (Article #1). Thus, we decided to perform our comprehensive sorting analysis with neurogenin 2 (Ngn2)-transgenic human WTC11 iPSCs that we differentiated into glutamatergic neurons (Article #4). We established high transduction rates of our exogenous Tau constructs with lentiviral delivery, and achieved efficient axonal sorting of exogenous Tau that was similar to endogenous Tau, an often-faced challenge in Tau sorting studies [366]. We could use the recently generated subclone of the mentioned iPSC line to avoid interfering effects of endogenous Tau [139].

Our sorting analysis provide novel insights into the Tau sorting process. We claim that the PRR2 domain is critical for successful axonal Tau sorting. In contrast, our data suggest that the N-terminal tail of Tau, the C-terminal repeat domains, and the general Tau microtubule affinity are not relevant for efficient Tau sorting. Thus, our study questions the role of axonal Tau retention mediated by axonal microtubule binding or by annexin interaction, that was claimed previously [255]. The PPR2 domain could control the sorting process in multiple ways: It could directly bind specific interactors that either guide Tau across the AIS or prevent retrograde retention. The PRR domain of Tau is well-known for its various cellular interactions,

including SH3-containing proteins, other signalling proteins, nucleic acids, and f-actin [22]. We aimed to identify interactors specific for sorting Tau isoforms by using TurboID-proximity labelling. We compared 0N3R and 0N4R with the somatically retained N-terminal-half. We found only few proteins that specifically interacted with sorting Tau. Among them was HSP110, an axonal activator of the major Tau phosphatase PP2A. Since PP2A mainly dephosphorylates KXGS residues of the repeat domains, a role of HSP110 in sorting efficiency appears unlikely. For future experiments, it appears more promising to choose another control construct than the N-terminal half with its peculiar distribution. The PRR2-lacking construct would be an obvious candidate in this regard.

There are several reasons why our interactome study had only limited success: Although the TurboID methods labels stable and transient binding partners, the enrichment of biotinylated interactors is inefficient [343]. So, there were probably many sorting-specific interactors that we did not detect due to insensitivity of the pulldown. The specific enrichment of axonal proteins is challenging since the majority of applied biotin will not reach distal axonal regions within the short incubation time, which in turn will overrepresent somatic proteins. Alternatively, proximity labelling could be done with other methods that have slower kinetic properties. The well-established BioID method labels less efficiently but allows labelling times of 18 hours, thus the proportion of axonal hits could be elevated. To avoid noise from somatic or dendritic interactions irrelevant for sorting, the neurons could be cultured in microfluidic chambers that allow distinct harvesting of axonal proteins. Another option would be laser microdissection microscopy that would allow to focus on even smaller regions, such as the AIS. Unravelling AIS-specific differences in interactions between efficiently sorting Tau and Tau constructs with impaired sorting would provide valuable new insights into the molecular basis of the sorting process.

In a next step, one could address the downstream effects of failed axonal sorting: Does PRR2-lacking Tau enrich in the dendrites? Does it mediated tau-mediated toxicity as seen in AD-related somatodendritic missorting? Or does this artificial construct lack prerequisites for toxicity, such as residues that are modified? Our iPSC-derived neurons, which we used successfully to address basic questions of axonal Tau sorting, might have limitations for answering these questions. The basal dendritic levels of all Tau^{HA} constructs and even endogenous Tau were rather high, which could mask possible effects of dendritic missorting. Further, the lack of synaptic spines makes it difficult to measure synaptic toxicity of Tau and to determine its impact on neuronal function. Further research on the maturity of human iPSC-derived neurons is needed to overcome these hurdles of Tau pathology research.

5.3 Tau isoform-specific interactors: Unravelling distinct roles in health and disease

Tau has diverse cellular functions beyond its role in microtubule stability. The six Tau isoforms, which are expressed in the human brain, show remarkable differences in intracellular sorting [185, 186], suggesting distinct contribution to these diverse functions. There is also evidence for isoform-specific roles in Tau pathology and disease progression [180]. However, knowledge about isoform-specific interactions and functions that could underly differential toxicity are sparse.

In this work, we investigated specific interactors of 0N4R- and 0N3R-Tau using the TurboID-based proximity labelling. Despite the comparably little number of found proteins, probably due to both more rigorous filtering for unspecific hits and technical challenges, such as limited axonal penetration of biotin (as detailed in 4.4.1 and 5.2), we revealed that 0N4R interacts with proteins important for synaptic plasticity. The 0N4R-interactors are involved in exocyst-mediated exocytosis, a major pathway of vesicle secretion, and postsynaptic plasticity via the CDC42 pathway. These findings corroborate the notion of isoform-specific cellular functions. Our study could not resolve whether the association with RAB11, MARCKS and other synaptic proteins is specific for 0N4R-Tau or a common feature of 4R isoforms. The interaction site of 0N4R-Tau with these proteins remains unclear but many signalling proteins such as calmodulin or 14-3-3 proteins bind to the repeat domains of Tau. In contrast, direct binding of synaptic vesicles via synaptogyrin-3 interaction is mediated by the N-terminus [430].

Synaptic dysfunction is a key process within Tau pathology, and normal function of both pre- and postsynaptic sites were shown to be impaired in AD and other tauopathies. Our data on 0N4R-specific synaptic interactors lead to the question whether 0N4R contributes to AD-related Tau toxicity both pre- and postsynaptically.

Overactivation of postsynaptic CDC42 leads to Tau hyperphosphorylation via GSK-3β recruitment. 0N4R specifically interacts with GSK-3β and MARCKS, an upstream activator of CDC42. Thus, 0N4R could presumably fulfil two key roles in the cascade of CDC42 pathology. The 0N4R-interactor RAB11 is reduced in many neurodegenerative diseases [474]. In AD, reduced RAB11 levels result in decreased endosomal uptake of β-secretase, the key enzyme for production of Aβ. If 0N4R-Tau regulates RAB11 activity in disease conditions, for instance by increased or reduced binding, it could indirectly deteriorate Aβ pathology. One could test this hypothesis by analysing the RAB11 interaction levels in pathological conditions, e.g. in Aβ-treated iPSC-derived neurons or in suitable AD mouse models [498, 499]. Using our *MAPT*-KO iPSC line, re-expression of individual isoforms under stress conditions could clarify whether synaptic dysfunction is mediated by certain isoforms. Recent studies revealed that FTD-mutant

Tau species bind to presynaptic vesicles via synaptogyrin-3 interaction with the N-terminus, and mediated Tau-induced dysfunction [430, 476].

Taken together, our study provides new insights how Tau domains and modifications affect axonal Tau sorting, suggesting anterograde Tau transport in a PRR2-dependent process. In order to unravel the mechanism of PRR2-mediated axonal Tau sorting, improved strategies for future interactome approaches are crucial. Thereby, our study may help to find new treatments that aim to restore physiological Tau sorting under disease conditions.
Our analysis of isoform-specific interactors revealed isoform-specific interactors that are involved in pre- and postsynaptic plasticity, and AD-associated synaptic dysfunction, such as CDC42 and RAB11. Thus, our interactome study support the idea of isoform-specific roles of Tau in health and disease. These findings might help to elucidate the roles of Tau isoforms in disease progression, and to design tailored strategies for the prevention of Tau-induced neuronal dysfunction.

6 References

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7 Appendix

7.1 Article #1

Bell, M., Bachmann, S., Klimek, J., Langerscheidt, F., Zempel, H. (2021). Axonal TAU Sorting Requires the C-terminus of TAU but is Independent of ANKG and TRIM46 Enrichment at the AIS. *Neuroscience*. 461: 155-171. doi: 10.1016/j.neuroscience.2021.01.041.

7.2 Article #2

Bell, M., Zempel, H. (2021). A simple human cell model for TAU trafficking and tauopathy-related TAU pathology. *Neural Regen Res*. 2022 Apr;17(4):770-772. doi: 10.4103/1673-5374.322450.

7.3 Article #3

Bell, M., Zempel, H. (2021). SH-SY5Y-derived neurons: A human neuronal model system for investigating TAU sorting and neuronal subtype-specific vulnerability. *Reviews in the Neurosciences*. doi: 10.1515/revneuro-2020-0152.

7.4 Article #4

Buchholz S#, Bell-Simons M#, Al Kabbani MA, Cakmak C, Klimek J, Laugsch M, Gan L, Zempel H. (2024). Cultivating, differentiating and transfecting human induced pluripotent stem cells for the investigation of axonal sorting of exogenous and endogenous human TAU. *Methods Mol Biol 2024*. doi: 10.1007/978-1-0716-3629-9_31. (# equal contribution)

7.5 Article #5

Bell-Simons M, Buchholz S, Klimek J, Zempel H. Laser-Induced Axotomy of Human iPSC-Derived and Murine Primary Neurons Decreases Somatic Tau and AT8 Tau Phosphorylation: A Single-Cell Approach to Study Effects of Acute Axonal Damage. Cell Mol Biol. doi: 10.1007/s10571-023-01359-z.

7.6 Article #6

Bell-Simons M, Buchholz S, Klimek J, Zempel H. Axonal Tau sorting depends on the PRR2 domain and 0N4R-specific interactions hint at distinct roles of Tau isoforms in synaptic plasticity. bioRxiv. doi: 10.1101/2024.06.28.601286.

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Gemäß der Promotionsordnung der Math.-Nat. Fakultät vom 12. März 2022

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