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Characterization of CDC25/String as novel phosphatase implicated in Tau modulation and neurodegeneration

Andreia Camila Oliveira

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Andreia Camila Monteiro Oliveira



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Orientadora – Doutora Carla S. Lopes

Investigadora

i3S - Instituto de Investigação e Inovação em Saúde

IBMC - Instituto de Biologia Molecular e Celular

Universidade do Porto, Portugal

Co-orientadora – Doutora Isabel Cardoso

Professora Auxiliar

i3S - Instituto de Investigação e Inovação em Saúde

IBMC - Instituto de Biologia Molecular e Celular

Universidade do Porto, Portugal

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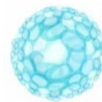
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DOCTORAL PROGRAM:

Molecular and Cell Biology

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Nothing in life is to be feared, it is only to be understood.

Marie Curie

Table of Contents

Financial Support.....	I
Acknowledgements / Agradecimientos	IX
Abstract	XI
Resumo	XIII
List of Publications	XV
List of Figures	XVII
List of Tables	XIX
List of abbreviations	XX
CHAPTER I	1
1. Introduction.....	2
1.1. Tauopathies	2
1.2. Tau protein.....	4
1.2.1. Tau physiological functions.....	6
1.2.2. MAPT/Tau mutations are linked with disease	7
1.2.3. Tau aggregation pathway	7
1.2.4. Tau post-translational modifications.....	8
1.2.5. Tau phosphorylation	10
1.2.6. Phosphorylated Tau as a therapeutic target.....	14
1.2.7. Secretion, uptake and spreading of Tau	16
1.3. <i>Drosophila</i> as a model organism to study tauopathies.....	19
1.4. <i>Drosophila</i> tauopathy models and readouts of neurodegeneration	21
1.4.1. Rough eye phenotype.....	23
1.4.2. Neuronal degeneration and cell death	24
1.4.3. Learning and memory.....	24
1.4.4. Lifespan.....	24
1.5. <i>Drosophila</i> contributions to unveiling the mechanisms underlying Tau-induced toxicity	25
1.6. Cdc25 phosphatases.....	26

1.7. Aims.....	28
CHAPTER II	29
2. Material and methods	30
2.1. Fly strains and genetics.....	30
2.2. Generation of transgenic flies.....	31
2.3. Protein extraction and analysis.....	31
2.4. Dot blot.....	32
2.5. Histology analysis	32
2.6. Immunofluorescence analysis	33
2.7. Proximity-ligation assay (PLA).....	34
2.8. Climbing assays	34
2.9. Scanning Electron Microscopy	35
2.10. Protein extraction for Tau immunoprecipitation	35
2.11. Tau PTMs detection by mass spectrometry analysis	35
2.12. Statistical analysis	36
CHAPTER III	38
3. String/Cdc25 phosphatase is a suppressor of Tau-associated neurodegeneration 39	
3.1. Results.....	39
3.1.1. Stg phosphatase activity suppresses Tau-induced phenotypes.	39
3.1.2. Stg activity reduces Tau phosphorylation levels.....	43
3.1.3. Endogenous Stg clusters with hTau in neurons	44
3.1.4. Stg suppresses Tau-driven neurodegeneration	46
3.1.5. Oligomerization and Tau spreading is impaired by Stg activity	50
3.1.6. Stg suppresses neurotoxicity after Tau aggregation is established	52
3.2. Discussion.....	56
CHAPTER IV	61
4. Analysis of Tau PTMs modified by Stg/Cdc25	62
4.1. Results.....	62

4.1.1.	Analysis of Tau PTMs by LC-MS/MS	62
4.1.2.	Phosphorylation sites identified on Tau.....	66
4.1.3.	Other PTMs identified in Tau	71
4.2.	Discussion.....	73
CHAPTER V		77
5.	Conclusions and future perspectives	78
6.	References	82

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Abstract

Tauopathies are neurodegenerative disorders characterized by the intracellular deposition of abnormal and aggregated forms of Tau protein in the brain. Tau is a microtubule-binding protein that promotes stabilization of axonal microtubules. Tau post-translational modifications (PTMs), in particular phosphorylation, alter its structure and solubility, making Tau more prone to aggregate. Hyperphosphorylated Tau is found in intracellular aggregates and is associated with Tau disassembly from microtubules and neuronal toxicity, in several models. Although phosphorylation is viewed as a major driver of aggregation, the exact pattern(s) of phosphorylation(s) that alter Tau conformation remains unclear. Unbalanced phosphatase (PPase) activity has been proposed as one of the mechanisms leading to Tau hyperphosphorylation in several neurodisorders.

In the present work, we used *Drosophila* as a model to address the molecular mechanisms underlying the interaction between Tau and the fly homologue of mammalian Cdc25 phosphatases String/Cdc25 (Stg/Cdc25) in the context of neurodegeneration. We show that Stg/Cdc25 overexpression rescues the rough eye phenotype associated with expression of human Tau (hTau) in the fly retina. In addition, expression of Stg/Cdc25 reduces brain vacuolization and locomotor deficits, two hallmarks of neurodegeneration in hTau-expressing flies. The amelioration of these phenotypes correlates with reduced Caspase-3 activity and Tau spreading in the fly brain. Importantly, our results show that Stg/Cdc25 phosphatase activity is required to suppress hTau- associated phenotypes, uncovering a role for Stg/Cdc25 as a novel Tau phosphatase. Indeed, we show that Stg/Cdc25 expression promotes reduction of hTau phosphorylation levels at disease-specific residues, Ser202, Thr205 and Thr231. Using a disease mimetic model, we provide evidence that Stg/Cdc25 reduces Tau phosphorylation levels independently of Tau aggregation status and delays neurodegeneration progression in the fly. These findings highlight the potential value of future Cdc25-based therapeutic approaches. Finally, and to uncover hTau neurotoxic PTMs modulated by Stg/Cdc25 activity we performed mass spectrometry analysis. Our results identified phosphorylation of hTau residues Thr231, Ser262 and Thr181 as specifically down-regulated in *hTau* ; *Stg*- expressing flies, suggesting these residues may be directly modified by Cdc25 phosphatase activity. Altogether, the present work uncovers a role for Stg/Cdc25 phosphatases as regulators of Tau biology, that extends beyond their well- characterized function as cell-cycle regulators during cell proliferation, and point-out Cdc25 based approaches as promising entry points to target abnormal Tau phosphorylation in the context of neurodegeneration.

Resumo

As tauopatias são um conjunto de doenças neurodegenerativas caracterizadas pela acumulação intracelular de agregados da proteína Tau no cérebro. A Tau é uma proteína associada aos microtúbulos que promove a estabilização dos microtúbulos axonais. Tau é alvo de diversas modificações pós-tradução (PTM) que alteram a sua estrutura e solubilidade. Estas modificações, em particular a fosforilação, aumentam a propensão para formar agregados. Formas hiperfosforiladas da proteína estão fortemente associadas à toxicidade neuronal e encontram-se presentes em agregados intracelulares em várias doenças neurodegenerativas. Embora a fosforilação de Tau seja considerada um dos principais eventos que conduz à agregação, o(s) padrão(ões) exato(s) de PTMs que alteram a conformação da Tau, estão ainda por determinar. Assim, a identificação de moduladores da fosforilação e agregação da Tau é crucial para uma melhor compreensão da progressão destas doenças bem como para o desenvolvimento de novas abordagens terapêuticas. No presente trabalho, utilizámos *Drosophila* como modelo para abordar os mecanismos moleculares subjacentes à interação entre Tau e o homólogo da mosca das fosfatases Cdc25 de mamíferos String/ Cdc25 (Stg/Cdc25) no contexto da neurodegeneração. Os resultados apresentados demonstram que o aumento da expressão da fosfatase Stg/Cdc25 resgata o fenótipo rugoso do olho, associado à expressão da proteína Tau humana (hTau) na retina da mosca, e reduz a vacuolização cerebral e o déficit locomotor, duas características de modelos de tauopatia em *Drosophila*. As melhorias observadas são acompanhadas por uma redução da atividade da Caspase-3 e propagação da Tau no tecido cerebral da mosca. Os nossos resultados mostram que a atividade fosfatase de Stg/Cdc25 é requerida para a supressão destes fenótipos, o que sugere que Tau é um novo alvo das fosfatases Cdc25. Os resultados obtidos mostram que a expressão de Stg/Cdc25 promove a redução dos níveis de fosforilação de hTau em resíduos específicos associados a tauopatias, nomeadamente Ser202, Thr205 e Thr231. Usando um modelo que mimetiza doença, mostramos que Stg/Cdc25 reduz os níveis de fosforilação de Tau pré-agregada e atrasa a progressão da neurodegeneração. Finalmente, e para identificar PTMs de Tau modificadas pela atividade de Stg/Cdc25, realizámos análise por espectrometria de massa. Os nossos resultados permitiram identificar a desfosforilação dos resíduos Thr231, Ser262 e Thr181 em moscas que expressam hTau; Stg, sugerindo que estes resíduos podem ser alvos diretos da fosfatase Cdc25. Em suma, o presente trabalho revela um papel para as fosfatases Stg/Cdc25 como reguladores da biologia da Tau, que se estende além de sua função bem caracterizada como reguladores do ciclo celular durante a proliferação celular, e

destaca o potencial de abordagens terapêuticas baseadas em Cdc25 como pontos de entrada promissores no tratamento de tauopatias.

List of Publications

Oliveira AC, Santos M, Pinho M, Lopes CS. String/Cdc25 phosphatase is a suppressor of Tau-associated neurodegeneration. *Dis Model Mech.* 2023;16(1): dmm049693. doi:10.1242/dmm.049693

List of Figures

Figure 1. Schematic representation of the human MAPT/Tau gene	5
Figure 2. Schematic representation of the longest Tau isoform (2N4R).	5
Figure 3. Schematic representation of Tau aggregation and NFT formation.	8
Figure 4- Schematic representation of the post-translational modifications (PTMs) identified in the longest Tau isoform (2N4R).	10
Figure 5. Schematic representation of Tau spreading mechanisms.	19
Figure 6. Schematic representation of GAL4-UAS system.	21
Figure 7. Stg suppresses the rough eye phenotype of hTau expressing flies.	40
Figure 8. Stg phosphatase suppresses the rough eye phenotype associated with hTau expression in the fly retina.	41
Figure 9. Stg RNAi does not suppress the rough eye phenotype associated with hTau expression in the fly retina	42
Figure 10. Stg reduces Tau phosphorylation levels.....	43
Figure 11. Stg interacts physically with hTau.	45
Figure 12. Graphical representation of the climbing assay with countercurrent apparatus.....	46
Figure 13. Stg reduces motor deficits associated with hTau expression.....	47
Figure 14. Stg improves locomotor deficits of hTau expressing flies.	48
Figure 15. Stg ameliorates retina morphology and reduces vacuolization	49
Figure 16. Stg reduces Tau spreading.	51
Figure 17. Stg promotes dephosphorylation of hTau toxic species.....	53
Figure 18. Stg reduces vacuolization in disease mimetic flies.	54
Figure 19. Stg expression delays neurodegeneration progression.	55
Figure 20. Western blot analysis of protein extracts from <i>elavG4; Tau-Flag; GFP</i> flies to validate immunoprecipitation (IP) of Flag-tagged hTau protein.	63
Figure 21. Schematic representation of immunoprecipitation followed by Mass-spectrometry workflow using anti-Flag magnetic beads.	63
Figure 22. Sequence coverage map of 2N4R hTau isoform.....	64
Figure 23. Schematic representation of Tau 2N4R protein and the PTMs identified in our analysis.	66

List of Tables

Table 1. Classification of tauopathies.....	3
Table 2. Protein kinases and phosphatases involved in regulation of Tau phosphorylation status.	13
Table 3. List of clinical trials with compounds targeting Tau phosphorylation	15
Table 4. GAL4-UAS models of tauopathy in <i>Drosophila</i>	22
Table 5. List of <i>Drosophila</i> lines used in this study.....	30
Table 6. List of the antibodies used in this study.	37
Table 7. Amino acid sequences obtained by mass spectrometry analysis of the longest hTau isoform (2N4R) and its associated PTMs.	65
Table 8. Abundance ratios of hTau2N4R isoform in samples from TauFlag; GFP, TauFlag; Stg and Tau; StgC379S – expressing flies according to proteome discoverer	66
Table 9. List of the eight phosphopeptides identified and their corresponding protein ratio in hTauFlag; Stg/ hTauFlag; GFP samples.	68
Table 10. List of the phosphopeptides identified and the corresponding protein ratio in hTauFlag; Stg ^{C379S} / hTauFlag;GFP samples.	68
Table 11. List of phosphopeptides identified and the corresponding protein ratio in hTauFlag; Stg/ hTauFlag; Stg ^{C379S} samples.	69
Table 12. Summary of the phosphoresidues identified in the three genotypes under analysis and their phosphorylation ratios comparisons.	70
Table 13. PSM values for each possible p-site identified for the phosphopeptide in sequence [68-87] and quantification of the ratio between the PSM of each p-site and the total PSM determined per sample.	72
Table 14. PSM values for each possible p-site identified for the phosphopeptide in sequence [396-406] and quantification of the ratio between the PSM of each p-site and the total PSM determined per sample.	72

List of abbreviations

α-syn	alpha-synuclein
Aβ	Amyloid beta
AD	Alzheimer's Disease
AMPK	5' adenosine monophosphate-activated protein kinase
BBB	blood-brain-barrier
Cdc25	cell division cycle 25
CDK1	cyclin-dependent kinase 1
CDK5	cyclin-dependent kinase 5
CNS	central nervous system
CSF	cerebrospinal fluid
GSK-3 β	glycogen synthase kinase-3 beta
HMW	high molecular weight
hTau	human Tau
ISF	interstitial fluid
LC-MS/MS	liquid chromatography with tandem mass spectrometry
MAP	microtubule-associated protein
MARK	microtubule affinity-regulating kinases
MBD	microtubule binding domain
MBR	microtubule binding repeats
MT	microtubule
NDD	neurodegenerative diseases
NFT	neurofibrillary tangles
UAS	upstream activator sequence
PD	Parkinson's disease
PHF	paired helical filaments

PLA	proximity ligation assay
PP1	Protein phosphatase 1
PP2	Protein phosphatase 2
Pro	Proline
RNA	ribonucleic acid
Sgg	Shaggy
Stg	String
Ser	Serine
TEM	Transmission electron microscopy
TTBK	Tau-tubulin kinases
Thr	Threonine
Tyr	Tyrosine
TNT	tunneling nanotubes
WHO	World Health Organization
WT	wild type

CHAPTER I

Introduction

1. Introduction

Human neurodegenerative diseases (NDD) are a group of age-dependent disorders characterized by progressive dysfunction of neurons and glial cells, for which there is no cure available. Neurons are incapable of self-renewal due to their differentiated nature. Therefore, loss of neurons and consequent disruption of the structure and function of neural network results in dementia and/ or motor deficits. Dementia is characterized by severe cognitive dysfunction, including impaired memory and behavior alterations, features of Alzheimer's disease (AD), frontotemporal dementia or dementia with Lewy bodies. Movement disorders are mainly characterized by the loss of motor control, in the form of lowered movements, rigidity (stiffness) and tremors, a condition called parkinsonism. Parkinson's disease (PD) and Huntington's disease (HD) belong to this category. However, dementia and movement disorders are not mutually exclusively, so dementias can also be accompanied by motor symptoms (Wilson et al., 2023).

The prevalence of these incurable diseases is increasing, in part, as a consequence of global population ageing (Heemels, 2016). Nowadays, about 55 million people live with a NDD and it is predictable that in 2060 this number will increase to 152 million. Dementia is currently the seventh leading cause of death and one of the major causes of disability and dependency among older people globally (WHO). In 2019, the annual global societal costs were estimated at 1189,42 billion EUR for people with dementia (Wimo et al., 2023). Besides ageing, NDD development is a result of genetic predisposition and environmental factors (Lampthey et al., 2022).

Neuropathological and genetic studies reveal that most NDDs are caused by misfolding, aggregation and accumulation of proteins in the brain, resulting in neuronal loss. This class of NDD are called proteinopathies and include amyloidoses, tauopathies and synucleinopathies (Bayer, 2015).

1.1. Tauopathies

Tauopathies comprise a group of NDD characterized by the intracellular accumulation of Tau/MAPT protein in the brain. So far were identified 28 Tau-associated diseases with overlapping, but distinct, clinical symptoms including dementia, parkinsonism, or a combination of both. The classification of tauopathies is based on cell types and anatomic regions affected and type of Tau isoforms involved (Ling, 2018). In different tauopathies, the aggregates are deposited in distinct brain regions including the hippocampus/entorhinal cortex, cortical regions, and mid/hindbrain. Although all have in common the presence of hyperphosphorylated and

misfolded Tau in aggregates, the protein isoforms involved can vary. At the neuropathological level, tauopathies are divided into primary - when Tau pathology is the major contributing factor for the disease- or secondary tauopathies – when associated to other pathologies besides Tau aggregation such as Amyloid β (A β) or α -Synuclein (α -Syn) deposition (Arendt et al., 2016). The first group includes several types of familiar or sporadic diseases including Progressive Supranuclear Palsy (PSP), Argyrophilic Grain Disease (AGD), Corticobasal degeneration (CBD), Pick's disease (PiD), Frontotemporal Dementia and Parkinsonism linked to chromosome 17 (FTDP-17), Postencephalitic Parkinsonism (PEP) and Parkinson's dementia complex (PDC) of Guam. On the other hand, AD and PD are included in the second group (Bussiere and Hof, 2000) (Table 1).

Depending on the Tau isoform identified in the intracellular inclusions, tauopathies are classified into 3R-tauopathies (PiD), 4R tauopathies (PSP and CBD) and 3R:4R tauopathies (FTDP-17 and AD) (Bartolome et al., 2022) (Table 1).

Table 1. Classification of tauopathies

Primary tauopathy	Predominant Tau isoform
Progressive Supranuclear Palsy (PSP)	4R
Argyrophilic Grain Disease (AGD),	4R
Corticobasal degeneration (CBD),	4R
Pick's disease (PiD)	3R
Frontotemporal Dementia and Parkinsonism linked to chromosome 17 (FTDP-17)	3R,4R, 3R+4R
Postencephalitic Parkinsonism (PEP)	3R+4R
Parkinson-dementia complex (PDC) of Guam	3R+4R
Globular glial tauopathy	4R
Secondary tauopathy	
Alzheimer's Disease (AD)	3R+4R
Parkinson's Disease (PD)	3R+4R
Down's syndrome	3R+4R
Dementia pugilistica	3R+4R
Familial British Dementia	-

1.2. Tau protein

Tau, initially discovered by Weingarten et al. (1975), is a microtubule (MT)-binding protein (Drechsel et al., 1992; Weingarten et al., 1975; Witman et al., 1976) that belongs to the microtubule-associated proteins (MAPs) family. Tau is the most abundant of all neuronal MAPs present in the central nervous system (CNS) (Barbier et al., 2019).

Human Tau (hTau) is encoded by the MAPT gene located in chromosome 17q21 (Neve et al., 1986). The gene contains a total of 16 exons. The alternative splicing of exons 2, 3 and 10 generates six different protein isoforms with a size range between 352 and 441 amino acids (aa) (Andreadis et al., 1992; Andreadis et al., 1995). The differences between the six isoforms rely on the number of amino-terminal inserts (29 amino acids each) that can be zero (0N), one (1N) or two (2N), and in the presence of three (3R) or four (4R) microtubule-binding domain repeats (Figure 1) (Martin et al., 2011). The expression of Tau isoforms is tissue specific and developmentally regulated revealing different physiological roles between them (Sergeant et al., 2005). Although only the shortest isoform (0N3R) is expressed during fetal stages, all the six isoforms are expressed in the adult healthy brain with similar concentrations of 3R and 4R (Kosik et al., 1989). However, the relative concentrations of N-terminal variations are different: ~37% of 0N; ~54% of 1N and ~9% of 2N (Goedert and Jakes, 1990; Kosik et al., 1989).

The MT-binding affinity is different depending on the Tau isoform. 4R Tau has a higher affinity for microtubules than 3R Tau, and consequently higher efficiency to promote microtubule assembly (Goedert and Jakes, 1990). mRNA and protein levels of Tau also vary in different brain regions, with levels two times higher in the neocortex than in white matter and cerebellum (Trabzuni et al., 2012). A high molecular weight Tau isoform, called “big Tau”, with 695 amino acids, is expressed predominantly in the peripheral nervous system. This isoform is identical to the 2N4R longest but contains an additional 254 amino acid insert in the amino-terminal region (Georgieff et al., 1991; Goedert et al., 1992).

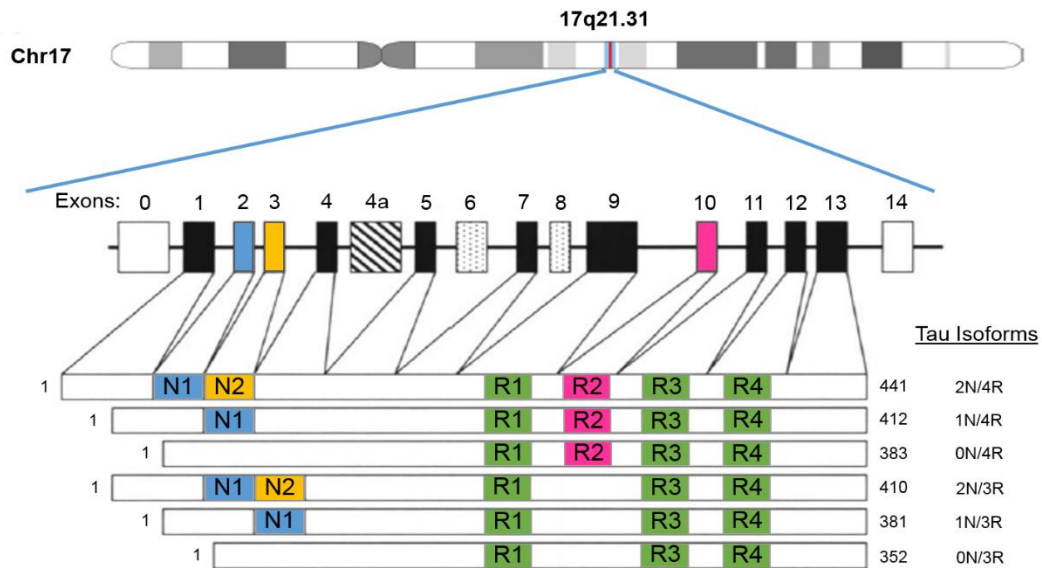


Figure 1. Schematic representation of the human MAPT/Tau gene. Tau is located in the longest arm of chromosome 17. It contains 16 exons but only 13 are transcribed. Combinations of the 13 exons give rise to six Tau isoforms that range from 352 to 441 amino acids. Exons 1,4,5,7,9,11,12 and 13, represented by black boxes, are present in all isoforms while exons 2,3 and 10 are alternatively spliced. Tau can contain zero (0N), one (1N) or two (2N) N-terminal inserts encoded by exons 2 and 3 (blue and yellow boxes), and three (3R) or four (4R) microtubule-binding repeats depending on the inclusion of exon 10 (pink box). Tau isoforms are described according to the domains included in the protein:2N4R, 1N4R, 0N4R, 2N3R, 1N3R and 0N3R. Adapted from (Arendt et al., 2016; Park et al., 2016).

Four protein domains are identified in Tau: 1) N-terminal, 2) proline-rich, 3) microtubule binding repeats (MBR) -that contains 4 repeat domains R1, R2, R3, and R4 - and 4) C-terminal domain (Cleveland et al., 1977). From the functional perspective, these domains are grouped into two: the projection domain, which consists in the N- terminal domain of the protein and is involved in microtubule spacing, membrane anchoring and Tau dimerization (Melo et al., 2016), and the microtubule binding domain (MBD), which spans from the proline-rich to the C-terminal domain and mediates interactions with microtubules (Figure 2) (Mukrasch et al., 2007).

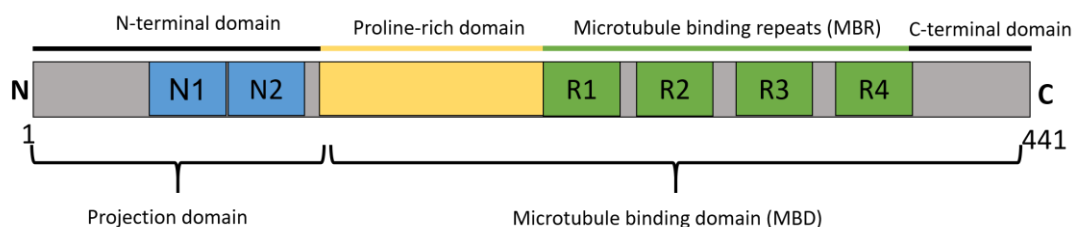


Figure 2. Schematic representation of the longest Tau isoform (2N4R). Tau protein can be subdivided into four protein domains: N-terminal, proline-rich, microtubule binding repeats (MBR) -containing the repeats R1, R2, R3 and R4- and the C-terminal domain. From the functional perspective, 2 domains are defined: the projection domain, and the microtubule binding domain (MBD).

In the human brain, Tau is mainly found in neurons (Binder et al., 1985), however, Tau can also be present, at lower levels, in dendrites and some types of glial cells such as astrocytes and oligodendrocytes (Lopresti et al., 1995; Migheli et al., 1988). In the neuron, Tau is predominantly localized in the cytoplasm and concentrated in the axon as its function suggests (Drubin and Kirschner, 1986). Tau can be also found in other compartments as in the nucleus, mitochondria, dendrites, synapses, and at the plasma membrane (reviewed in (Brunello et al., 2019)).

1.2.1. Tau physiological functions

Initial studies revealed that Tau modulates and stabilizes the assembly of microtubules (Cleveland et al., 1977; Weingarten et al., 1975), in a phosphorylation-dependent manner, promoting MT nucleation, growth and bundling (Drewes et al., 1995), and also reducing MT dynamics and instability (Drubin and Kirschner, 1986). However, Tau has numerous other functions. As Tau interacts with microtubules in a dynamic way, it regulates the dynamic reorganization of the cytoskeleton promoting neurite outgrowth and stabilization (Feinstein and Wilson, 2005). Tau is also important to cytoskeleton organization as it binds to microtubules and actin simultaneously, promoting microtubule-actin co-alignment (Elie et al., 2015).

Additionally, Tau is also implicated in axonal transport (Wang and Mandelkow, 2016) by influencing the motor function of dynein and kinesin which transport cargoes towards the minus ends (towards the cell body) and plus ends of microtubules (towards axonal terminus), respectively (Stamer et al., 2002).

It was suggested that the small amount of Tau present in dendrites and dendritic spines may regulate synaptic plasticity. Using hippocampal slices and *in vitro* mouse neurons it was demonstrated that pharmacological synaptic activation induces translocation of endogenous Tau from the dendritic shaft to excitatory postsynaptic compartments (Frandsen et al. 2014). It was also proposed that Tau can mediate alterations in the cytoskeletal structure of dendrites and spines through its interaction with partners such as tubulin, F-actin and Scr family kinases (Morris et al. 2011). Kimura et al. (2014) demonstrated that knocking down Tau in mice affects long-term depression mechanisms.

The nuclear role of Tau remains unclear despite it was demonstrated that Tau can bind directly to DNA and RNA (Antón-Fernández et al., 2023; Sultan et al., 2011). Nuclear Tau binds to DNA through the proline rich and microtubule binding domains (Camero et al., 2014) contributing to DNA protection in both physiological and in stress conditions (Maina et al., 2018; Sultan et al., 2011; Violet et al., 2014). Nuclear Tau has also been shown to regulate gene expression. Tau binds to the AT-rich minor groove of

DNA, causing a conformational change that affects the activity of multi-protein DNA complexes that control gene transcription (Rossi et al., 2013; Violet et al., 2014). Finally, nuclear Tau has also been reported to be involved in chromosome stability and processing/silencing of rRNA (Rossi et al., 2013).

1.2.2. MAPT/Tau mutations are linked with disease

The identification of MAPT mutations in some neurodegenerative diseases like Frontotemporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17) strongly implicates that Tau dysfunction is sufficient to cause neurodegeneration. The first MAPT mutations were reported In 1998, in exons 9, 10 and 13, and in intron 10 (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Since then, more than 50 MAPT mutations were identified including missense, silent, deletion or intronic mutations (Ghetti et al., 2015). Most of the mutations are located in exon 10 or in proximal regions, disrupting the ratio between 3R and 4R Tau isoforms. MAPT mutations reduce Tau- microtubule binding affinity and tubulin polymerization which in turn affects cytoskeletal functions. Exonic mutations increase the propensity of Tau to post-translational modifications (PTMs), particularly phosphorylation. Although alterations in 3R/4R ratio are linked with mutations on MAPT, the unbalance between Tau isoforms is also present in sporadic diseases. Therefore, other mechanisms rather than MAPT transcription are involved in the regulation of Tau splicing (Guo et al., 2017).

1.2.3. Tau agregation pathway

In healthy neurons, Tau is an unfolded and highly soluble protein that holds a relaxed conformation with a low tendency for secondary structure formation. Under pathological conditions, such as genetic mutations, post-translational modifications, tau proteins can misfold, starting as monomers with an altered conformation prone to aggregation (Kontaxi et al., 2017). These monomers can aggregate into small toxic clusters called tau oligomers, which further assemble into larger tau filaments termed paired helical filaments (PHF). The accumulation of these filaments within neurons leads to the formation of neurofibrillary tangles (NFT), disrupting cellular processes and contributing to neuronal dysfunction and cell death (Cowan and Mudher, 2013; Meraz-Rios et al., 2010) (Figure 3). This process has been suggested to occur in a “prion-like” manner as Tau can seed further aggregation of Tau. This process can lead to the progressive spread of tau pathology from one brain region to another and is believed to contribute to the progression of neurodegenerative diseases (Ayers et al., 2018).

The mechanisms behind Tau aggregation are not completely understood. It is well established that Tau aggregates are enriched in hyperphosphorylated Tau and thus phosphorylation has been viewed as the trigger in tauopathy (Kontaxi et al., 2017; Ping et al., 2018). However, other PTMs such as acetylation, glycosylation, glycation, methylation and truncation (Martin et al., 2011) and MAPT mutations (Guo et al., 2017) have also been implicated in Tau conformational alterations that enhance Tau aggregation propensity.

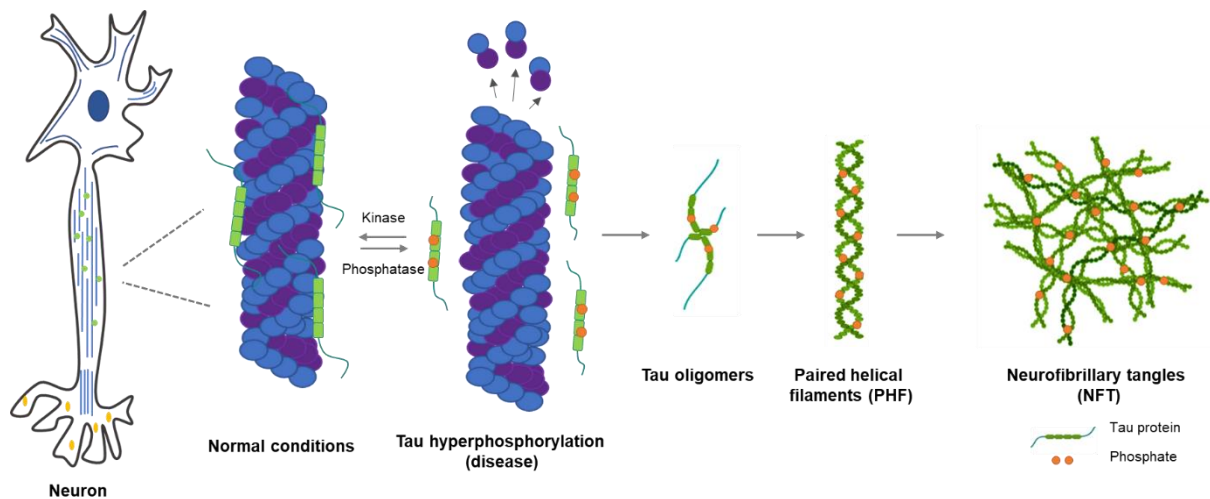


Figure 3. Schematic representation of Tau aggregation and NFT formation. Tau dynamic interaction with microtubules is regulated by phosphorylation. Tau hyperphosphorylation (p-Tau) reduces its microtubule binding affinity and promotes conformational changes that favour Tau dimerization and aggregation. Tau oligomers are formed upon assembly of Tau dimers. Oligomers assemble into PHFs, which in turn lead to NFT formation.

1.2.4. Tau post-translational modifications

Post-translational modifications refer to protein modifications that occur just after their translation by ribosomes or after their folding and localization are complete. PTMs alter the charge and hydrophobicity of proteins, which results in structural changes that alter the stability and cellular localization, protein-protein interactions, and/ or aggregation propensity (Alquezar et al., 2021).

The lack of tertiary structure makes Tau more susceptible to PTMs. A variety of PTMs, including phosphorylation, acetylation, ubiquitination, methylation, oxidation, deamination, proteolysis, SUMOylation, glycation, O-GlcNAcylation, N-glycosylation, isomerization and nitration have been identified in Tau (reviewed in (Alquezar et al., 2021; Ye et al., 2022). Around 35 % of Tau residues are susceptible to modification, including serine (Ser), threonine (Thr), tyrosine (Tyr), lysine (Lys), arginine (Arg),

asparagine (Asn), histidine (His), and cysteine (Cys) (Alquezar et al., 2021). The observation that certain PTMs dispute the same amino acid residues indicates that Tau function, localization and solubility can be regulated by crosstalk between different PTMs (Alquezar et al., 2021; Limorenko and Lashuel, 2022) (Figure 4).

Although Tau PTMs are important to its physiological function, some PTMs are only found in Tau aggregates associated with disease, suggesting that these modifications can drive Tau pathogenesis. In summary, Tau PTMs alters its solubility, localization, and interaction capacity which enhances Tau propensity to assemble into β -sheet structures and consequently form aggregates. It is accepted that transformation from a “normal” to a pathogenic state is not one-step event but a consequence of intrinsic structural alterations combined with extrinsic cellular conditions. In fact, both Tau function and aggregation are a consequence of different PTMs that can modify the protein at distinct residues or compete for the same residue. Serine and threonine residues can be modified by phosphorylation and O-GlcNAcylation. Several studies revealed that O-GlcNAcylation levels of Tau are decreased in AD brain compared with normal brain, which in turn leads to an enrichment in Tau phosphorylation and consequently Tau detachment from MTs and aggregation (Gong et al., 2016; Liu et al., 2004). Lysine can be modified by acetylation, ubiquitination, methylation, SUMOylation, and glycation. Depending on the modification the consequences on Tau structure, function and aggregation be different. Luo et al. (2014) observed that SUMOylation at Lys340 competes with its ubiquitination, which inhibits Tau degradation and consequently leads to Tau accumulation (Luo et al., 2014). Another example is Lys254, which can be modified by methylation and ubiquitination. It is reported that methylation at Lys254 is a common feature of PHF-Tau suggesting that this modification blocks Tau ubiquitination-associated degradation (Thomas et al., 2012). *In vivo* studies showed that accumulation of acetylated-Tau blocks Tau proteasome-mediated degradation (Min et al., 2010). Additionally, certain PTMs render Tau more prone for further modifications. For example, methylation at Lys267 precedes and predisposes Tau to be phosphorylated at Ser262, which can result in Tau hyperphosphorylation. On the other hand, phosphorylation can also affect the propensity of Tau to undergo other PTMs. It was shown, *in vitro*, that pseudo phosphorylation at Ser422 precedes and blocks the cleavage of Tau at Asp421 by Caspase-3 (Guillozet-Bongaarts et al., 2006).

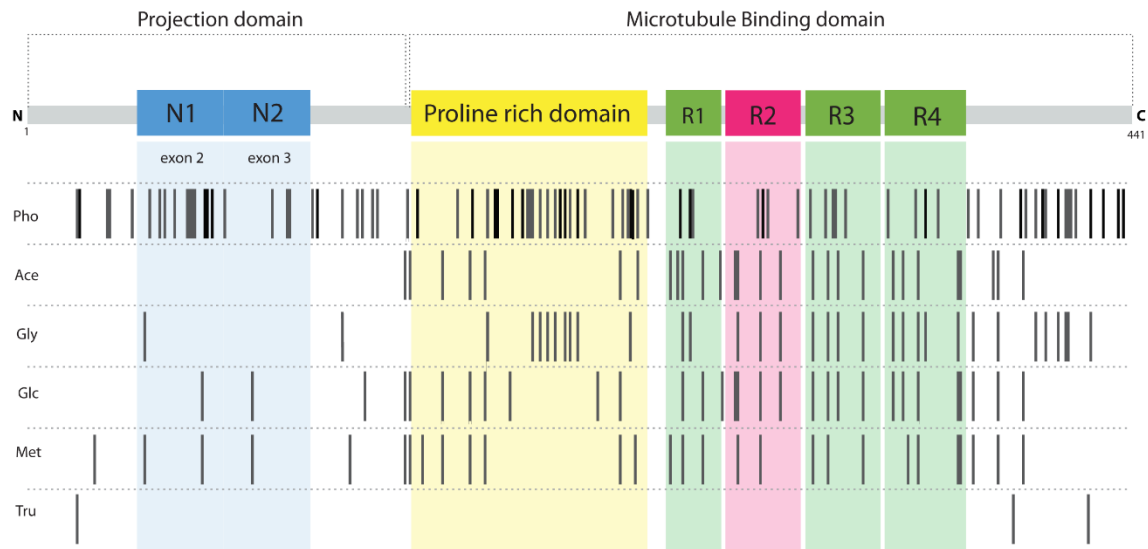


Figure 4- Schematic representation of the post-translational modifications (PTMs) identified in the longest Tau isoform (2N4R). Each bar represents an amino acid residue. The PTMs represented were identified in brain samples, *in vivo* studies using tauopathy models and *in vitro* approaches, and represent modifications occurring in normal and pathologic conditions. The black bars highlight phosphorylated residues observed only in disease conditions. The different domains of Tau are highlighted. Pho-phosphorylation; Ace-acetylation; Gly- glycosylation; Gly-glycation; Met- methylation; Tru-truncation (yellow)

1.2.5. Tau phosphorylation

Phosphorylation of Tau is the most frequent PTM studied to date (Guo et al., 2017). There are 85 putative phosphorylation sites in the longest Tau isoform, comprising 45 serine, 34 threonine and 5 tyrosine residues. Thus, almost 20% of the protein has the potential to be phosphorylated (Arendt et al., 2016; Chun and Johnson, 2007; Guo et al., 2017). The majority of these sites are clustered in the proline-rich domain of Tau, flanking the microtubule-binding domain (Arendt et al., 2016; Noble et al., 2003) (Figure 4). Therefore, it is understandable that phosphorylation has a huge impact on Tau physiology.

Tau phosphorylation status is important to control cellular processes in which Tau participates in a variety of metabolic and intracellular pathways. During embryogenesis and early development, fetal Tau is highly phosphorylated to support cytoskeleton plasticity, specially at Thr181, Ser199, Ser202, Thr205, Thr231, Ser396 and Ser404 (Yu et al., 2009). In contrast, under physiological conditions, the levels of Tau phosphorylation in the adult brain are reduced. Phosphorylation of Tau also regulates its own cellular localization. In neurons, Tau localizes mainly in the axon, where it exhibits a gradient distribution, with dephosphorylated Tau localizing preferentially at the distal region (Mandell and Banker, 1996). Tau also localizes to the nucleus, where is predominantly found in the dephosphorylated form (Loomis et al.,

1990); dephosphorylated Tau was also found at the plasma membrane (Pooler et al., 2012). Tau Phosphorylation at Ser202/Thr205 residues is responsible for its translocation to dendrites and dendritic spines (Ittner et al., 2016; Zempel et al., 2010). Tau phosphorylation regulates neuronal functions such as axonogenesis and neurite outgrowth through MT binding (Biernat and Mandelkow, 1999; Mandell and Banker, 1996; Yu et al., 2009).

Increased Tau phosphorylation leads to decreased Tau affinity to axonal microtubules, which in turn results in cytoskeleton destabilization, in neurons (Guo et al., 2017). It was demonstrated that phosphorylation at Thr231, Ser262, Ser396, or Ser404 reduces the affinity of Tau to bind Mts and consequently affects MT dynamic stability (Forman et al., 2000; Kiris et al., 2011; Melo et al., 2016; Sengupta et al., 1998). When Tau is dissociated from MTs, in consequence of hyperphosphorylation, is more prone to form aggregates. In agreement, it was shown that phosphorylation of Tau is three to four-fold higher in disease brains compared with normal brains (Kopke et al., 1993), and Tau hyperphosphorylation is described as the main driver of NFTs formation (Iqbal et al., 2005; Sengupta et al., 1998).

Phosphorylation at residues Thr231, Ser235 and Ser262 was the first to be identified in AD brains. Since then, several residues have been identified and nowadays there are more than 40 Tau phosphorylatable sites associated with several tauopathies (such as AD, PD and DLW) (Kimura et al., 2018; Martin et al., 2011). Although some of these phospho residues are essential for Tau neuronal function and are found in non-disease brain, the majority is exclusive of disease conditions (Martin et al., 2011).

The status of Tau phosphorylation depends on the balance between the activity of protein kinases and phosphatases (Iqbal et al., 2005). Three main classes of kinases act on Tau (Table 2):

1) Proline-directed kinases: Glycogen Synthase kinase-3 beta (GSK-3 β) (Hanger et al., 1992; Mudher et al., 2004; Noble et al., 2005); Cyclin-Dependent kinase 5 (cdk5) (Hanger et al., 1992); 5' Adenosine Monophosphate-activated Protein kinase (AMPK) (Thornton et al., 2011), Mitogen-activated protein kinase (MAPK), including p38, Extracellular signal-regulated kinases 1 and 2 (Erk1/2) and c-Jun N-terminal kinases (JNK) (Drewes et al., 1992);

2) Non-proline-directed kinases: Casein kinase 1 (CK1); CK2 (Takahashi et al., 1995); Microtubule Affinity-regulating kinases (MARKs) (Lee et al., 2012; Trinczek et al., 1995); Tau-tubulin kinases (TTBKs) (Takahashi et al., 1995; Taylor et al., 2018); cyclic AMP-dependent Protein Kinase A (PKA); PKB; PKC (Andorfer and Davies,

2000); and dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK-1A) (Woods et al., 2001);

3) Tyrosine kinases: Fyn (Williamson et al., 2002); Abl (Cancino et al., 2011); and Syk (Lebouvier et al., 2008).

The expression and/or activity of some of these proteins was shown to be deregulated in several tauopathies (Kimura et al., 2014). GSK3 β is highly expressed in the brain, particularly in neurons (Krishnankutty et al., 2017), where it is known to interact with microtubules. This made GSK3 β a prime candidate involved in abnormal Tau phosphorylation. In agreement, in AD brains, increased activity of GSK3 β correlates with the formation of NFTs (Leroy et al., 2002). Several studies revealed that upon GSK3 β overexpression, the number of Tau phosphorylated sites increases (Guo et al., 2017; Ittner et al., 2016). In mice, overexpression of GSK3 β increases Tau phosphorylation levels, which in turn leads to a neurodegenerative phenotype (Lucas et al., 2001). In contrast, the inhibition of GSK3 β reduces Tau phosphorylation levels, and axonal degeneration and rescues neuronal loss, also in mouse models (Caccamo et al., 2007; Noble et al., 2013; Sereno et al., 2009). These results highlight inhibition of GSK3 β as a good therapeutic approach for Tau-associated neurodegenerative disorders. Following this hypothesis, Jhang and colleagues (2017) promoted GSK3 β downregulation via resveratrol - a natural polyphenolic phytoestrogen –and achieved a reduction in Tau phosphorylation, at Ser396, in rat hippocampal slices (Jhang et al., 2017).

As observed for GSK3 β there are evidences suggesting that CDK5 co-localizes with Tau aggregates and its activity is increased in primary tauopathies, as well as in AD (Liu et al., 2003). The abnormal expression of CDK5, in mice overexpressing a mutant isoform of hTau, enhances Tau phosphorylation and leads to NFT formation (Cruz et al., 2003; Noble et al., 2003).

Phosphatases can counteract the action of Tau kinases. Tau is directly dephosphorylated by several phosphatases, including Protein Phosphatase 1 (PP1), 2A (PP2A), 2B (PP2B) and 5 (PP5) (Tian and Wang, 2002) (Table 2). The expression and/or activity of some of these phosphatases is reduced in the damaged area of AD brains (Lian et al., 2001; Liu et al., 2005; Sontag et al., 2004; Vogelsberg-Ragaglia et al., 2001). For example, PP2A expression was found reduced to half in AD brains (Liu et al., 2005). *in vitro* studies showed that PP2A, which is responsible for approximately 70% of Tau dephosphorylation, can promote dissociation of Tau from PHFs formed in AD samples, restoring the ability to bind microtubules (Wang et al., 1995; Wang et al., 2007). The activity of PP2A is controlled by Inhibitor-1 (I1PP2A) and Inhibitor-2 (I2PP2A), which bind to the catalytic subunit of PP2A, leading to its inhibition.

Interestingly, both, I1PP2A and I2PP2A are upregulated in AD brains (Tanimukai et al., 2005). Due to its structure and composition PP2A -a holoenzyme with several regulatory subunits- is a challenging therapeutic target (Wang et al., 2013). Moreover, PP2A regulates the activity of proteins of the MAPK cascade that activate GSK3 β , suggesting a potential feedback loop (Kamat et al., 2014).

In summary, increased levels of Tau kinases and decreased activity of Tau phosphatases are associated with abnormal phosphorylation patterns observed in tauopathies (Guo et al., 2017). Therefore, comprehensive and mechanistic studies involving Tau kinases and phosphatases are essential to uncover and explore novel therapeutic targets and approaches.

Table 2. Protein kinases and phosphatases involved in regulation of Tau phosphorylation status.

Proline directed protein kinases (PDPK)	
Kinases	Glycogen Synthase kinase-3 beta (GSK-3 β)
	Cyclin-Dependent kinase 5 (cdk5)
	5' Adenosine Monophosphate-activated Protein kinase (AMPK)
	Mitogen activated protein kinase (MAPK)
	Non-Proline directed protein kinases (NPDPK)
	Casein kinase 1 (CK1)
	Microtubule Affinity-regulating kinases (MARKs)
	Tau-tubulin kinases (TTBK)
	cyclic AMP-dependent Protein Kinase A (PKA)
	dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK-1A)
	Tyrosine kinases
	Fyn
	Abl
	Sky
Phosphatases	Protein phosphatase 1 (PP1)
	Protein phosphatase 2A (PP2A)
	Protein phosphatase 2B (PP2B)
	Protein phosphatase 5 (PP5)

1.2.6. Phosphorylated Tau as a therapeutic target

We still lack effective treatments for Tau-associated disorders. However, new therapeutic approaches aiming to target Tau phosphorylation have been developed recently. This can be a promising approach since Tau phosphorylation is key in early disease progression and there are several phosphorylatable sites to target. The current therapeutic approaches targeting Tau phosphorylation are based in three main strategies: kinase inhibitors, phosphatase activators, and immunotherapy. These strategies have in common the reduction of Tau phosphorylation by the direct reduction of the levels of p-Tau (with the use of kinase inhibitors and phosphatase inhibitors) or the clearance of p-Tau (p-Tau immunotherapies) (Shahpasand et al., 2018; Xia et al., 2021).

Tideglusib, Lithium, Valproate and Nilotinib are kinase inhibitory drugs currently used to treat some tauopathies (reviewed in (Xia et al., 2021). Tideglusib (Domínguez et al., 2012) and Lithium (Hong et al., 1997; Noble et al., 2005) act as inhibitors of GSK3- β and are capable of decreasing Tau hyperphosphorylation and consequently slow neurodegeneration progression in AD. Valproate is used to treat bipolar disorder and acts by inhibiting the two main Tau kinases: GSK3- β and Cdk5 (Cipriani et al., 2013; Long et al., 2015). Nilotinib was first approved to treat chronic myeloid leukemia (Sacha and Saglio, 2019). However, it can be also used as a therapeutic in AD because it inhibits Abl kinase (La Barbera et al., 2021). In the other hand, sodium selenate acts on Tau dephosphorylation by promoting the activation of PP2 (Domínguez et al., 2012) (Table 3).

Nowadays, immunotherapies targeting phosphorylated Tau are currently viewed as the most promising therapeutic approaches. There are two main strategies regarding p-Tau immunotherapies: active immunotherapies, which involves the administration of a vaccine to cause an immune response against the epitope; and passive immunotherapy, which relies on the administration of a specific antibody that will target a particular antigen (Shahpasand et al., 2018; Xia et al., 2021). The last one is viewed as the most promising strategy because, in animal models, the administration of Tau-targeting antibodies lead to the clearance of Tau toxic species and aggregates. However, this approach implicates continuous administration of the antibody, in contrast to vaccine administration (Xia et al., 2021). A number of compounds of Tau protein-targeted immunotherapies are under clinical trials or have been recently approved (reviewed in (Shahpasand et al., 2018; Xia et al., 2021) (Table 3).

Table 3. List of clinical trials with compounds targeting Tau phosphorylation

Type	Drug name	Mechanism	Clinical Trial ID	Status
Kinase Inhibitor	Tideglusib	Inhibits GSK3- β	NCT01350362	Completed
			NCT01049399	Completed
			NCT00948259	Completed
	Lithium	Inhibits GSK3- β	ISRCTN7204646 2	Completed
			NCT00088387	Completed
			NCT01055392	Completed
			NCT02601859	Completed
			NCT03185208	In Progress
	Valproate	Inhibits GSK3- β	NCT01729598	Completed
			NCT00088387	Completed
			NCT00071721	Completed
	Nilotinib	Inhibits tyrosine kinase	NCT02947893	Completed
Phosphatase Activator	Sodium Selenate	Activates PP2A	ACTRN 12611001200976	Completed
			ACTRN 12620000236998	In Progress
	J-63733657	Antibody against	NCT03375697	Completed

Type	Drug name	Mechanism	Clinical Trial ID	Status
Immunotherapy		pThr217	NCT03689153	Completed
			NCT04619420	In Progress
	PNT001	Antibody against Cis isomer of pThr231	NCT04677829	In Progress
			NCT04096287	In Progress
	Lu AF87908	Antibody against pSer396	NCT04149860	In Progress
	RO6926496	Antibody against pSer404	NCT02281786	Completed
	ACI-35	Antibody against pSer404	NCT04445831	In Progress

1.2.7. Secretion, uptake and spreading of Tau

Tau propagation is a multi-step process that involves secretion of pathological Tau, cellular uptake from the recipient cells and formation of new intercellular aggregates (Zhang et al., 2021). Tau was always described as an intracellular protein, however, recent studies showed that Tau can also be found in the extracellular space (reviewed in Yamada, 2017). Although initially believed that Tau release occurs as a consequence of cell death, it is already demonstrated that extracellular Tau can exist independently of cell death or neurodegeneration (Fuster-Matanzo et al., 2018). *In vitro* studies showed that in conditions of Tau overexpression, the full-length or truncated forms, can be secreted by neuronal and non-neuronal cells (Pérez et al., 2016; Saman et al., 2012; Simón et al., 2012). Moreover, microdialysis-based measurements in mice and humans allowed the detection of Tau in the interstitial fluid (ISF) and cerebrospinal fluid (CSF) (Magnoni et al., 2012; Yamada et al., 2011). Although the physiological function of extracellular Tau remains poorly understood, it is in the spotlight of investigators due to the potential role in transcellular propagation.

Frost et al. (2009) showed that contrary to monomeric Tau, extracellular Tau aggregates can be uptaken by cells in culture. Moreover, the internalized aggregates can induce fibrilization of intracellular full-length Tau (Frost et al., 2009). These findings suggest a possible role for extracellular toxic species in Tau pathogenesis. In agreement, subsequent studies showed that mouse cortical neurons can only efficiently uptake high molecular-weight (HMW) Tau species, and the use of cell chambers with a microfluidic chip demonstrated that Tau oligomers are easily propagated in neuronal cells when compared to Tau monomers (Takeda et al., 2015; Wu et al., 2013). Michel et al. (2014) proposed that monomeric exogenous Tau is endocytosed by SH-SY5Y cells (Michel et al., 2014). However, Mirbaha et al. (2015) demonstrated that a minimum of 3-unit assemblies of Tau is required to induce intracellular Tau aggregation in HEK293 cells (Mirbaha et al., 2015).

Besides *in vitro* models, studies in mouse models showed that extracellular Tau aggregates can be uptaken by cells. Clavaguera and colleagues (2009) injected brain extracts from mutant P301S Tau-expressing mice into the brain of transgenic wild-type Tau-expressing animals and observed that wild-type human Tau (hTau) formed filaments and spread from the site of injection to adjacent brain regions (Clavaguera et al., 2009). In 2012, three independent groups used mouse models that overexpressed a human mutant Tau isoform (P301L) under control of a specific promoter that restricts its expression to the entorhinal cortex. They detected the presence of hTau not only in neurons of the entorhinal cortex but also in other brain regions, like the dentate gyrus (De Calignon et al., 2012; Harris et al., 2012; Liu et al., 2012). These studies suggest that pathological Tau isoforms can spread from entorhinal cortex to dentate gyrus neurons. Moreover, other investigations showed that injection of Tau synthetic fibrils or mouse brain extract containing Tau aggregates on mice that overexpressed P301S hTau results in formation of NFT-like structures that propagate to distal regions (Ahmed et al., 2014; Iba et al., 2013). In both studies the investigators concluded that the appearance of Tau pathology in other brain regions is dependent on synaptic connectivity rather than spatial proximity suggesting that Tau propagation can occur via synaptic transmission.

Studies showed that Tau propagation can occur by synaptic and non-synaptic transmission. (Wu et al., 2013).

Propagation of Tau involves, essentially, monomeric and oligomeric forms of the protein, which are the most susceptible to aberrant folding and PTMs such as phosphorylation (Arriagada et al., 1992). Tau spreading is now viewed as a trigger of Tau pathology progression. However, this is a complex mechanism, which is dependent on Tau strains and aggregation state. Therefore, uncovering the

mechanisms underlying Tau spreading is crucial to implement new therapeutic approaches for the different tauopathies.

The current knowledge suggests that Tau secretion occurs mainly in synapses (Sokolow et al., 2015) through (Figure 5):

1) Direct translocation across the plasma membrane in a free form. Tau can be secreted in a free form directly into the extracellular space outside the neuron. This process is thought to be favored by hyperphosphorylation, as hyperphosphorylated Tau detaches from MTs and remains free in the cytoplasm (Katsinelos et al., 2018; Sayas et al., 2019). Tau release can also be favored by its binding to proteins such as end-binding proteins, which belong to the group of microtubule plus-end tracking proteins (Sayas et al., 2019) or phosphoinositides (PIPs) such as PI(4,5)P2 (Katsinelos et al., 2018).

2) Release from synaptic vesicles. Studies suggest that Tau release in synaptic vesicles is Ca²⁺ dependent and influenced by synaptic activity. Moreover this release is dependent on AMPA, but not NMDA glutamatergic receptors (Pooler et al., 2013; Zhou et al., 2017).

3) Secretion in extracellular vesicles such as exosomes and ectosomes. Exosomes are small membranous vesicles produced by molecules' endocytosis (Dujardin et al., 2014). Saman et al. (2012) identified the presence of exosome-associated Tau in human CSF samples of AD patients (Saman et al., 2012). Exosomes were enriched with Tau phosphorylated at Thr181, an established biomarker in AD. Winston et al. (2016) observed that peripheral exosomes extracted from AD patients are able to propagate Tau pathology in the CNS of normal mice (Winston et al., 2016). Ectosomes are larger extracellular vesicles compared with exosomes that are directly shed from cells by plasma membrane budding (Dujardin et al., 2014). Tau release by ectosomes is dependent of calcium intracellular levels, inflammatory molecules and/or oxidative stress (Doeuvre et al., 2009; Piccin et al., 2007).

4) Transport through tunneling nanotubes (TNTs). TNTs are filamentous bridges that directly connect cells. Several studies suggest that Tau, as other proteins, can spread through TNTs. Abounit et al. (2016) observed that internalized fibrillar Tau is found in TNTs of Hela and CAD cells (Abounit et al., 2016). Tardivel et al. (2016) observed that in primary cultures of rat embryonic cortical neurons, extracellular Tau leads to formation of TNTs, which in turn facilitate the spreading of pathological Tau (Tardivel et al., 2016).

Once released, Tau can be uptaken by other neurons (Figure 5):

5) Interaction with muscarinic receptors, such as M1 and M3 (Gómez-Ramos et al., 2008; Gómez-Ramos et al., 2009);

6) Clathrin- or dynamin-mediated endocytosis (Calafate et al., 2016; Wu et al., 2013);

7) Micropinocytosis mediated by heparan sulfate proteoglycan (HSPGs) or low-density lipoprotein receptor-related protein 1 (LRP1s) (Rauch et al., 2020; Song et al., 2021);

8) Fusion of extracellular vesicles with plasma membrane (Perea et al., 2020).

In addition to these mechanisms, oligodendrocytes (Narasimhan et al., 2017), astrocytes (Kovacs et al., 2018) and microglia (Asai et al., 2015) can also play a role in Tau internalization.

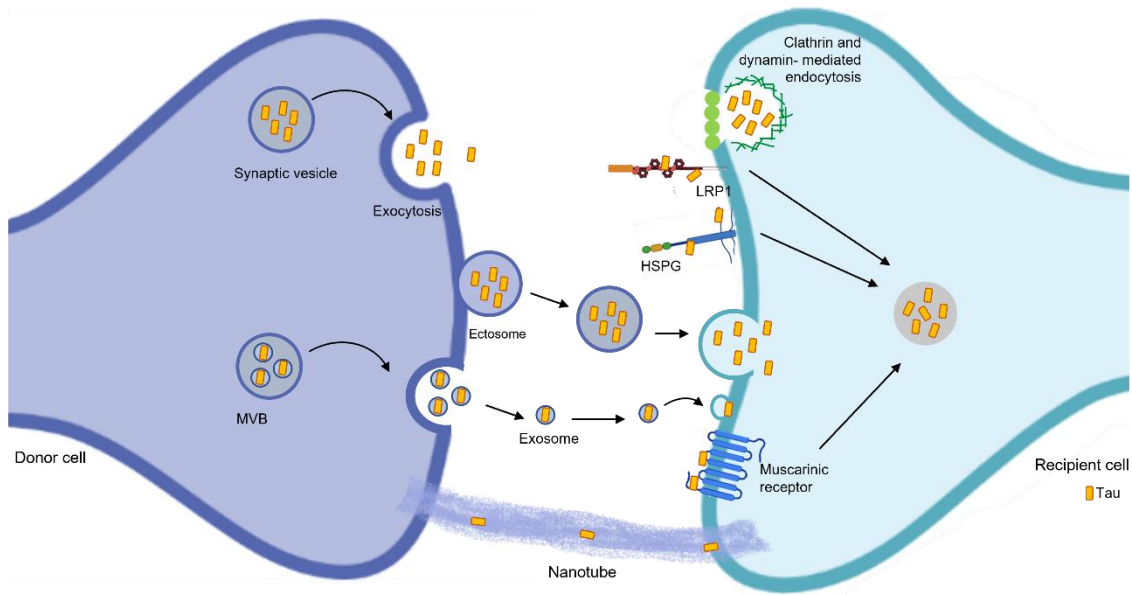


Figure 5. Schematic representation of Tau spreading mechanisms. Tau can be released from the donor cell to the extracellular space through (from top to bottom) synaptic vesicles, ectosomes, exosomes or in a free form. Tau can be uptaken by recipient cells through (from top to bottom) clathrin- and dynamin-mediated endocytosis, micropinocytosis mediated by heparan sulfate proteoglycan (HSPGs) or low-density lipoprotein receptor-related protein 1 (LRP1s), extracellular vesicles (ectosomes and exosomes) that fuse to the membrane and muscarinic receptor-mediated endocytosis. Additionally, Tau can propagate via nanotubes, which are actin-bridges that connect cells.

1.3. *Drosophila* as a model organism to study tauopathies

Drosophila melanogaster, known as the fruit fly, is a powerful organism that has been used as a genetic model to study complex biological questions for more than a century, including neurodegenerative diseases. Besides the obvious characteristics of its small size, high fecundity, short life span, easy handling, and low-cost maintenance, the fruit fly has numerous technical advantages. The genome of *Drosophila* is fully

sequenced and highly annotated (Adams et al., 2000). There are two key factors that contribute to the success of *Drosophila* as a model organism in the study of human-associated neurodegenerative diseases: the high conservation between fruit flies and humans, since over 75% of genes implicated in human disease, have at least one *Drosophila* orthologue (Reiter et al., 2001); and the common evolutionary origin of the CNS of invertebrates and vertebrates (Tardivel et al., 2016). Despite less complex than vertebrates, the central nervous system of the fly is also composed by neurons and glia and equally protected by a blood-brain-barrier (BBB) (Edwards and Meinertzhagen, 2010). Moreover, *Drosophila* and vertebrates' CNS share some features as eyes, olfactory organs, gustatory organs, auditory organs, a ventral nerve cord (spinal cord analog), peripheral sensory neurons for proprioception and pain, and a brain (Hirth, 2010). The well-organized brain of fruit fly allows the study of complex behaviors such as learning and memory, functions known to be impaired in neurodegenerative disorders, including tauopathies. In fact, *Drosophila* models of tauopathy have provided valuable insights into the mechanisms that lead to neurodegeneration.

Modeling human neurodegenerative diseases in flies may require the expression of the human genes associated with the disease. A major tool in *Drosophila* research is the binary GAL4/UAS system, which allows the expression of a genetic responder construct downstream of an Upstream Activator Sequence (UAS) driven by tissue-specific expression of the yeast GAL4 transcription factor (Figure 6A) (Brand and Perrimon, 1993). To generate tissue specific UAS-transgene expression, GAL4 is placed under the control of a tissue-specific promoter. There is a huge collection of GAL4 transgenes that express in single cells, groups of cells or entire tissues, commonly called “drivers”, which are available from *Drosophila* public stock centers (e.g. Bloomington *Drosophila* Stock Center at Indiana University). Thus, the expression of the protein of interest can be controlled both, in time and space, depending on the presence of GAL4 in cells and tissues. The majority of the GAL4 drivers activates UAS-transgene expression during development. However, to model adult-onset neurodegenerative diseases it is useful to restrict expression to adulthood. A modification of the GAL4/UAS system uses a temperature-sensitive variant of GAL4-GAL80 temperature-sensitive (GAL80^{ts}) (Figure 6B). At 18°C (permissive temperature), GAL80^{ts} is active and inhibits GAL4 activity. After eclosion, flies are shifted to 29°C (restrictive temperature). At this temperature the GAL80^{ts} becomes inactive and permits the expression of the UAS-transgene driven by GAL4 expression (Suster et al., 2004) (Figure 6).

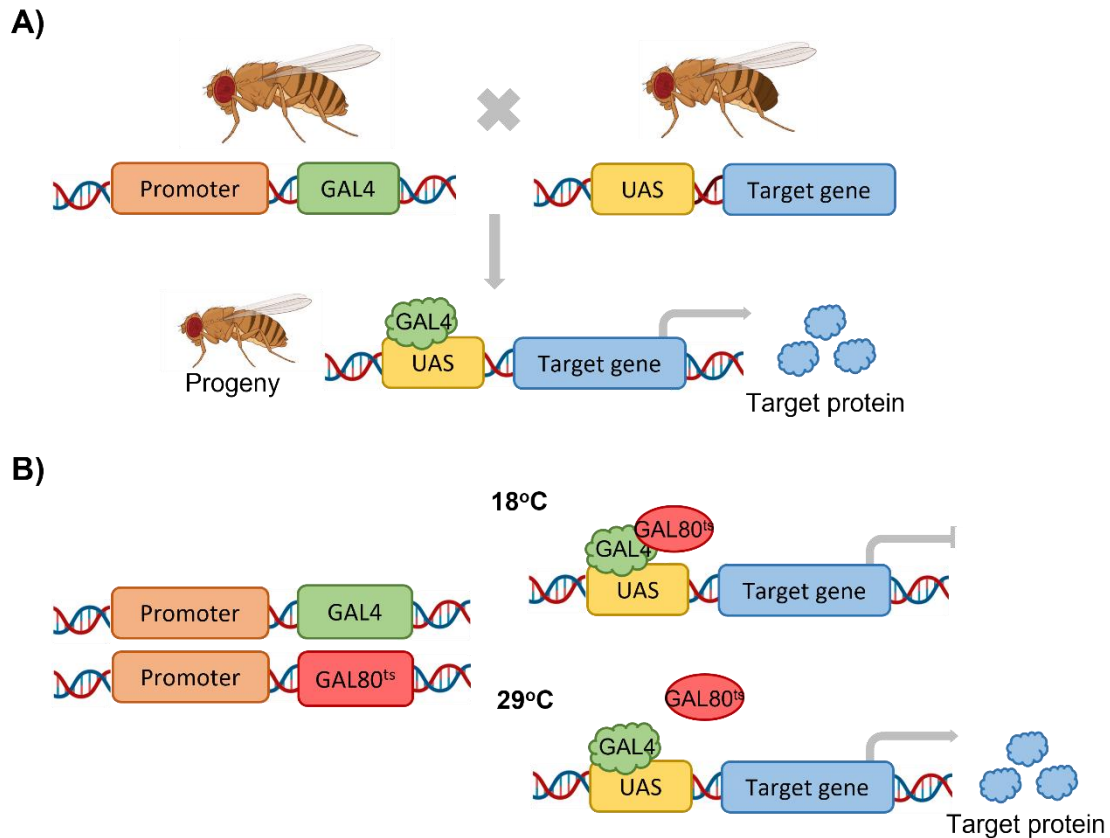


Figure 6. Schematic representation of GAL4-UAS system. (A) The GAL4/UAS system is based in the cross of two independent transgenic fly lines: one carries the yeast transcriptional activator GAL4 and the other its high-affinity binding site, the upstream activating sequence (UAS) fused to a gene of interest. The progeny carries both, the GAL4 and UAS components and will express the gene of interest in an activator-specific manner. **(B)** The GAL4-UAS system with temperature - sensitive GAL80 (GAL80^{ts}). At 18°C gene transcription is repressed by GAL80^{ts} binding to GAL4. At 29°C GAL80^{ts} is inactive, allowing the transcription of the target gene.

1.4. *Drosophila* tauopathy models and readouts of neurodegeneration

Over the last 20 years, several *Drosophila* models expressing different human Tau isoforms have been generated. These are based on the expression of the wild-type hTau 0N3R, 0N4R, and 2N4R isoforms or mutant hTau, such as hTauR406W, hTauV337M, and hTauP301L (Table 4). Additionally, to study specific mechanisms models were generated that carry transgenes with target mutations and/or truncations, mimicking for example hTau phosphorylation or cleavage. The expression of hTau in flies leads to tauopathy-related phenotypes such as rough eye phenotype, learning and memory defects, reduced lifespan, neuromuscular junction and locomotion defects, axonal transport and synaptic impairment brain vacuolization and cell death. These phenotypes can be scored and used as readouts to identify genetic modifiers of Tau (Sun and Chen, 2015).

Table 4. GAL4-UAS models of tauopathy in *Drosophila*.

Tau Isoform	hTau Constructs (UAS)	GAL4 Driver	Phenotypes	References
2N4R	WT	<i>GMR-GAL4, elav-GAL4</i>	REP, ND	(Jackson et al., 2002)
	P301L	<i>GMR-GAL4</i>	REP, ND	(Karsten et al., 2006)
	S2A (S262A/S356A)	<i>GMR-GAL4</i>	REP, ND	(Chatterjee et al., 2009)
	S11A S46A/ T50A/ S199A/ S202A/ S205A/ T212A/ S214A/ T231A/ S235A/ S396A/ S404A	<i>GMR-GAL4</i>	REP, ND	Kosmidis et al., 2010
	WT:FLAG	<i>elav-GAL4, OK107-GAL4</i>	ND, LMD	Kosmidis et al., 2010
	STA:FLAG (S238A/ T245A)	<i>elav-GAL4, OK107-GAL4</i>	ND, LMD	Kosmidis et al., 2010
0N4R	R406W	<i>elav-GAL4 OK-G-GAL4</i>	ND, RL, TL, MT	Kosmidis et al., 2010
	V337M	<i>elav-GAL4</i> <i>OK6-GAL4</i>	RL, ND TL, MT	(Kosmidis et al., 2010)
	R406W/S2A	<i>GMR-GAL4</i>	REP, ND	(Nishimura et al., 2004)
	S262A	<i>GMR-GAL4</i>	REP, ND	(Iijima-Ando et al., 2010)
	TauAP T111A/ T153A/ T175A/ T181A/ T199A/ T217A/ S202A/ S205A/ T212A/ S214A/ T231A/ S235A/ S422A/ S396A/ S404A	<i>elav-GAL4, OK6GAL4</i>	MT defects	(Talmat-Amar et al., 2011)
	E14 T111E/ T153E/ T175E/ T181E/ S199E/ S202E/ S205E/ T212E/ T217E/ T231E/ S235E/ S396E/ S404E/ S422E	<i>GMR-GAL4</i>	REP, pTL ND	(Khurana et al., 2006)

	K44Q/R230Q 44–230	<i>GMR-GAL4</i>	REP, calpain activity	(Reinecke et al., 2011)
	1–421	<i>elav-GAL4</i>	RL, ND, pTL	(Khurana et al., 2010)
	PH-Tau	<i>elav-GAL4</i> , <i>MB-GAL4</i>	climbing, MB defects, LMD	(Mershin et al., 2004)
0N3R	WT	<i>C161-GAL4</i>	sensory neurons defects	(Williams et al., 2000)
		<i>D42-GAL4</i>	locomotion, axon, NMJ defects	(Mudher et al., 2004)
		<i>elav-GAL4</i> ; <i>D42-GAL4</i>	pTL, MT defects	(Cowan et al., 2011)

GMR-GAL4: eye-specific GAL4; *elav*-GAL4: pan-neural GAL4; OK-GAL4: motor neuron-specific GAL4; MB-GAL4: Mushroom Body-specific GAL4; C161-GAL4: sensory neuron-specific GAL4; D42-GAL4: motor neuron-specific GAL4. hTauWT: human Tau wild-type; LMD: learning and memory defects; MT: microtubule; ND: neurodegeneration; NMJ: neuromuscular junction; pTL: pTau levels; REP: rough eye phenotype; RL: reduced lifespan.

1.4.1. Rough eye phenotype

Expression of either, wild-type (0N4R and 2N4R) or mutant hTau isoforms (V337M and R406W) under the control of the eye-specific driver- GMR-GAL4- leads to a rough-eye phenotype (Feuillette et al., 2010; Jackson et al., 2002; Shulman and Feany, 2003). Shulman and Feany (2003) were the first to perform an exhaustive genetic modifier screen in a *Drosophila* model that expresses the mutant hTau V337M under the control of GMR-GAL4. They identified numerous enhancers and suppressors of the rough eye phenotype caused by the expression of hTauV337M, including kinases such as Par-1/MARK and phosphatases as PP1, PP2 and Stg/Cdc25 (Shulman and Feany, 2003). Using a similar tauopathy model, Blard et al. (2007) performed a genetic screen with 1250 mutant *Drosophila* lines and identified Tau modifiers that are involved in synaptic transmission and cytoskeletal regulation (Blard et al., 2007). Ambegaokar and Jackson (2011) performed a genetic screen using loss-of-function and gain-of-function alleles in flies that express wild type full length- hTau (2N4R). They identified numerous kinases

and cytoskeleton elements already associated with tauopathy as novel modifiers of the Tau-induced rough eye phenotype (Ambegaokar and Jackson, 2011).

1.4.2. Neuronal degeneration and cell death

Several studies reported *Drosophila* neuronal death after hTau expression. This phenotype can be visualized by the presence of brain vacuolization and abnormal swelling of nuclei (Jackson et al., 2002; Mershin et al., 2004; Wittmann et al., 2001). This kind of neurotoxicity can be both, autonomous and non-autonomous, since neuronal death is reported in cells that express hTau but also in other neuronal populations, including cholinergic neurons, sensory neurons, and mushroom bodies (Kosmidis et al., 2010; Wittmann et al., 2001; Yeh et al., 2010). Williams et al., (2000) observed neuronal degeneration accompanied by abnormal axon bundling, reduced arborization, axonal swelling or loss of axonal projections, and beading on flies that express different Tau constructs (human WT ON3R, bovine, and rodent). Khurana et al., (2006) demonstrated that, in the context of tauopathy, cell death could be explained by the activation of TOR in a cell cycle - dependent manner. Furthermore, they suggest a possible role for cell-cycle activation downstream of Tau phosphorylation, directly preceding apoptosis (Khurana et al., 2006).

1.4.3. Learning and memory

Drosophila mushroom body (MB) is the structure homologous to the hippocampus of mammals, which is the epicenter of regulation of learning and memory (Roman and Davis, 2001). By testing responses to attractive and repulsive odours, Mershin et al. (2004) demonstrated that expression of different Tau transgenes strongly compromised associative olfactory learning and memory (Mershin et al., 2004). Pan-neuronal expression, using the *elav-GAL4* driver, of pathological forms of hTau also affects olfactory learning and memory functions in flies (Kosmidis et al., 2010). Ali et al. (2010) used an aversive phototaxis suppression assay to demonstrate that overexpression of WT or R406W Tau affects the learning capacity and memory of 20-day-old flies (Ali et al., 2012).

1.4.4. Lifespan

The ultimate consequence of neurotoxicity is the reduction of lifespan in hTau-expressing flies. In normal and controlled conditions, WT flies can live approximately 60 to 80 days. The expression of WT or mutant hTau isoforms decreases flies' lifespan according to Tau toxicity (Sealey et al., 2017; Wittmann et al., 2001).

Altogether, these phenotypes can be used to identify genetic modifiers of Tau and to better understand the mechanisms behind Tau toxicity.

1.5. *Drosophila* contributions to unveiling the mechanisms underlying Tau-induced toxicity

Drosophila models of tauopathy have provided important insights about the role of Tau phosphorylation in mediating neuronal toxicity. Jackson et al. (2002) demonstrated that overexpression of Shaggy (Sgg), the fly homologue of GSK3 β , enhanced neurodegenerative phenotypes associated with hTau expression and lead to the formation of neurofibrillary tangle-like structures. They also revealed that this interaction could not go through the canonical Wnt pathway (Jackson et al., 2002). Nishimura et al. (2004) showed that PAR-1 kinase directly phosphorylates Tau at Ser262 and Ser356, triggering toxicity in flies. In agreement, they observed that downregulation of PAR-1 or mutation of PAR-1 phosphorylation sites (TauS2A) reduced toxicity associated with hTau (0N4R-R406W) expression. These authors suggested that, in disease, Tau phosphorylation occurs in a sequential manner, in which Tau Ser262 and Ser356 are primary events that facilitate further phosphorylation by downstream kinases, such as GSK-3 β /Sgg and Cdk5 (Nishimura et al., 2004). Chatterjee and colleagues (2009) demonstrated that PAR-1 acts as Tau kinase reducing the toxicity associated with expression of hTau (2N4R). However, they observed that a Tau mutant, resistant to phosphorylation by GSK-3 β /Sgg, retained substantial toxicity (Chatterjee et al., 2009). The different conclusions about Sgg action suggest that Tau phosphorylation toxicity might be dependent on isoform expression. Mudher et al. (2004) observed that expression of Tau 0N3R lead to axonal transport impairment and this phenotype was enhanced by Sgg co-expression. Using two GSK-3 β inhibitors, Lithium and AR-A014418, they showed that axonal transport was restored, demonstrating that the pathological effects of Tau are phosphorylation dependent (Mudher et al., 2004).

Other pathways distinct from GSK3 β signaling as JAK/STAT signaling pathway (Colodner and Feany, 2010), and Nuak1, an AMP-activated protein kinase (AMPK)-related kinase (Lasagna-Reeves et al., 2016) were shown to be involved in Tau hyperphosphorylation and consequently Tau toxicity, in flies. Feany' group generated 0N4R hTau constructs in which 14 Serine-Proline (SP) and/or Threonine- Proline (TP) residues were mutated to phosphoresistant alanine. They observed that blocking phosphorylation at these motifs reduces Tau toxicity *in vivo*. Moreover, they found that CDK5 kinase and PP2A phosphatase modify Tau neurotoxicity via these SP/TP

phosphorylation sites (Steinhilb et al., 2007a). In a different study, the same group analyzed the 14 mutated residues individually to determine which were responsible for Tau neurotoxicity. They concluded that toxicity relays on the cooperation between phosphorylation of different SP/TP sites rather than on individual phosphosites (Steinhilb et al., 2007b). The expression of phosphomimetic Tau (Tau^{E14}) was further described to be toxic in *Drosophila* mushroom bodies, in contrast to the phosphodeficient versions TauAP, 0N4R TauR406W/S2A, and 2N4R TauS2. The authors also identified two novel phosphorylation sites - Ser238 and Thr245- that are essential for Tau toxicity (Steinhilb et al., 2007b).

Drosophila is also a valuable model to screen for pharmacological compounds to be used in tauopathy treatments. Recently, Ro 31-8220, described as a PKC α inhibitor, was tested in a fly model of frontotemporal dementia (FTD). The authors showed that this small molecule reduced eye roughness and Tau phosphorylation and improved memory and motor deficits (Shim et al., 2019).

1.6. Cdc25 phosphatases

Cell division cycle 25 (Cdc25) is a dual specificity phosphatase that belongs to the class of protein tyrosine phosphatases (Rudolph, 2007). Cdc25 was first discovered in the fission yeast *Schizosaccharomyces pombe* as a dose dependent inducer of mitosis (Russell and Nurse, 1986). *Drosophila* has two Cdc25 homologues: String (Stg) and Twine (Twi), which control mitosis and meiosis, respectively. In mammals three homologous are described, Cdc25A, Cdc25B and Cdc25C (Galaktionov and Beach, 1991).

Cdc25 proteins have two domains: the C-terminal domain and the N-terminal domain. The C-terminal domain has around 60% of homology between isoforms and contains the catalytic site. The N-terminal domain displays lower similarity between isoforms and contains the nuclear localization sequence and the nuclear export sequence, which determine the subcellular localization of the protein. Moreover, it contains specific phosphorylation and ubiquitination sites that regulate the activity of the phosphatase (Rudolph, 2007). Cdc25s play a conserved function in cell-cycle by dephosphorylation of crucial tyrosine and threonine residues on inactivated CDKs. These phosphatases are also checkpoint regulatory proteins, preventing CDK/cyclin activation in response to DNA damage. Although sharing a common mechanism of action, Cdc25 isoforms have distinct roles in cell cycle progression. Cdc25A has a comprehensive function in the cell cycle, regulating G1/S transition, S phase and G2/M transition, whereas Cdc25B and Cdc25C control G2 to M transition (Donzelli and Draetta, 2003). In agreement, their localization also differs: Cdc25A is mainly found in

the nucleus; Cdc25B, also known as the “initiator” phosphatase shifts its localization between nucleus and cytoplasm. During interphase is present in the nucleus and shifts to cytoplasm during G2 to activate the Cyclin B1/ CDK1 complex and then re-enters the nucleus to promote mitosis initial steps. Cdc25C is present in the cytoplasm during interphase and then shuttles to the nucleus. Cdc25 is considered a proto- oncogene and is found overexpressed in numerous types of cancer as neuroblastoma, breast, ovarian, gastric, lung, and oral cancer, among others. Overexpression of Cdc25, specially Cdc25A and Cdc25B, is correlated with malignancy poor prognosis in cancer patients (reviewed in (Kristjánsdóttir and Rudolph, 2004).

Cell cycle phosphatases, as other mitotic regulators, are inactivated in post mitotic neurons, that are in G₀ state. However, some studies revealed the presence of cell cycle molecules in neurons undergoing degeneration in AD, including Cdc25 (Chang et al., 2012; Ding et al., 2000; Vincent et al., 2001). Ding et al. observed the presence of Cdc25A in normal and AD brains, independently of the presence of NFT structures. Moreover, they detected Cdc25 phosphatase activity in both types of samples analyzed, with an increase of enzymatic activity in AD samples (Ding et al., 2000). Vincent et al. detected the presence of Cdc25B in neurons from control and AD brains. They argued that although with low levels of activity, Cdc25 phosphatase could be a resident molecule in adult brain neurons. The observation of increased enzymatic activity on AD samples led them to propose that Cdc25B activates mitotic events that lead to neurodegeneration (Vincent et al., 2001).

1.7. Aims

According to World Health Organization WHO, more than 55 million people live with dementia, including tauopathy. Tau hyperphosphorylation is viewed as a trigger of tauopathy. Therefore, the identification of Tau phosphorylation modifiers is crucial for the development of therapeutic approaches. In this project we used a *Drosophila* tauopathy model, in which the longest WT isoform of Tau (2N4R) was expressed, to study the relevance of Stg/Cdc25-Tau interaction, following the hypothesis that Stg/Cdc25 acts as Tau phosphatase. To address this topic our specific aims were:

1. Address Stg/Cdc25 ability to suppress hTau-induced neurodegeneration phenotypes.
2. Evaluate the impact of Stg/Cdc25 in Tau phosphorylation levels.
3. Characterize the nature of Stg/Cdc25 – Tau interaction.
4. Assess Tau oligomerization and spreading in Stg overexpressing flies.
5. Evaluate Stg/Cdc25 impact in a disease mimetic model.
6. Identify hTau residues modified by Stg/Cdc25 activity.

Overall, we expect to validate Stg/Cdc25 as novel Tau phosphatases with impact on disease progression and uncover their potential value in new therapeutic approaches.

CHAPTER II

Material and methods

2. Material and methods

2.1. Fly strains and genetics

Flies were maintained in standard media, at 25°C under a 12:12 light-dark cycle, unless indicated. The strains *elav^{C155}*GAL4 (BDSC 458), *UAS-Stg* (BDSC 4777), *UAS-Stg.HA* (BDSC 56562), *elav^{C155}*GAL4; *GMR-hTau* (BDSC 51360), *tubGAI80^{ts}* (BDSC 7017), *UAS-StgRNAi* (BDSC 29556), *UAS-StgRNAi* (BDSC 34831), *UAS-StgRNAi* (BDSC 36094), *Stg-GFP* (BDSC 50879), *GMR::myrRFP* (BDSC 7121), *UAS-mCherry* (BDSC 35787), *UAS-mCherryRNAi* (BDSC 35785), Oregon R were obtained from the Bloomington *Drosophila* Stock Centre (BDSC;NIH P40OP018537). *UAS-hTau-Flag* was previously described (Kosmidis et al., 2010) (Table 5). Standard genetic techniques and fly lines carrying balancers on the second and third chromosomes were used to generate the different genetic backgrounds. Control flies in all experiments were as closely related to the experimental flies as possible. *elav^{C155}*GAL4 flies were crossed to *Oregon R* to generate heterozygous control alleles. Flies of genotypes containing the GAL4/UAS/GAL80^{ts} constructs were grown at 18°C and transferred to 29°C as 1-3 day-old adults, to allow expression of the Gal4, and aged until use in histological or protein analysis. The phenotype of adult retinas was imaged in a stereomicroscope Stemi 2000 Zeiss equipped with a Nikon camera.

Table 5. List of *Drosophila* lines used in this study.

Stock	Genotype	source
GMR-Gal 4		In house
ELAV-Gal4	P{w[+mW.hs]=GawB}elav[C115];;	BDSC (#458)
ELAV-Gal 4; GMR::hTau	P{w[+mW.hs]=GawB}elav[C155]; P{w[+mC]=GMR-htau.Ex}1.1	BDSC (#51360)
UAS-String	w[1118]; P{w[+mC]=UAS-Stg.N}16/CyO,P{ry[+t7.2]=sevRas1.V12}FK1	BDSC (#4777)
UAS- String RNAi	y[1] v[1]; P{y[+t7.7]v[+t1.8]=TRiP.JF03235}attP2	BDSC (#29556)
UAS- String RNAi	y[1] sc[*] v[1] sev[21]; P{y[+t7.7]v[+t1.8]=TRiP.GL00513}attP40	BDSC (#36094)
UAS- String RNAi	y[1] sc[*] v[1]; P{y[+t7.7]v[+t1.8]=TRiP.HMS00146}attP2	BDSC (#34831)
UAS- String phosphatase-dead	W; Sp/Cyo ; P{w[+mC]=UAS-Stg ^{C379S} /TM6B	In house
OR-R	Oregon-R	In house

UAS-Stg/CyO; TubGal80^{ts}/TM 6B	W; UAS-Stg/CyO; TubGal80 ^{ts} /TM6B	In house
TubGal80^{ts}	w[*]; P{w[+mC]=tubP-GAL80[ts]}2/TM2	BDSC (#7017)
Stg-GFP/TM3	y[1] w[*]; PBac{y[+mDint2]=HpaI-GFP.A}stg[YD0685]/TM6C, Sb[1	BDSC (#50879)
UAS-m-CD8GFP	y[1] w[*]; betaTub60D[Pin-Yt]/CyO; P{w[+mC]=UAS-mCD8::GFP.L}LL6	BDSC (#5130)
UAS-mCherry	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=UAS-mCherry.VALIUM10}attP2	BDSC (#35787)
UAS-mCherryRNAi	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=VALIUM20-mCherry}attP2	BDSC (#35785)

BDSC-Bloomington *Drosophila* Stock Center

2.2. Generation of transgenic flies

The *UAS-Stg*^{C379S} transgenic flies were generated by replacing the conserved cysteine within the catalytic domain to serine using the NZY Mutagenesis kit according to manufacturer's protocol. The following oligonucleotides were used to amplify *Stg* coding sequence from LD47579 plasmid, *Stg*PPAseFW (5'-CAACATCATTATCTTCCACGCCGAATTCTCCTCGGAGCGT-3') and *Stg*PPAseRW (5'-ACGCTCCGAGGAGAATTCGGCGTGGAAGATAATGATGTTG-3'), followed by DpnI digestion of parental DNA (LD47579). After transformation of DH5α competent cells, plasmid DNA was amplified and at least three clones were selected for sequencing analysis. *EcoR1-XhoI* digestion was used to clone *Stg*^{C379S} sequence into *pUAST-attB*. Transgenic flies were obtained after site-specific integration of *UAST-Stg*^{C379S} on the 3rd chromosome (3R-86F) (Bischof et al., 2007).

2.3. Protein extraction and analysis

For protein analysis, adult flies of the appropriate genotypes were aged for 8-15 days, at appropriate temperature, flash frozen in liquid nitrogen and stored at -80°C. Flies were decapitated by vigorously vortexing for 15 seconds, and heads were homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton), supplemented with protease and phosphatase inhibitors (Roche) in dry ice. Extracts were incubated for 1h at 4°C with rotation and centrifuged at 2000 g for 20 minutes, to separate soluble from insoluble fractions. Total protein content was quantified by the Lowry Method (DC™ Protein Assay, Bio-Rad, CA, USA). Fifteen micrograms of soluble extract were loaded in 10% SDS- polyacrylamide gels and transferred onto nitrocellulose

(Amersham, GE Healthcare) membranes, for western blot analysis. Extracts were loaded in replicated gels and probed in parallel with total Tau and phosphorylation-specific Tau antibodies. Membranes were blocked for 1 hour with TBS 0,1% Tween containing either 5% low-fat milk or 5% BSA (Sigma). Primary antibodies were diluted in TBST 0,1%Tween with 3% blocking agent: phospho-Tau (Ser202, Thr205) (AT8) (1:2000, Termofisher Scientific), phospho-Tau (Thr231) (AT180) (1:2000, Termofisher Scientific), total Tau (5A6) (1:6000, DSHB), anti-Syntaxin (1:500, DSHB) and anti-Tubulin (1:10000, DSHB). Goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham) were diluted at 1:10000 and 1:15000, respectively, in TBST 0,1% Tween containing 3% low-fat milk. Signal was detected using Clarity Western ECL Substrate reagent (Bio-Rad Laboratories) according to manufacturer's instructions. A GS-800 calibrated densitometer with Quantity One 1-D Analysis Software 4.6 (Bio-Rad Laboratories) was used for quantitative analysis of protein levels. Western blots were repeated at least 3 times with biological replicates and representative blots are shown. The antibodies used were registered in Table 6.

2.4. Dot blot

For dot blot analysis, 10 fly heads were homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors. After a 5 min centrifugation at 10000 g, samples were diluted in 1% Glycerol/PBS. 3µl of protein extract were dotted in the nitrocellulose membrane. After blocking in 5% low-fat milk /TBS-0,1%, the membrane was incubated with anti-Amyloid oligomer A11 antibody (1:500, AB9234 Merck), at 4 °C, and handled for protein detection following the protocol described above. Images were acquired in ChemiDoc XRS (Bio-Rad).

2.5. Histology analysis

Female flies were anesthetized on ice, immobilized in a histology collar and fixed with Carnoy's fixative (6 ethanol: 3 chloroform: 1 acetic acid), overnight at 4°C. Tissue was dehydrated in ethanol series. Methyl Benzoate (Sigma) was used to clear the samples prior to paraffin embedding and microtome sectioning as described in (Sunderhaus and Kretzschmar, 2016). Serial sections (5 µm) from the entire head were stained with hematoxylin and eosin and examined by bright-field microscopy. Images were captured with an Olympus DP72 microscope. Total vacuole number and vacuole

area were quantified, in the central brain, retina and lamina, in at least 8 flies per genotype.

For the immunocytochemical study, head sections were mounted on adhesive microscope slides (StarFrost, Knittel Glass) and immunostained using the modified avidin–biotin–peroxidase complex (ABC) method. Antigen retrieval was performed by microwave treatment in 0.01 M-citrate buffer at pH 6.0, for 10 min. Endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide in methanol for 20 min. After washing with PBS 0,05% Tween, the sections were incubated for 20 min in a humid chamber with normal rabbit serum (X092, Dako) diluted 1:5 in PBS 0,05% Tween with 10% BSA. The sections were then incubated overnight at 4 °C with phospho-Tau (AT8; 1:500) antibody diluted in PBS 0,05% Tween with 5% BSA. Tissue was incubated for 30 min with a biotin-labelled rabbit anti-mouse secondary antibody (EO35301-2, Dako) diluted 1:200 in PBS 0,05% Tween, 5% BSA followed by incubation in an avidin-biotin-peroxidase complex (ABC) (1:100 dilution in 5% BSA; Vector Laboratories) for additional 30 min. The slides were incubated with DAB substrate (3,3'-Diaminobenzidine tetrahydrochloride, Dako). Tissue sections were counterstained with Mayer's hematoxylin (HX390929, Merck) and dehydrated slides were mounted in Entellan.

2.6. Immunofluorescence analysis

Third instar larval tissues were dissected and fixed with 3,7% formaldehyde in PBS for 20 min, followed by washes in PBS 0.1% Triton. The primary antibodies used were: anti-GFP (A11122, 1:1000, Invitrogen) and anti-Tau (5A6, 1:5000, DSHB). The secondary antibodies were conjugated with Alexa Fluor dyes (ThermoFisher Scientific). Brains from 10 day -old flies were dissected in cold PBS and fixed in 3.7% formaldehyde in PBS for 30 min. Samples were washed three times in PBT 0.5% [1 x PBS, 0.5% Triton X-100 (Sigma-Aldrich)] and incubated in blocking buffer (PBT 0.5%, 0.5% BSA, 0.5% FBS) for 1 h 30 min at room temperature. Incubation with primary antibodies anti- cleaved Caspase 3 (Asp 175) (1:50, Cell Signaling) and anti-ELAV (1:400, DSHB) was performed in blocking buffer overnight at 4 °C. Secondary antibodies conjugated with Alexa Fluor dyes (ThermoFisher Scientific) were diluted 1:500 in PBT 0.5% and incubated for 3 h at room temperature. Tissues were mounted in Fluoromount-G™ Mounting Medium (ThermoFisher Scientific). Samples were imaged on a Leica SP5 confocal microscope and images were processed with ImageJ (NIH). The antibodies used are described in Table 6.

2.7. Proximity-ligation assay (PLA)

The Duolink In Situ Red system (92101, Merck) was used to detect Stg-Tau interaction *in vivo*, following the manufacturer's protocol. Briefly, third instar larvae eye imaginal discs were dissected on ice and fixed with 3,7% formaldehyde in PBS for 20 min, followed by washes in PBS 0.1% Triton. Eye discs were incubated with Duolink Blocking Solution for 1 h at 37°C. Incubation with the primary antibodies anti-GFP (A11122, Invitrogen) and anti-hTau (5A6, 1:5000, DSHB) was performed ON at 4°C. Samples were washed twice with Duolink Wash Buffer A, and incubated with MINUS (anti-mouse) and PLUS (anti-rabbit) Duolink PLA Probes for 1 h at 37°C. Samples were washed with Duolink Wash Buffer A prior to the ligation (1h at 37°C) and amplification (1h30h at 37°C) steps. Eye imaginal discs were mounted in Duolink In Situ Mounting Medium with DAPI (MERCK). Images were acquired using Leica SP5 confocal.

2.8. Climbing assays

Climbing assays were performed in a counter-current apparatus equipped with six chambers, as described by (Inagaki et al., 2010). Newly hatched flies of the appropriate genotypes were allowed to mate for 2 days, and after separated by gender in groups of 10-20 flies and aged at 25°C. At each time point, flies were placed into the first chamber, tapped vigorously to the bottom and given 15 secs to climb upwards (approximately 10 cm), reaching the upper tube. The flies that successfully reached the upper tube were shifted to a new chamber, and both sets of flies were given another opportunity to climb upwards, in 15 secs. This procedure was repeated for a total of five times, and the number of flies in each chamber was counted. Flies were classified into three performance groups according to the locomotor ability displayed: flies that remained in tubes 1 and 2 were considered to have severe locomotor impairment (group I); those remaining in tubes 3 and 4 were considered to have moderate locomotor impairment (group II), while those that reached the last tubes, tubes 5 and 6, - group III - performed well in the climbing assay. Climbing assays were performed at days 8, 15 and 22 (Figure 13 and 14) or at 3, 8 and 15 (Figure 17), and partition coefficient (Cf) was calculated, according to (Inagaki et al., 2010). The partition coefficient (Cf) represents the probability of flies to climb up. At least 100 flies were used per genotype.

2.9. Scanning Electron Microscopy

For SEM analysis, 1-day old flies were dehydrated through incubation in ethanol series (25%, 50%, 75%, 100%), incubated with hexamethyldisilazane (HMDS, Sigma), air-dried, mounted in SEM stubs and coated with Au/Pd thin film, by sputtering using the SPI Module Sputter Coater equipment. Imaging was performed using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-ray microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM/EDAX Genesis X4M. The images were acquired with 400X and 1000X magnification.

2.10. Protein extraction for Tau immunoprecipitation

To analyze Tau PTMs, flies were aged for 8-15 days at 25°C, flash frozen in liquid nitrogen and stored at -80°C until protein extraction. For each Tau immunoprecipitation protocol, 200 fly heads were homogenized in RIPA Buffer (50 mM Tris-HCL, 150 mM NaCl, 1% Triton), using a ratio of 1,5 uL per head, supplemented with protease (Roche) and phosphatase (Roche) inhibitors, in dry ice. Homogenates were placed at 4° in rotation during 1 hour and then centrifuged at 2000g for 20 minutes. The supernatant was incubated with 25 µl Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich) at RT for 4 hours. After 5 washes in PBS 0,1% Triton supplemented with protease and phosphatase inhibitors and one additional wash in PBS, the beads were eluted in elution buffer (100 mM Tris, 1% sodium deoxycholate (SDC), 10 mM TCEP, 40 mM 2-chloroacetamide (CAA). After running into a gel to confirm the success of the immunoprecipitation protocol, the purified protein was analyzed by mass spectrometry (LC-MS/MS).

2.11. Tau PTMs detection by mass spectrometry analysis

The eluted protein from the genotypes under analysis was digested with trypsin, overnight at 37 °C with agitation, 1000 rpm. The resulting peptides were analyzed by Liquid Chromatography—Mass Spectrometry (LC-MS) using an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Five hundred nanograms of peptides of each sample were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 µm i.d., 160454, Thermo Scientific, Bremen, Germany) in a mobile phase of 2% ACN, 0.1% FA at 10 µL/min. After 3 min loading, the trap column was switched in-line to a 50 cm × 75 µm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific, Bremen, Germany) at 300 nL/min.

Separation was achieved by mixing A: 0.1% FA and B: 80% ACN, 0.1% FA with the following gradient: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B), and 10 min (hold 99% B). Subsequently, the column was equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany).

The mass spectrometer was operated in the data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380-1580) and subsequent HCD MS/MS of the 10 most intense peaks from a full scan (normalized collision energy of 27%). The ESI spray voltage was 1.9 kV. The global settings were as follows: use lock masses best (m/z 445.12003), lock mass injection Full MS and chrom. peak width (FWHM) of 15 s. The full scan settings were as follows: 70 k resolution (m/z 200), AGC target 3×10^6 , maximum injection time 120 ms; dd settings: minimum AGC target 8×10^3 , intensity threshold 7.3×10^4 , charge exclusion: unassigned, 1, 8, >8, peptide match preferred, exclude isotopes on, and dynamic exclusion 45 s. The MS2 settings were as follows: microscans 1, resolution 35 k (m/z 200), AGC target 2×10^5 , maximum injection time 110 ms, isolation window 2.0 m/z , isolation offset 0.0 m/z , dynamic first mass, and spectrum data type profile.

The raw data were processed using the Proteome Discoverer 3.0 software (Thermo Scientific, Bremen, Germany). Protein identification analysis was performed with the data available in the UniProt protein sequence database for the *Drosophila melanogaster* and *Homo sapiens* Proteome.

2.12. Statistical analysis

For western blot analysis, significance was determined using an unpaired t- test with Welch's correction, two-tailed. For the climbing assays, the significance was measured using two-way ANOVA with Tukey's multiple comparison test. For vacuole analysis, one-way ANOVA with Tukey's multiple comparison test (Figures 13 and 14) or unpaired t- test with Welch's correction, two-tailed (Figure 19) were used to determine significance. All statistical analyses were performed using GraphPad Prism, version 6.0 (GraphPad Software). All experiments were performed with biological triplicates. Significance is defined as $p < 0.05$ and bars in graphs represent the mean $\pm$ SD.

Table 6. List of the antibodies used in this study.

Antigen	Symbol	Type	Dilution		Source
			IF	WB/DB	
Phospho-Tau (Ser202, Thr295)	AT8	Mouse /IgG1	-	1:2000	Thermofisher
Phospho-Tau (Thr231)	AT180	Mouse /IgG1	-	1:2000	Thermofisher
Tau	5A6	Mouse /IgG1	1:5000	1:7000	DSHB
GFP	A11122	Rabbit / IgG	1:1000	-	Thermofisher
anti- Cleaved caspase 3 (Asp 175)	-	Rabbit	1:50	-	Cell Signaling
ELAV	7E8A10	Rat/ IgG2a	1:400	-	DSHB
Amyloid Oligomer	A11	Rabbit	-	1:500	Merck
α-Tubulin	EP1332Y	Rabbit / IgG	-	1:10000	DSHB
Syntaxin	8C3	Mouse /IgG1	-	1:500	DSHB

IF- Immunofluorescence. WB- Western blot. DT- Dot blot.

CHAPTER III

String/Cdc25 phosphatase is a suppressor of Tau-associated neurodegeneration

3. String/Cdc25 phosphatase is a suppressor of Tau-associated neurodegeneration

Tauopathies, a class of neurodegenerative disorders, are characterized by the deposition of Tau inclusions in neurons and/or glial cells. Tau aggregates are enriched in aberrant and hyperphosphorylated forms of the protein. Therefore, Tau's abnormal phosphorylation has been postulated to be the origin of the formation of Tau aggregates. The phosphorylation of Tau is regulated by the balance between kinase and phosphatase activities and so a disruption of this equilibrium leads to abnormal Tau phosphorylation. Thus, it is crucial to identify the proteins that act on Tau to understand the causes that lead to the formation of Tau aggregates. Although PP1, PP2 and PP5 are recognized to carry out most of the phosphatase activity in the brain, it was shown that different members of the Cdc25 phosphatases family are active in the adult brain. However, the role of Cdc25s in neurons remains to be established.

In this work, we used the *Drosophila* retina as a model to address the molecular mechanisms underlying Stg/Cdc25-Tau interaction, following the hypothesis that Stg/Cdc25 act as Tau phosphatases. We also evaluated the impact of Stg/Cdc25 function in the context of neurodegeneration.

3.1. Results

3.1.1. Stg phosphatase activity suppresses Tau-induced phenotypes.

To explore the genetic interaction between Stg and Tau, we used the well-established fly tauopathy model *GMR-hTau2N4R* (hereafter *GMR-hTau*), which is based on the expression of the longest hTau isoform (2N4R) under the control of the Glass Multimer Reporter (GMR) regulatory sequences, which drive transcription in all cell types posterior to the morphogenetic furrow in the developing eye (Hay et al., 1994; Jackson et al., 2002). Accordingly, hTau is expressed in all cells of the developing retina, including photoreceptor neurons (Ellis et al., 1993). To validate Stg as a neuron-specific modifier of hTau toxicity we used a *GMR-hTau* fly line that contains the pan-neuronal driver *elav-GAL4* in the genetic background (*elav;GMR-hTau*). In the retina, *elav-GAL4* will drive expression of UAS-sequences in photoreceptors and neurons associated with inter-ommatidia bristles, where hTau is co-expressed.

We used the external morphology of the retina as a readout of Stg - hTau interaction (Figure 7A - D). As previously reported (Jackson et al., 2002), when

compared to control flies (*elav/+*; Figure 7A, A'), hTau expressing flies (*elav*; *GMR-hTau*) display a rough eye phenotype with disordered ommatidia and missing or irregular mechanosensory bristles (Figure 7B, B'). The overexpression of Stg (*elav*; *GMR-hTau*; *Stg* and *elav*; *GMR-hTau*; *StgHA*) during retina development is sufficient to rescue the adult rough eye phenotype induced by *GMR-hTau* expression (Figure 7C, D).

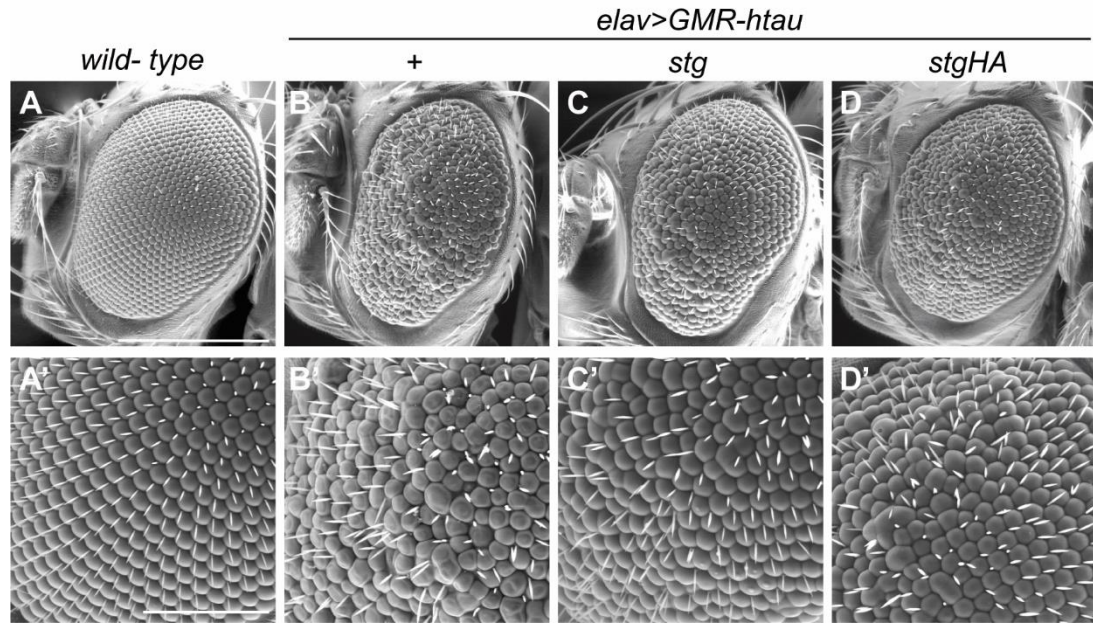


Figure 7. Stg suppresses the rough eye phenotype of hTau expressing flies. (A – D) Representative images of the retina of (A) control flies, (B) flies expressing one copy of hTau (*elavG4*; *GMR-hTau*) and (C,D) flies co-expressing Stg and hTau (*elavG4*; *GMR-hTau*; *Stg* and *elavG4*; *GMR-hTau*; *Stg-HA*). Scale bar is 300 μ m (A'– D') magnified views of A, B, C and D, respectively. Scale bar is 100 μ m

Morphometric analysis measuring the circularity and length of the anterior-posterior (A-P) axis of the retina showed that the roughness observed in *GMR-hTau* flies is accompanied by a reduction in eye size (Figure 8B, F, G), compared to control (Figure 8A, F, G). The retina size is restored in Stg overexpressing flies with circularity and A-P length undistinguishable from control flies (Figure 8C, F, G). In contrast, knocking down Stg using RNA interference in hTau-expressing flies (*elav*; *GMR-hTau*; *StgRNAi*) has a minor impact on the retina size and morphology of hTau - expressing flies (Figure 8D, F, G). As Stg is a phosphatase we asked if its enzymatic activity was required for the suppression of the hTau rough eye phenotype. The active site of Cdc25 phosphatases is highly conserved, and modification of the conserved cysteine in the active HCX5R site, was shown to abolish phosphatase activity in different models (Dunphy and Kumagai, 1991; Gautier et al., 1991; Rudolph, 2007; Sohn et al.,

2004). Accordingly, we generated a Stg allele harboring a cysteine to serine (C379S) modification on Stg conserved active site (*UAS-Stg^{C379S}*; Stg phosphatase-dead). Our results show that hTau; Stg^{C379S} flies show severe ommatidia disorganization, without significant changes in the retina size compared to hTau expressing flies (Figure 8E, F, G). Altogether, our findings confirm the Stg-hTau genetic interaction and show that Stg phosphatase activity is required to suppress Tau-associated phenotypes, leading us to propose that Stg/Cdc25 phosphatases may act as regulators of Tau toxicity *in vivo*.

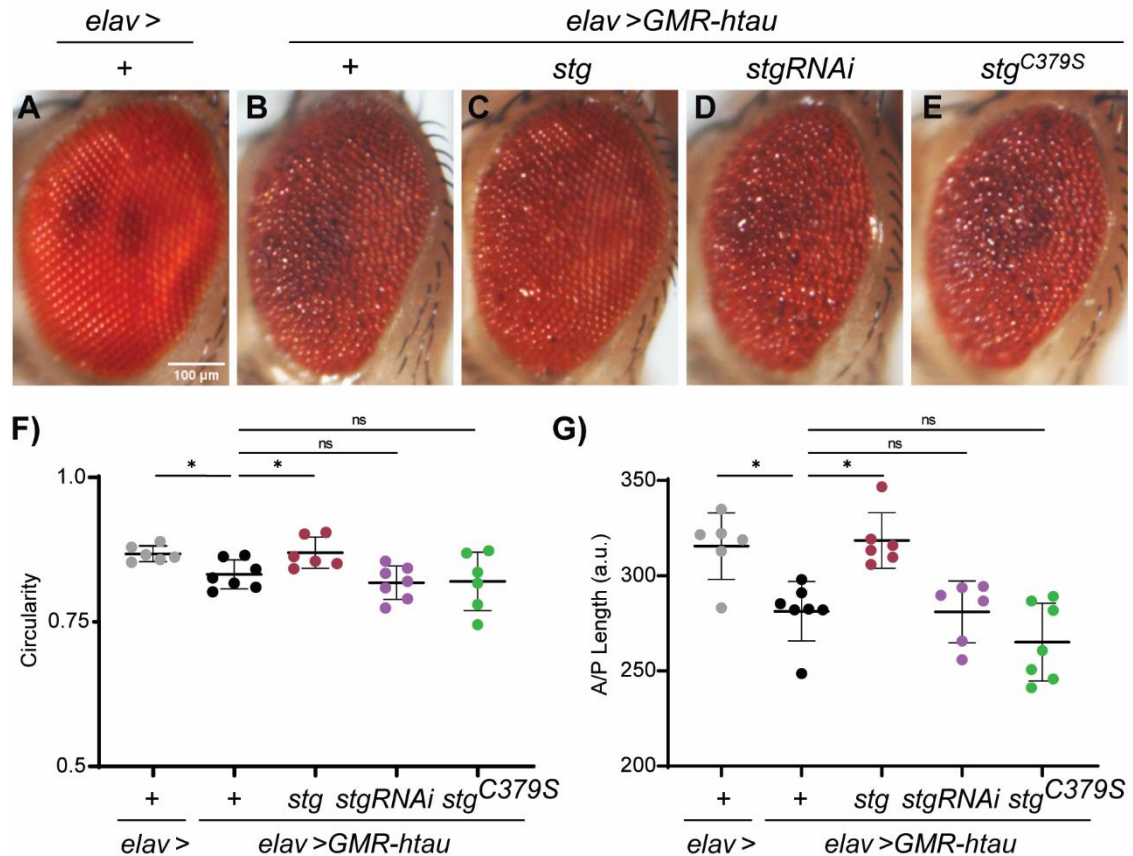


Figure 8. Stg phosphatase suppresses the rough eye phenotype associated with hTau expression in the fly retina. (A-E) Representative images of adult retinas (A) carrying *elav*-GAL4 or expressing (B) one copy of hTau (*elav;GMR-hTau*), (C) hTau and Stg (*elav;GMR-hTau;Stg*) (D) Stg RNAi (*elav;GMR-hTau; StgRNAi*) or (E) a phosphatase dead Stg allele (*elav> GMR-hTau; Stg^{C379S}*) (F) Quantification of circularity of the retina of flies for the indicated genotypes. (G) Quantification of the anterior-posterior (A-P) axis length for the indicated genotypes. Image quantification was performed on Fiji-ImageJ and the results were analyzed using one-way ANOVA with multiple comparisons; $n \geq 6$. Error bars denote SD; n.s. indicates non-significant; * $p < 0.05$.

To validate our results, we used two additional *Stg* RNAi lines (Figure 9B, C) and also a control for the RNAi insertion site (Figure 9D, E). Eye Circularity and size of *elav; GMR-hTau; StgRNAi*, *elav;GMR-hTau; mCherry* and *elav; GMR-hTau; mCherryRNAi* were similar to *elav; GMR-hTau* flies (Figure 9F, G). Additionally, to confirm that the phenotypes observed were dependent on hTau expression, we expressed all the UAS

lines used with *elav*-Gal4 driver. Expression of the individual lines did not lead to a rough eye phenotype (Figure 9H- L).

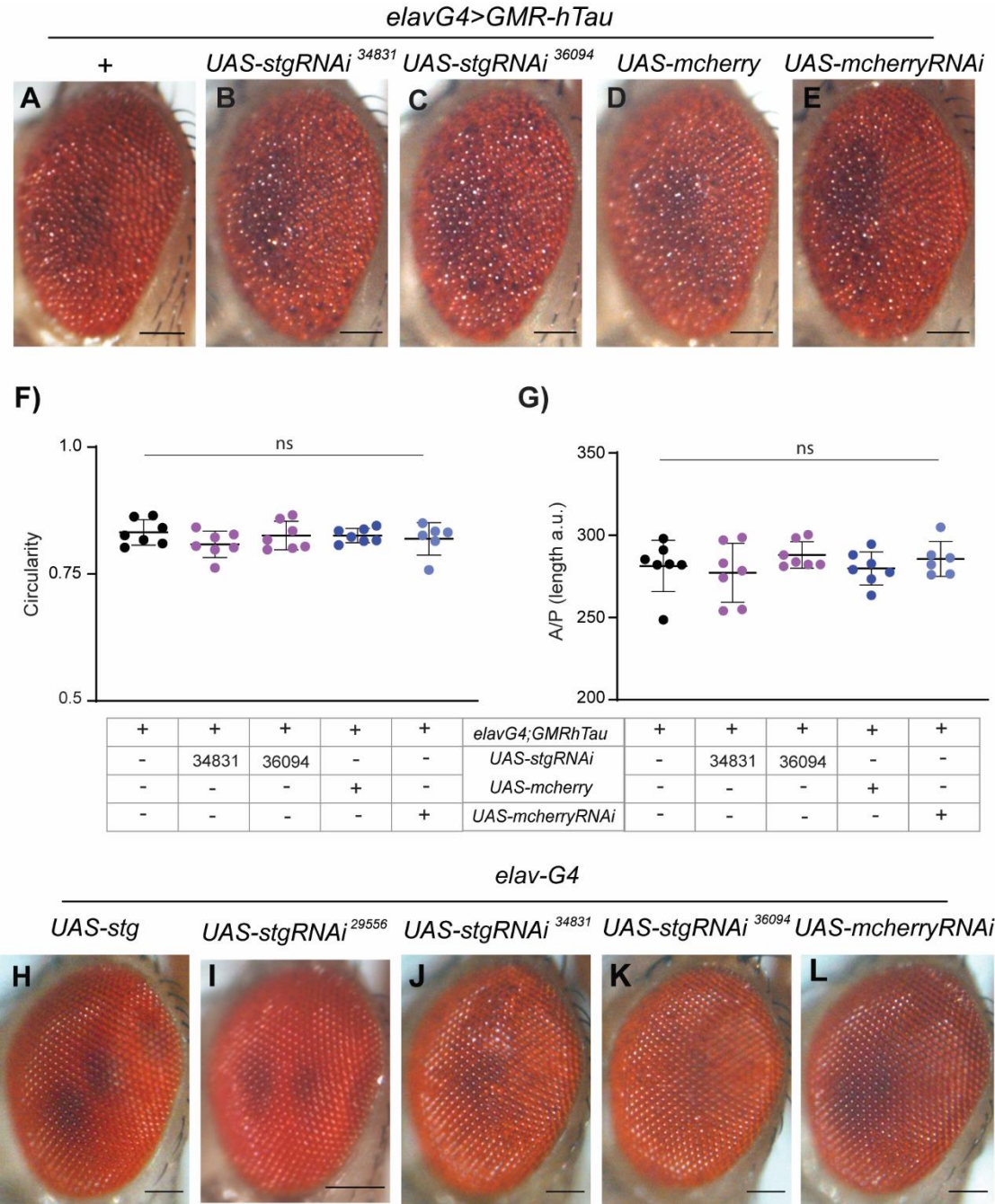


Figure 9. Stg RNAi does not suppress the rough eye phenotype associated with hTau expression in the fly retina (A-E) Representative images of the retina from (A) flies expressing one copy of hTau (*elav*; *GMR-hTau*; n=7), (B, C) *GMR-hTau* flies co-expressing *UAS-StgRNAi* (*elav*; *GMR-hTau*; *StgRNAi*; n=7) and *elav*; *GMR-hTau* flies co-expressing *UAS* control lines (D) *UAS-mCherry* (n=7) and (E) *UAS-mCherryRNAi* (n=6). (F, G) Quantification of the circularity (F) and anterior-posterior axis length (G) in the indicated genotypes. Image quantification was performed on Fiji-ImageJ and the results were analyzed using one-way ANOVA, multiple comparisons. (H-L) Representative images of the retina of flies expressing *UAS-Stg* (*elav*; *Stg*) (H) and *UAS-StgRNAi* alleles (*elav*; *StgRNAi*) (I-K) under the control of *elav*-Gal4. *UAS-mCherryRNAi* (L) was used as control. Scale bar is 100 μ m.

3.1.2. Stg activity reduces Tau phosphorylation levels

The results mentioned above suggest that Stg acts as a Tau phosphatase. Therefore, we investigated the impact of Stg levels on Tau phosphorylation. Our analysis focused on phosphorylation of Ser202/Thr205 (AT8 antibody) and Thr231 (AT180 antibody) residues, which correlate with increased hTau aggregation propensity, AD clinical progression, and are used for diagnostic purposes (Bibow et al., 2011; Hanger and Noble, 2011; Jeganathan et al., 2008; Wegmann et al., 2021). Accordingly, we compared the levels of total and phosphorylated Tau in *GMR-hTau* and *GMR-hTau; Stg* expressing flies. For the residues under study, western blot analysis shows that hTau phosphorylation levels are reduced by the co-expression of Stg in fly tissues, without significantly affecting Tau total levels. (Figure 10). Quantification of the ratio between phosphorylated and total hTau showed that Stg overexpression reduces phosphorylation to 69% at Ser202/Thr205 and to 52 % at Thr231 residues. (Figure 10E, F). These results clearly show that Stg is able to counteract hTau phosphorylation *in vivo*.

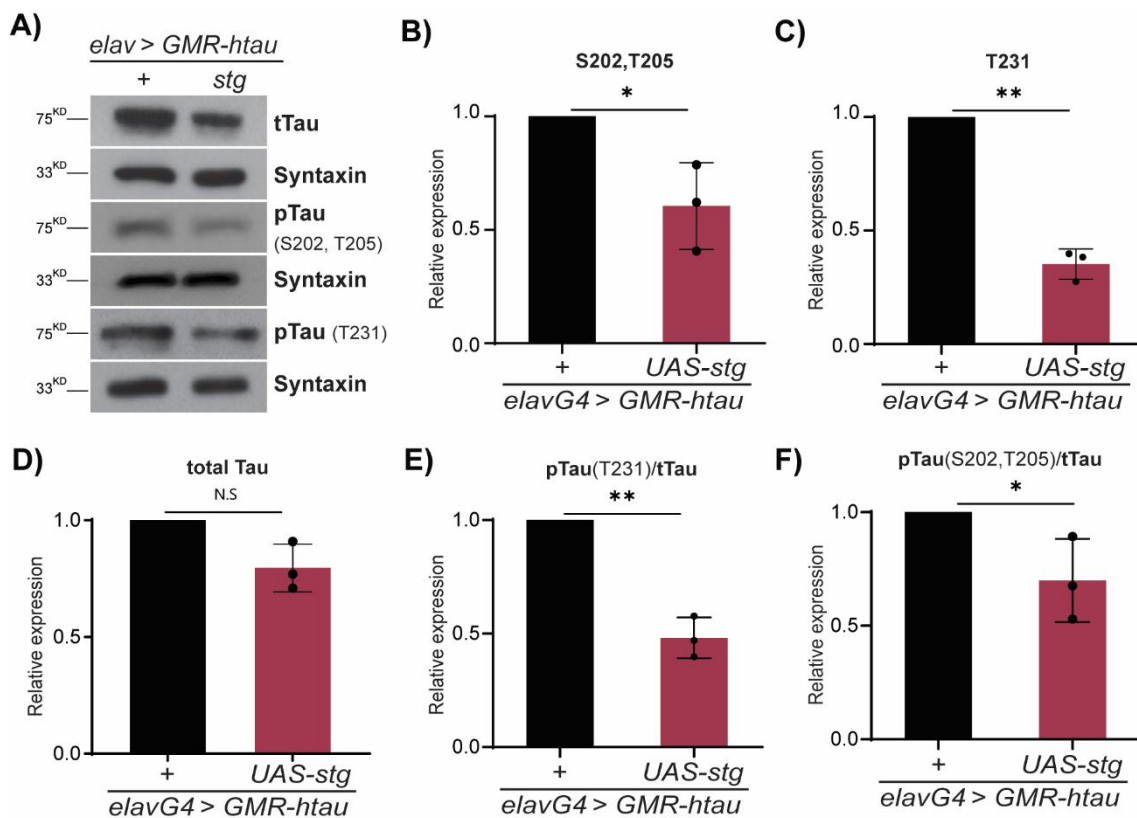


Figure 10. Stg reduces Tau phosphorylation levels. (A) Western blot on protein extracts from fly heads of the indicated genotypes blotted with total Tau and Tau phosphorylated at S202/T205 and T231. Syntaxin was used as loading control. (B, C, D) Quantification of the levels of phosphorylated Tau at S202/T205 (B) and T231 (C) and total Tau (D). (E, F) Ratio between Tau phosphorylated at S202/T205 (E) and T231 (F) and total Tau levels. Data refers to

three biological replicates. Data were analyzed using unpaired t- test with Welch's correction. Error bars denote SD; n.s. indicates non-significant; * $p < 0.05$; ** $p < 0.01$.

3.1.3. Endogenous Stg clusters with hTau in neurons

The reduced hTau phosphorylation levels detected upon Stg expression can be explained by direct Stg-hTau interaction. Alternatively, Stg can regulate hTau phosphorylation status indirectly, through the regulation of Tau kinases and phosphatases. Since the association of protein phosphatases Stg/Cdc25 with their substrates is transient and difficult to detect (Fahs et al., 2016; Sohn et al., 2004), we used *in situ* proximity ligation assay (*in situ* PLA) to probe Stg-hTau interaction. We used a *stg* allele expressing Stg - GFP fusion under *stg* endogenous promoter (*stg-GFP*, *protein trap*) to evaluate Stg-Tau proximity in photoreceptors, avoiding false-positive interactions due to Stg overexpression. In third-instar eye imaginal discs Stg-GFP is expressed at high levels in the proliferative/anterior domain and immediately posterior to the morphogenetic furrow (Figure 11A). Immunolocalization studies in *elav*; *GMR-hTau*/ *stg-GFP* larvae show that hTau is expressed in cells posterior to the morphogenetic furrow, following the expression pattern of GMR promoter and overlapping Stg-GFP expression domain (Figure 11B). PLA assays reveal the presence of *in situ* PLA signals in the domain co-expressing Stg and hTau (Figure 11C), which indicates that Stg-GFP and hTau are found in close proximity in these cells. In contrast, PLA signal is not detected in eye-imaginal discs from *GMR-hTau*; *TM3,Ser* larvae confirming the specificity of *in situ* PLA-detection (Figure 11D).

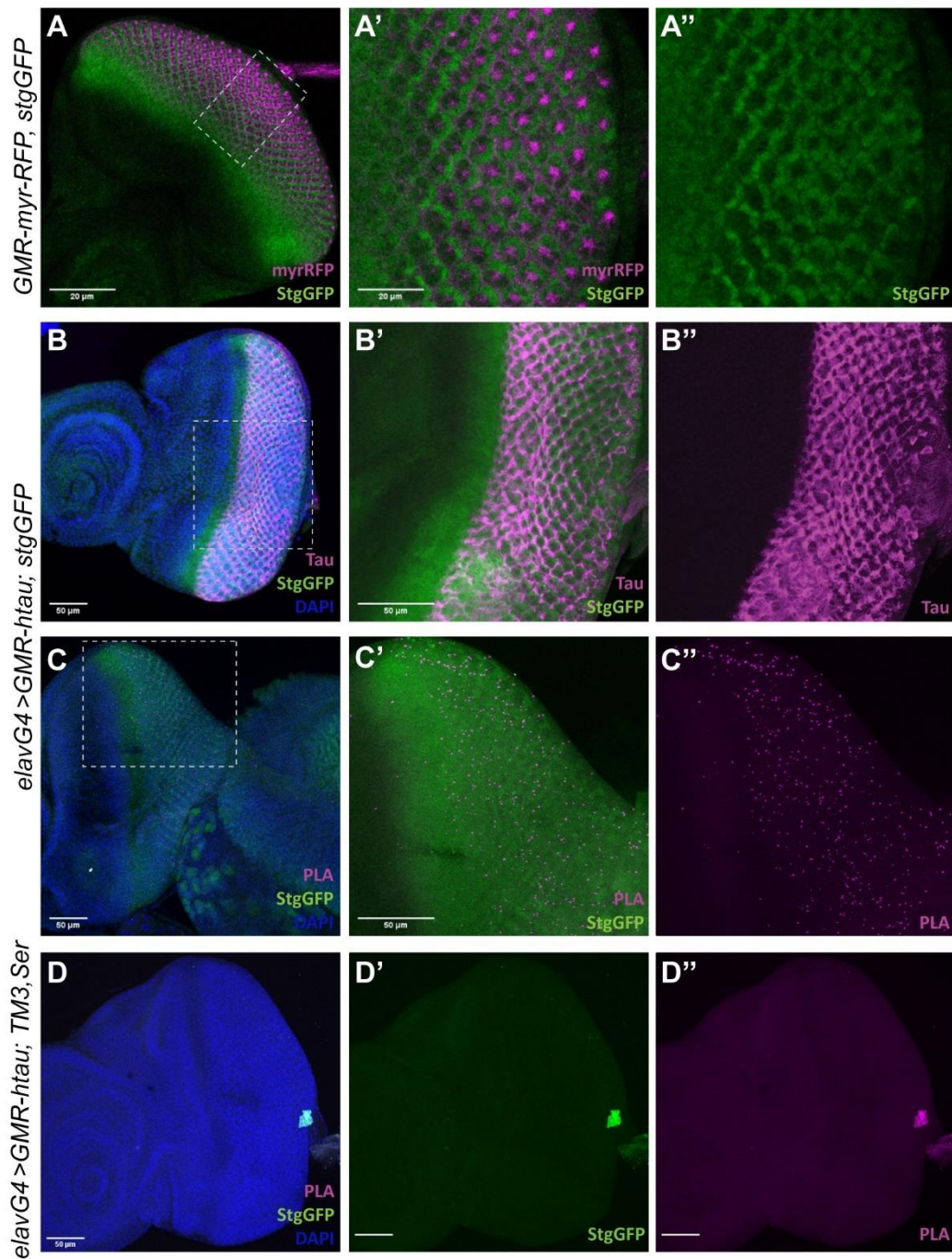


Figure 11. Stg interacts physically with hTau. (A) Representative third-instar eye imaginal disc from *GMR-myRFP; stg-GFP* stained for GFP (green) to visualize Stg expression pattern. *GMR-myRFP* (magenta) labels the GMR expression domain. (A', A'') Magnification of the region delimited by the dashed lines in A. (B) *GMR-hTau; stg-GFP* third-instar eye imaginal disc, showing the expression pattern of hTau (magenta) and Stg-GFP (green). DNA (blue) was counterstained with DAPI. (B, B'') Detail from the posterior domain of disc shown in B. (C) L3 eye imaginal disc from *elavG4>GMR-hTau; stg-GFP* labeled for Stg (green) and showing PLA puncta (magenta), a readout of the interaction between endogenous Stg (green) and hTau. DAPI (blue) was used to counterstain DNA. (C, C'') Magnification of the posterior region of the disc showing PLA puncta (magenta). In all images anterior is to the left. (D- D2'') Representative images of eye imaginal discs from *elavG4>GMR-hTau; TM3,Ser* larvae as a negative control of the PLA experiment.

3.1.4. Stg suppresses Tau-driven neurodegeneration

To uncover the biological significance of Tau dephosphorylation by Stg/Cdc25 we assayed neurodegeneration phenotypes in flies. Cell death (Jackson et al., 2002; Wittmann et al., 2001), brain vacuolization (Khurana et al., 2006; Mershin et al., 2004; Wittmann et al., 2001) and locomotor impairment (Sealey et al., 2017; Ubhi et al., 2007) are well-established neurodegeneration phenotypes strongly associated with pan-neural expression of hTau and phosphorylation status (Nishimura et al., 2004). Therefore, we asked if Stg expression would modify these neurodegeneration signatures in hTau-expressing flies.

To assay locomotor function, we performed climbing assays in 8, 15- and 22-day-old flies using a countercurrent apparatus (Figure 12). In this assay we evaluate the ability of flies to climb-up a tube, in a set period of time, in 5 consecutive attempts (Inagaki et al., 2010). Flies that successfully climb-up the tube are transferred to a new tube and are challenged again. Thus, flies reaching the last tubes, tubes 5 and 6 (group III), have increased climbing ability compared to those retained in tubes 1 and 2 (group I), despite being given the same five climbing attempts.

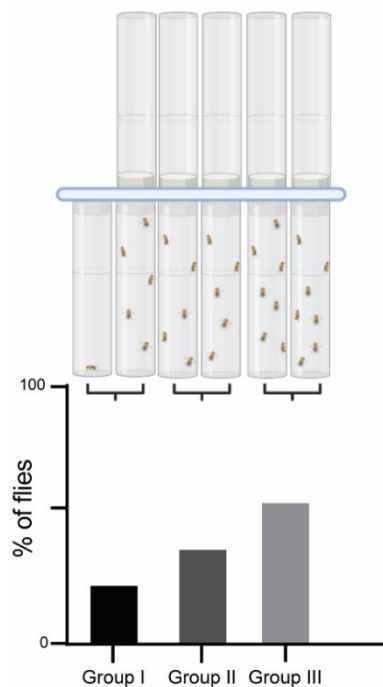


Figure 12. Graphical representation of the climbing assay with countercurrent apparatus. (A) Final distribution of the flies in the countercurrent apparatus is represented as a bar graph. Flies with severe motor deficits remain in the first 2 tubes, the group I. Flies that climb to the 3rd and 4th tubes are categorized into group II and flies that reach the last 2 tubes of the apparatus (the fittest flies) are represented by group III.

We observed that Stg improves motor ability of hTau- expressing flies over time, although without statistical impact. By day 8, only 37% of GMR-hTau expressing flies showed the ability to climb towards tubes 5 and 6 (Figure 13A, group III). This percentage improves to almost 50% upon co-expression of Stg. The results are even more significant in 22-day old flies, where the percentage of flies in group III increased

by more than twofold after *Stg* expression (Figure 13C), from 7,1% to 15,3 . In agreement, analysis of the climbing-up probability, given by the partition coefficient (Cf) shows that *GMR-hTau*; *Stg* flies have a higher capability to reach the last tube at all time points compared to *GMR -hTau* flies (Figure 13D).

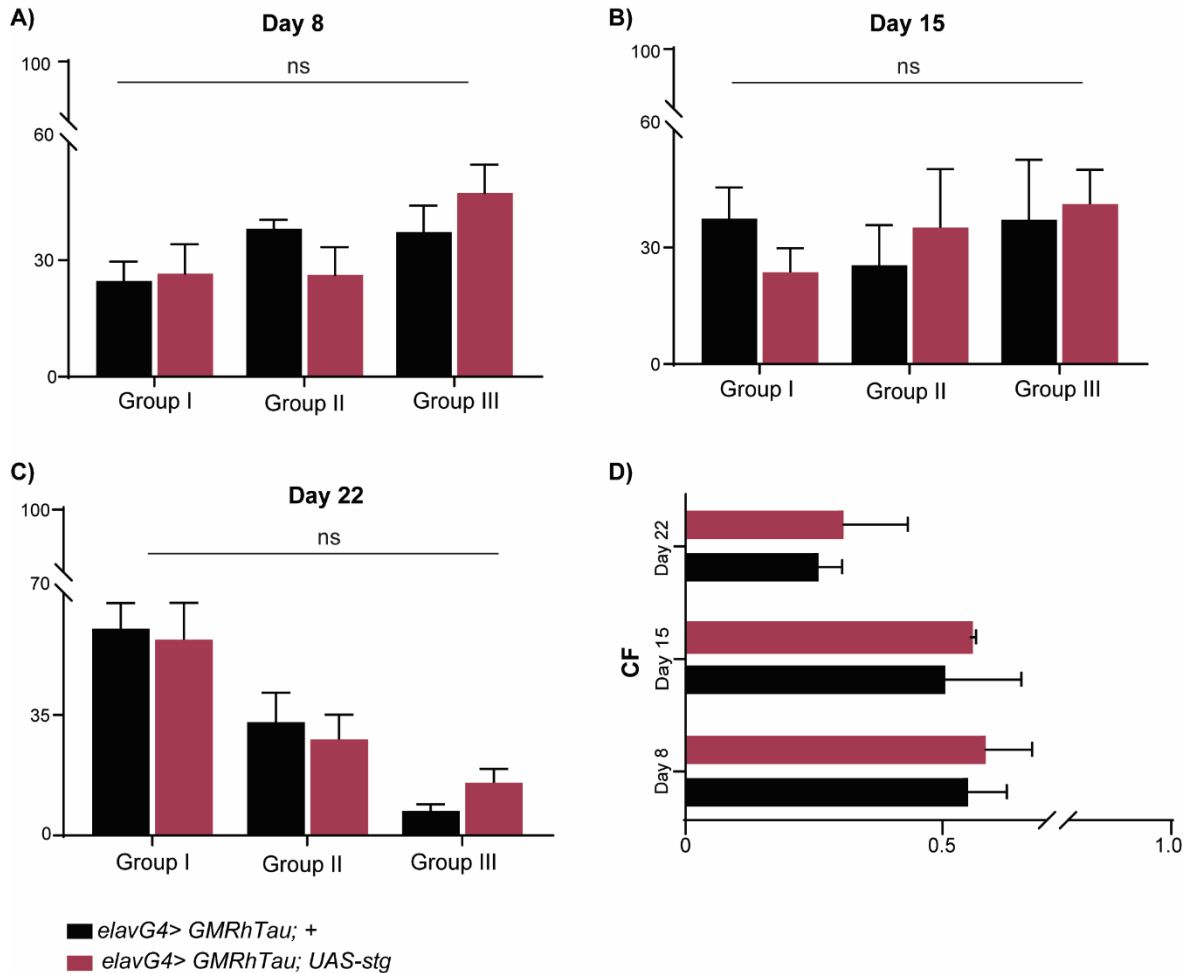


Figure 13. *Stg* reduces motor deficits associated with hTau expression. (A-C) Quantification of the percentage of flies retained in groups I, II and III at day 8 (A), 15 (B) and 22 (C), for the genotypes indicated. (D) Graphic representation of the index CF for 8, 15 and 22-day old flies. Statistical significance was calculated using two-way ANOVA. Error bars denote SD; n.s. indicates non-significant.

Since we observed an improvement in locomotor function upon *Stg* in a model in which hTau is only expressed in the fly retina, we decided to perform the same experiment using the pan-neural *elav-GAL4*, a broader driver to express hTau and *Stg*, to ensure more neurons, including motor neurons, are affected and reinforce our findings.

We observed that the climbing performance of hTau-expressing (*elavG4*; *UAS-hTauFlag*) flies is significantly impaired compared with control (*elav-GAL4*) (Figure 14),

in agreement with previous observations (Sealey et al., 2017). By day 8, only 3% of hTau expressing flies reach tubes 5 and 6 (group III), while 77% are retained in group I (tubes 1 and 2). In contrast, at the same timepoint, more than 10% of hTau; Stg expressing flies complete the assay (group III) (Figure 14A). Their behavior is similar to those observed in control flies. Although the climbing performance decreased with time in all genotypes, as expected, due to age-related deterioration, the same pattern is observed on days 15 and 22 (Figure 14B, C). Moreover, the climbing -up probability for each genotype, given by the partition coefficient (Cf), shows that over time hTau; Stg flies perform better than hTau flies (Figure 14D). The improved locomotor function associated with Stg expression is sustained in time and by day 22 hTau; Stg expressing flies remain indistinguishable from controls. Stg co-expression increased Cf to values similar to control, in all time points analyzed (Figure 14D).

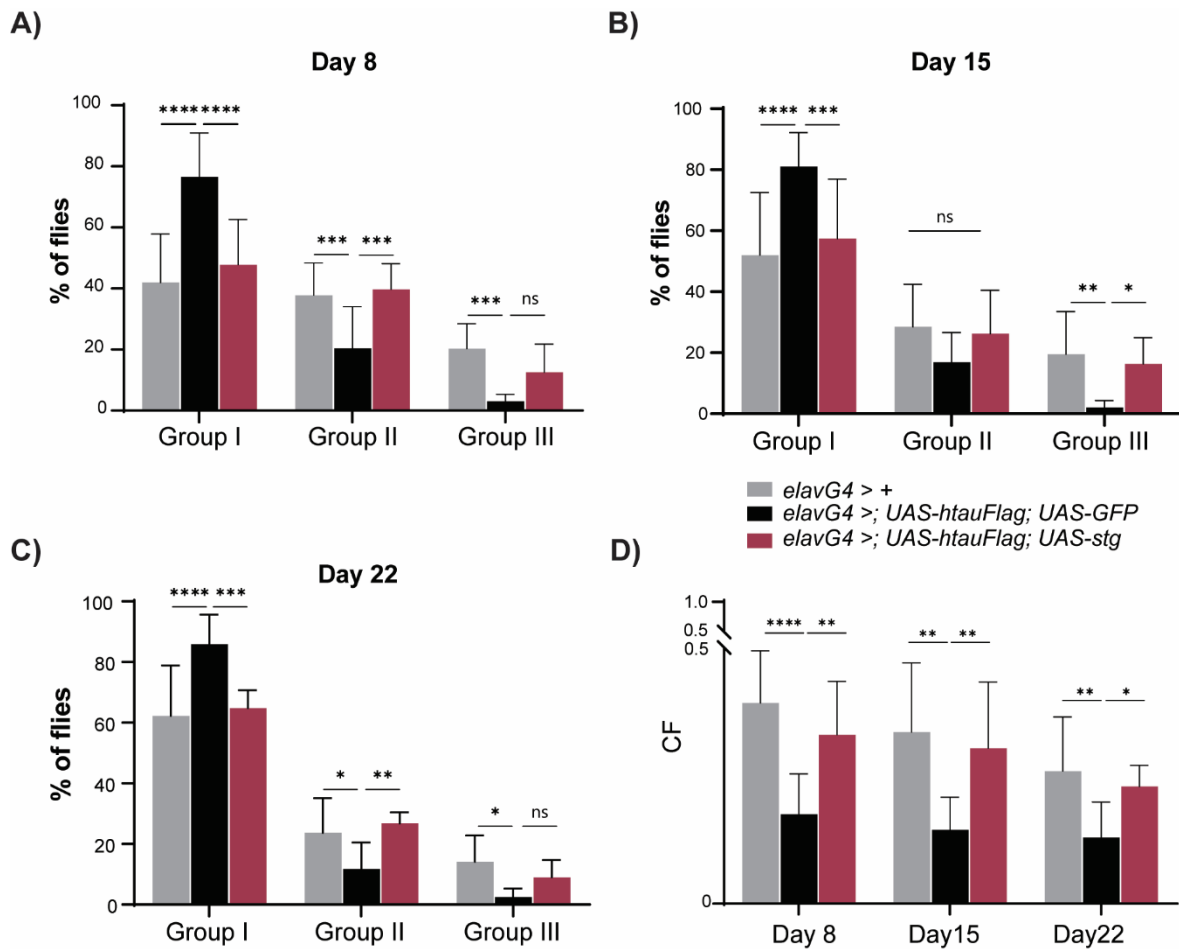


Figure 14. Stg improves locomotor deficits of hTau expressing flies. (A, B) Quantification of the percentage of flies retained in groups I, II and III at day 8 (A), 15 (B) and 22 (C), for the genotypes indicated. (D) Graphic representation of the climbing index for 8, 15 and 22 days for the indicated genotypes. Statistical significance was calculated using two-way ANOVA, multiple comparisons. Error bars denote SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

To evaluate the internal morphology of the retina we used standard hematoxylin-eosin staining (Figure 15). Analysis of adult head sections from *GMR-hTau* flies reveals severe disruption of the internal structure of the visual system, with strong vacuolization in both retina and lamina regions. Importantly, the expression of Stg in photoreceptors significantly improves the internal structure of the retina of hTau expressing flies (Figure 15A). In addition, quantification of the number of vacuoles between hTau and hTau; Stg expressing flies shows that the number of vacuoles is reduced in *elav; GMR-hTau; Stg* 10-day old flies (Figure 15B). We also analyzed vacuole area in the retina of these flies. Although not statistically significant, we observed bigger vacuoles in *GMR-hTau* flies compared to control. The co-expression of Stg in *GMR-hTau* flies reduced vacuole area (Figure 15C).

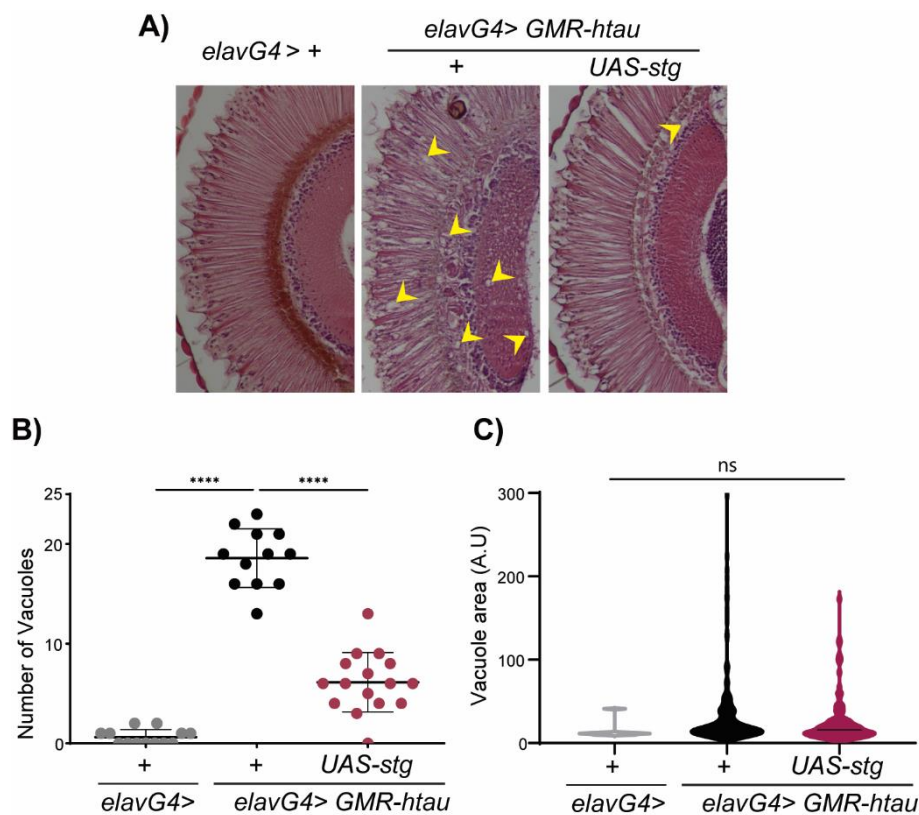


Figure 15. Stg ameliorates retina morphology and reduces vacuolization (A) Representative cross-sections of adult retinas from control (*elav* /+), *elav; GMR-hTau* and *elav; GMR-hTau; Stg* expressing flies, stained with hematoxylin and eosin. Yellow arrowheads indicate vacuoles. (B) Quantification of the number of vacuoles in the lamina region with a diameter equal or superior to 3,5 μ m for the genotypes indicated. (C) Vacuole area. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison test; $n \geq 12$. Error bars denote SD; n.s. indicates non-significant; **** $p < 0.0001$.

These observations indicate that Stg expression suppresses Tau- associated neurodegeneration phenotypes and suggest that the dephosphorylation mediated by Stg reduces hTau cellular toxicity.

3.1.5. Oligomerization and Tau spreading is impaired by Stg activity

During the analysis of adult head sections, we consistently detected the presence of vacuoles in the central brain region of *GMR-hTau* flies (Figure 16A). This was unexpected since hTau expression is limited to cells within the posterior domain of the eye imaginal disc (Figure 11B) and is not expressed in brain neurons. The occurrence of vacuolization in the central brain area is suggestive of spreading of toxic forms of hTau from the retina to neighboring regions in the fly head. To address this hypothesis, we quantified the number of vacuoles and apoptotic cells using the cleaved Caspase 3 positive cells, a readout of apoptosis, in the central brain region in all genotypes under study (Figure 16D). Our analysis shows that Stg expression reduces vacuole number in hTau expressing flies (Figure 16A, B). Quantification of cleaved -Caspase 3 positive cells, reveals increased apoptosis in *GMR-hTau* relative to control flies. Importantly, the number of apoptotic cells is significantly reduced upon Stg expression (Figure 16C, D). These findings support the hypothesis that hTau toxicity extends beyond the retina and imply spreading of hTau toxic forms in *GMR-hTau* tauopathy model. Accordingly, we evaluated the presence of oligomers in extracts from hTau expressing flies, as these are strongly implicated in hTau toxicity and the most amenable for spreading (Mudher et al., 2017). Dot blot analysis with A11, an oligomer specific antibody (Abskharon et al., 2020), reveals strong reactivity with *GMR-hTau* extracts (Figure 16E). This contrasts with lower reactivity, in *GMR-hTau; Stg* extracts, for equivalent levels of total protein. The dot blot analysis was performed using the total extract of fly heads and A11 antibody recognizes oligomers from different proteins. To overcome these questions and perform a more specific analysis for Tau oligomers we stained histological brain sections with AT8 antibody to detect Tau species outside of the hTau expressing domain. As expected, we observed positive staining in the eye component, more specifically in photoreceptors, as it corresponds to the domain of GMR expression. Moreover, we also detected Tau deposits in optic lobe and central brain of both genotypes under study (*GMR-hTau* and *GMR-hTau; Stg*).

Using this methodological approach, we clearly show that hTau expressed in photoreceptors can spread to distal regions in the head, as the central brain (Figure 16F). However, we were only able to detect Tau deposits in 2 heads of hTau and hTau; Stg flies (out of 5). These experiments should be repeated to increase the *n* available and enable statistical analysis in order to explore in detail the role Stg activity in the modulation of hTau spreading.

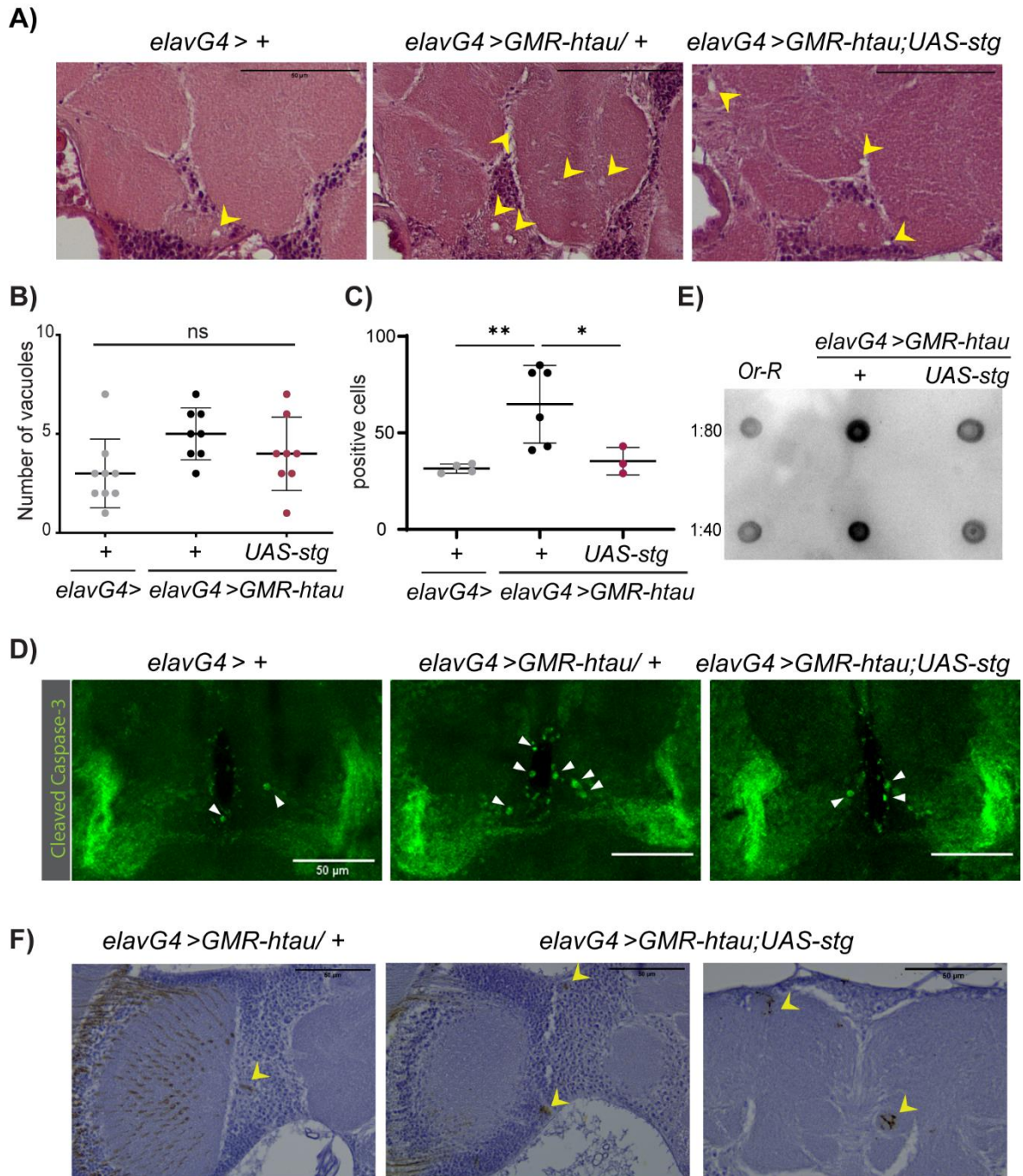


Figure 16. Stg reduces Tau spreading. (A) Representative cross-sections of adult heads from control (*elav*/+), *elav*; *GMR-hTau* and *elavG4*; *GMR-hTau*; *Stg* expressing flies, stained with hematoxylin and eosin. Vacuoles are indicated by yellow arrowheads. (B) Quantification of the number of vacuoles in the central brain region of 10-day old flies for the genotypes indicated; $n \geq 8$. (C) Quantification of Caspase-3 positive cells in brain of 10-day old flies; $n \geq 3$. (D) Representative images of adult fly brains stained for Caspase-3. Caspase-3 positive cells are indicated by white arrowheads. (E) Dot-blot analysis of oligomeric species in the soluble fraction of extracts from fly heads, for the indicated genotypes. Two dilutions of extract were analyzed. (F) Analysis of phosphorylated Tau (Ser202/Thr205; AT8 antibody) in crosssections of adult heads for the indicated genotypes. Yellow arrowheads indicate Tau deposition outside the domain of *GMR-hTau* expression. Scale bar is 50 μ m. Statistical significance was calculated

using one-way (B, C) ANOVA. Error bars denote SD; n.s. indicates non-significant; * $p < 0.05$; ** $p < 0.01$.

Altogether, these findings show that Stg impact on hTau phosphorylation correlates with reduced oligomerization potential and neurodegeneration.

3.1.6. Stg suppresses neurotoxicity after Tau aggregation is established

The results presented thus far highlight Stg phosphatase as a regulator of hTau phosphorylation and toxicity with effective physiological improvement in neurodegeneration progression. We next asked whether the up-regulation of Stg activity could be an advantage in a disease context, as this could promote the development of therapeutic approaches based on Tau regulation by Stg/Cdc25 phosphatases. To test this possibility, we assessed if overexpression of Stg after Tau hyperphosphorylation and aggregation are established could reduce hTau – associated toxicity. We used the GAL80 temperature-sensitive (hereafter GAL80^{ts}) system to block the expression of UAS-Stg until *elav*; *GMR-hTau* individuals reach the adult stage. Accordingly, fly crosses were maintained at 18°C until flies eclose, since at this temperature the GAL80 binds to DNA and inhibits GAL4 mediated transcription. Adult flies (day 1) were transferred to 29°C, at which temperature, GAL80 no longer acts as repressor and GAL4 can bind to the UAS sequence, promoting Stg expression. Using this model, we induced Stg overexpression during adult stages in flies that constitutively express hTau and exhibit Tau hyperphosphorylation and aggregation. Consequently, these flies accumulate hTau since early developmental stages, mimicking a disease condition.

Analysis of protein levels in head extracts from 10-day old flies showed a significant reduction in hTau phosphorylated at Ser202/Thr205 residues after Stg upregulation (Figure 17). This clearly showed that Stg phosphatase can dephosphorylate hyperphosphorylated oligomeric forms of hTau *in vivo*.

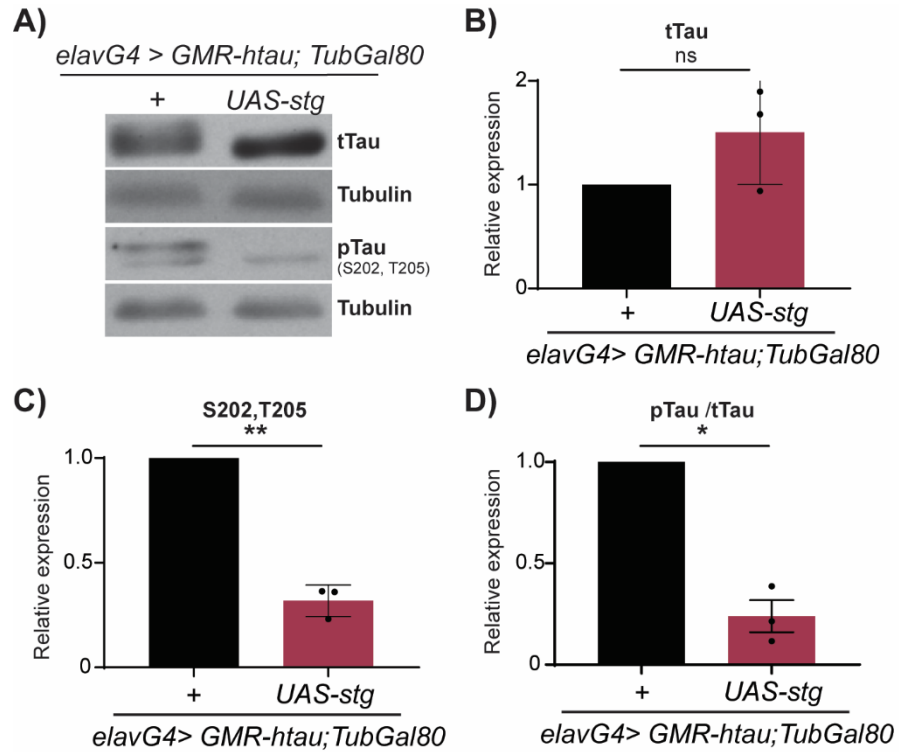


Figure 17. Stg promotes dephosphorylation of hTau toxic species. A) Representative immunoblots of extracts from 10-day-old adult flies expressing hTau (*elavG4; GMR-hTau; GAL80^{ts}*) or hTau; Stg (*elavG4; GMR-hTau/ Stg; GAL80^{ts}*), probed with total and phosphorylated Tau. (B, C) Quantification of total and phosphorylated Tau levels. (D) Ratio between Tau phosphorylated at Ser202/Thr205 and total hTau levels. Tubulin was used as loading control. Data represents three biological replicates. Data were analyzed using unpaired t- test with Welch's correction. Error bars denote SD; n.s. indicates non-significant; * $p < 0.05$; ** $p < 0.01$.

We next asked if the lower levels of phosphorylated hTau correlate with slower disease progression, using brain and retina vacuolization and climbing performance as readouts. As retina is completely formed when flies eclose, Stg overexpression failed to restore the internal structure of the retina in 10-day- old flies (Figure 18A), as expected. However, it was sufficient to promote a reduction in the vacuole area (Figure 18B). Analysis of the brain region indicated that Stg expression significantly reduced the number of vacuoles detected (from 3,5 to 0,75 vacuoles, on average) (Figure 18C, D).

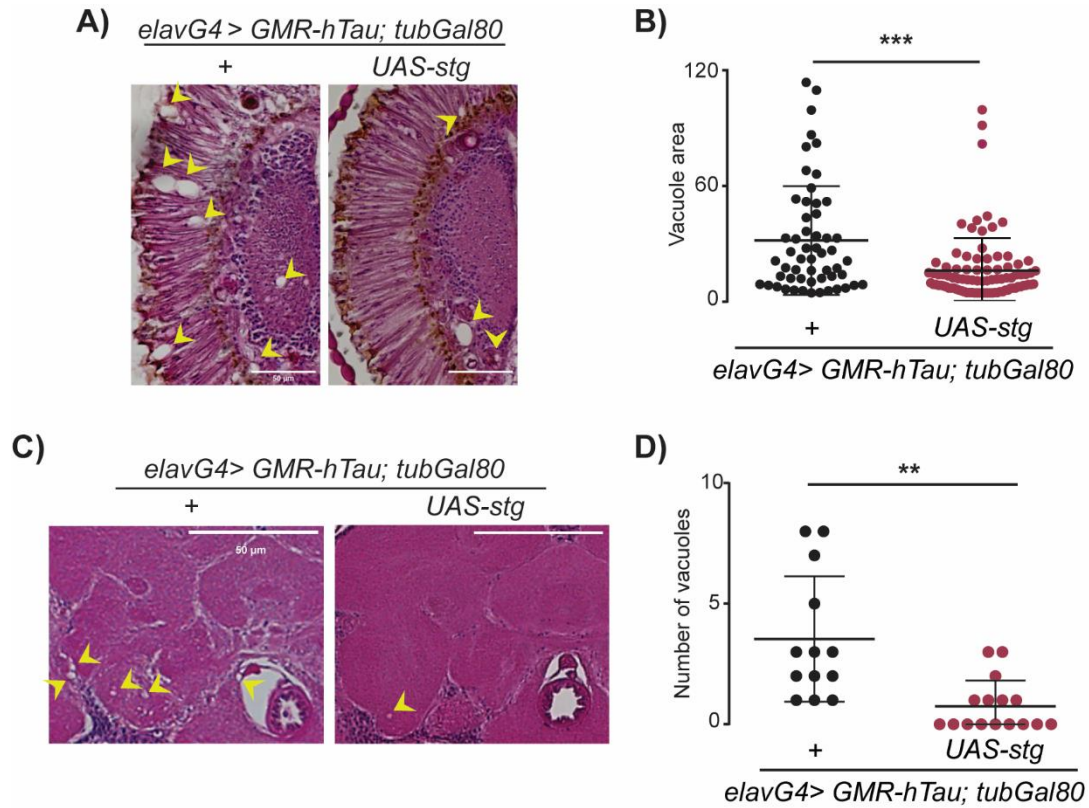


Figure 18. Stg reduces vacuolization in disease mimetic flies. Representative images from (A) retina and (C) central brain of 10-day-old flies of the indicated genotypes, stained with hematoxylin and eosin. Vacuoles are indicated by yellow arrowheads. Scale bar 50 μ m. (B) Quantification of vacuole size (diameter $\geq 3,5\mu$ m) in the retina and lamina regions. (D) Quantification of the number of vacuoles in the central brain region of the genotypes indicated. The results were analyzed using unpaired Student's t-test with Welch's correction, two-tailed ($n \geq 13$). Error bars denote s.d.; ** $P < 0.01$; *** $P < 0.001$.

We next assayed the climbing ability of hTau (*GMR-hTau; GAL80^{ts}*) individuals at days 3, 8 and 15 after Stg upregulation, to evaluate the impact of Stg activity on neurodegeneration progression. Climbing ability analysis showed that hTau and hTau; Stg individuals behave identically at day 3 (Fig 19 A, D). This was expected as UAS-Stg expression only occurs after flies eclose. However, following Stg continuous expression by days 8 and 15, hTau; Stg expressing flies performed significantly better than hTau flies (Figure 19 B, D). Indeed, for all time points, the climbing probability (CF) of hTau; Stg flies is higher than that of hTau -expressing flies (Figure 19D), suggesting that increased Stg levels and activity prevent the decay of locomotor function in hTau flies. In addition, analysis by performance groups showed that by day 8, 48% of *GMR-hTau; Stg* expressing flies reach tubes 5 and 6 (group III) versus 22 % of hTau (*GMR-hTau; GAL80^{ts}*) individuals. This difference increases with time, and by day 15 almost 70% of hTau flies is retained in group I and only 10% is able to complete the assay. In contrast, at this timepoint, almost 40% of *GMR-hTau; Stg* individuals reach tubes 5 and 6 (group III).

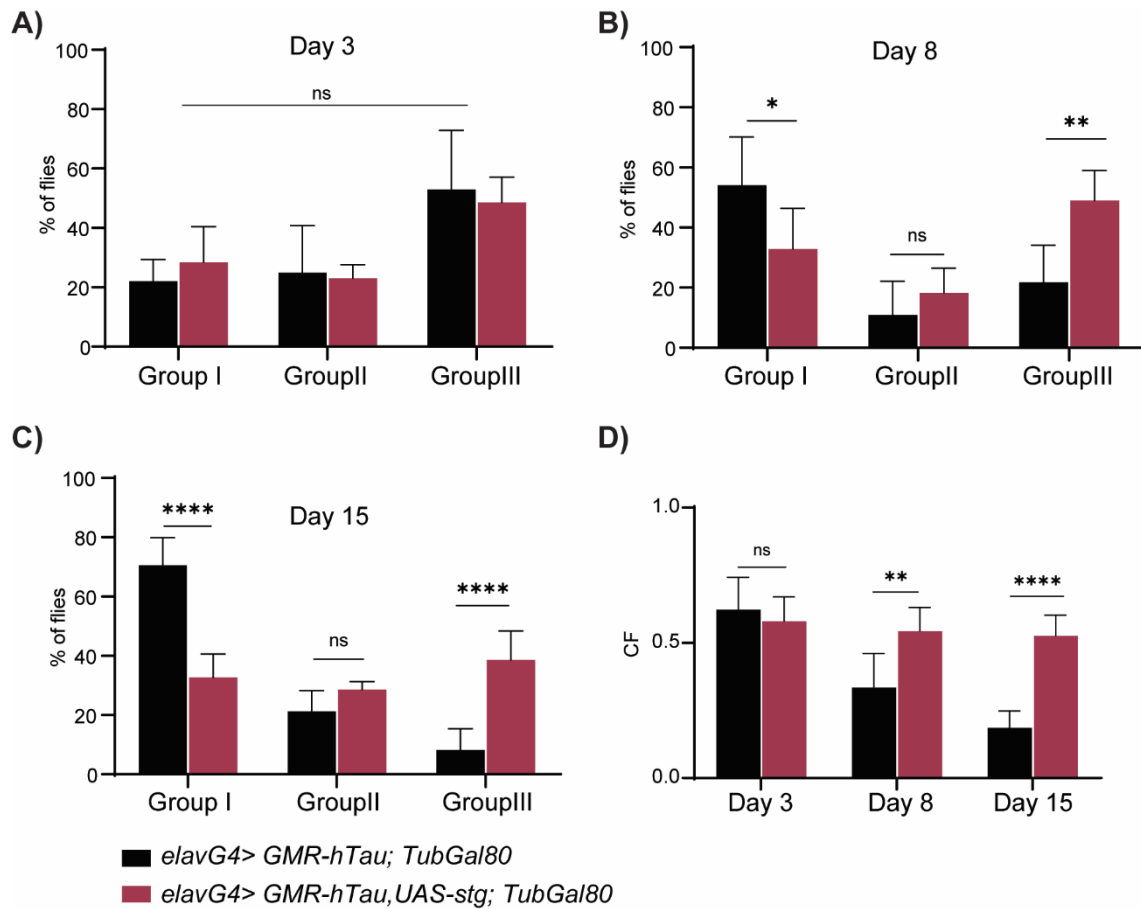


Figure 19. Stg expression delays neurodegeneration progression. (A-C) Quantification of the percentage of flies retained in group I (severe climbing impairment), II (moderate climbing defects) and III (reduced climbing defects) at days 3 (A), 8 (B) and 15 (F) of the genotypes indicated. (D) Graphic representation of the climbing index at 3, 8 and 15 days of the indicated genotypes. Statistical significance was calculated using two-way ANOVA, multiple comparisons ($n \geq 191$). Error bars denote SD; ns- non-significant; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Overall, our findings show that Stg is able to promote dephosphorylation of aggregated and abnormally phosphorylated hTau, and this has a physiological impact, as indicated by improved locomotor function of hTau flies.

3.2. Discussion

According to WHO approximately 55.2 million people worldwide live with dementia, with more than 7 million new cases per year (WHO, 2023). AD is the most common NDD, which currently has no available cure (Soria Lopez et al., 2019). Together with AD, all tauopathies share the presence of abnormal and hyperphosphorylated Tau deposits ordered as beta-sheet fibrils that can spread through the brain. Therefore, uncovering the phosphorylation patterns that favor Tau aggregation and propagation is key to develop new therapeutic approaches.

In the present study we validate Stg/Cdc25 as a novel Tau phosphatase implicated in disease. We used a tauopathy model that allowed us to constitutively express hTau under the control of an eye-specific promoter – GMR. In agreement with previous studies, the expression of hTau isoforms in the *Drosophila* eye leads to a rough phenotype, loss of bristles, and alterations in eye size and circularity (Chatterjee et al., 2009; Karsten et al., 2006; Nishimura et al., 2004). This phenotype is advantageous to look for genetic modifiers of hTau. We observed that the pan-neuronal overexpression of Stg, the fly homologue of Cdc25, is able to rescue the phenotypic alterations associated with 2N4R hTau expression. We provide evidence for an unambiguous Stg - Tau genetic interaction, expanding the former identification of Stg as a genetic modifier of TauV337M phenotype, a mutation associated with familial frontotemporal dementia (Shulman and Feany, 2003).

Cdc25 phosphatases (Cdc25A/ B/ C) are dual specific phosphatases that play essential roles during normal cell division as regulators of Cdk activity. Abnormal and increased Cdc25 activity is associated with cell proliferation and tumor progression (Boutros et al., 2006; Boutros et al., 2007). Interestingly, increased expression of Cdc5A and Cdc25B was detected in samples from AD brains, and both were shown to accumulate in the cytoplasm of degenerating neurons (Ding et al., 2000; Vincent et al., 2001). Cdc25 up-regulation correlated with increased phosphatase activity towards Cdk1, and the occurrence of mitotic figures in degenerating neurons (Ding et al., 2000; Vincent et al., 2001). Likewise, increased activity of Cdc25A was linked to hypoxia-induced neuronal death, and correlated with increased RB phosphorylation (Iyirhiar et al., 2017). The up-regulation of cell cycle genes, including *Cdc25*, is regarded as a neuronal response to insults, as DNA damage, that promotes aberrant cell-cycle re-entry, and ultimately leads to neuronal death (Chang et al., 2012; Marlier et al., 2020). Chatterjee et al (2016) demonstrated NGF deprivation and A β treatment or expression increased the expression and phosphatase activity of Cdc25A (Chatterjee et al., 2016). However, our findings suggest that increased expression of Cdc25 in neurons can be

neuroprotective. In agreement, Cdc25 overexpression promotes regeneration in *Drosophila* sensory neurons after physical injury, whereas Cdc25 knockdown impedes regeneration (Li et al., 2021). In fact, Cdc25 participates in the evolutionary conserved Piezo-Atr-Chek1-Cdc25 inhibitory regeneration pathway. Thus, we propose that Cdc25 may play a neuroprotective role during the early steps of Tau-induced neurodegeneration, in response to compromised axonal dynamics and homeostasis, while at later stages of the disease the co-occurrence of DNA damage elicits cell-cycle re-entry culminating in neuronal death.

Consistent with a potential homeostatic function for Cdc25 phosphatases in neurons, Cdc25A and Cdc25B expression has been reported in human brain samples (Ding et al., 2000; Vincent et al., 2001), mouse (Iyirhiar et al., 2017) and rat (Chang et al., 2012). The Stg/Cdc25-Tau genetic interaction, herein described, supports a function as cytoskeleton regulators. Importantly, the functional conservation between Stg and mammalian Cdc25 phosphatases predicts that Stg- Tau interaction is likely conserved throughout evolution. Accordingly, we propose that Stg/Cdc25 comprise novel Tau phosphatases with a strong impact on Tau biology and pathology, whose activity deserves further studies. Thus, Stg/Cdc25 adds to the list of key Tau regulators like Shaggy/GSK3-B (Jackson et al., 2002), Par-1/MARK (Ambegaokar and Jackson, 2011; Nishimura et al., 2004), MAPK and PP2A phosphatases (Shulman and Feany, 2003) identified in flies (reviewed in Gistelink et al., 2012; Hannan et al., 2016; Limorenko and Lashuel, 2022).

Our findings show that the Stg/Cdc25 phosphatase counteracts hTau-associated toxicity. We detected a correlation between reduction of Tau phosphorylation levels mediated by Stg and suppression of neurodegeneration phenotypes. Considering Stg/Cdc25 role as a phosphatase, we propose that Stg interacts directly with hTau promoting hTau dephosphorylation. Our results show that overexpression of a Stg phosphatase dead allele does not modify the rough-eye phenotype of hTau- expressing flies, showing that Stg/Cdc25 phosphatase activity is required to modify the phenotype associated with hTau expression. Moreover, phospho-specific antibodies, we showed that Stg co-expression is able to decrease Tau phosphorylation at disease- associated residues. Both AT8 and AT180 are specific for PHF-Tau and commonly used as biomarkers of AD, since they recognize abnormal Tau phosphorylation at Ser202/Thr205 and Thr231, respectively. Moreover, the close proximity detected among Stg and hTau, alludes to a direct interaction between the two proteins. Therefore, we put forward the hypothesis that Tau dephosphorylation is likely the outcome of direct Stg/Cdc25 activity. We showed that this is likely the case. Using a proximity ligation assay, with an endogenous *stg-GFP*, to avoid the possible false

positives due to Stg overexpression, we were able to demonstrate that hTau interacts with Stg/Cdc25. However, indirect regulation of Tau phosphorylation status, through modulation of Tau kinase(s) activity cannot be ruled out.

We demonstrated that Stg - Tau interaction is biologically relevant as Stg overexpression is able to counteract neurodegenerative phenotypes characteristic of hTau expressing flies. The expression of hTau, either wild-type or mutant isoforms leads to a reduced lifespan, locomotor deficits, brain vacuolization, and neuronal death. We used two models to address the impact of Stg/Cdc25 in hTau expressing flies; one where hTau expression is restricted to the retina, *elavG4; GMR-hTau; Stg*, and another where hTau expression occurs pan-neurally, *elavG4; UAS-hTauFlag; UAS-Stg*. In agreement with previous studies, we showed that expression of the longest hTau isoform in neurons impairs flies climbing ability (Passarella and Goedert, 2018). In both models we observed that Stg overexpression ameliorates the climbing ability of hTau expressing flies. Moreover, we observed an improvement of climbing performance in flies that express hTau only in the eye (*elav; GMR-hTau; Stg*). This observation lead us to propose that hTau expression in the eye also impacts brain neurons, leading to neurodegeneration phenotypes that could be rescued by Stg co-expression.

Brain vacuolization is a characteristic of aging in *Drosophila* that is drastically exacerbated by the expression of human genes associated with neurodegeneration (Wittmann et al., 2001). As we are expressing hTau in the retina we started by measuring the number of vacuoles in the retina. As expected, hTau expression increased retinal vacuolization that is reduced in hTau:Stg expressing flies. However, upon analysis of histological sections of flies heads we systematically detected vacuoles in the central brain region of *GMR-hTau* flies. The increased number of vacuoles detected in hTau flies correlates with an increase of Caspase-3 positive cells in the central brain. This finding is quite interesting since hTau expression is restricted to photoreceptors plus accessory cells and, in flies, only the lamina and medulla neuropils receive direct input from photoreceptor neurons (Fischbach and Dittrich, 1989; Sanes and Zipursky, 2010). Thus, the increased brain vacuolization detected in hTau-expressing flies could be explained by spreading of toxic forms of Tau, such as oligomers. In fact, we detected stronger reactivity of the A11 antibody in protein extracts from hTau flies compared with control samples. The A11 antibody recognizes protein oligomers, but not monomers or mature fibers of proteins or peptides. Although A11 recognizes different protein oligomers, and is unique for Tau, it is a good indicator that protein oligomers accumulate, in the model used in this study (*elavG4;GMR-hTau*), and spread to other regions of the fly head. To confirm spreading of Tau species we stained histological sections with the AT8 antibody that targets specifically PHF-Tau.

We detected positive PHF-Tau species in the optic lobe and central brain regions of hTau and hTau; Stg expressing flies, an evidence of Tau spreading. The observation PHF-Tau aggregates in the central brain of hTau;Stg individuals indicates that although Stg/Cdc25 reduces Tau phosphorylation, it is not sufficient to prevent Tau spreading. However, it should be noted that PHF structures are not considered the most toxic species (Niewiadomska et al., 2021). Therefore, we propose that Stg/Cdc25 is able to slow down disease progression through Tau dephosphorylation, including the propagation of the most toxic aggregates. However, unequivocal conclusions require analysis of a higher number of samples and further experiments, with a complementary battery of antibodies detecting aggregated Tau. Similar findings were reported in mice (Hu et al., 2016) where it was shown that dephosphorylation of AD- associated hyperphosphorylated Tau, by PP2A, leads to a reduction of Tau propagation in the brain. To date, mouse models have been the most credible *in vivo* models to investigate Tau spreading. These models are used to understand how Tau is delivered and/or uptaken by adjacent cells and can be divided in three main categories: transgenic expression of hTau restricted to the entorhinal cortex (EC); inducible Tau expression by adeno-associated virus (AAV); and intracranial delivery of pathological material as recombinant oligomeric/fibrillar Tau or human/mouse brain-derived Tau fibrils (reviewed in Colin et al., 2020; Robert et al., 2021). In this work, we characterize a *Drosophila* model that can be used as a powerful tool to identify key regulators of Tau spreading. Taking advantage of the easy manipulation of *Drosophila*, *elavG4;GMR-hTau* flies could be used to address the association of several proteins in hTau spreading mechanism. Likewise, other hTau isoforms could be tested.

We clearly demonstrated that Stg/Cdc25 dephosphorylates hTau and counteracts its associated toxicity. Importantly, our observations unveil the potential of therapeutic approaches based on Cdc25-Tau interaction. To address this possibility, we took advantage of the GAL80^{ts}/UAS system, to regulate the temporal activity of GAL4. In contrast to studies based on co-expression approaches, during fly development or restricted to adult stages (Cowan et al., 2010; Fernandez-Funez et al., 2015; Hannan et al., 2016), here we use a paradigm of disease and induce Stg/Cdc25 expression after high levels of phosphorylated Tau build-up in neurons. This approach enabled us to show that Stg/Cdc25 is able to promote dephosphorylation of highly-phosphorylated toxic forms of hTau. Moreover, we observed a reduction in vacuolization levels in the brain of 8- and 15-day old flies, in support of lagged neurodegeneration. This occurs albeit the maintenance of high levels of hTau protein. These findings show that it is the presence of highly phosphorylated soluble hTau that

is detrimental to neurons, in agreement with other studies in flies and mice (Cowan et al., 2015; Feuillette et al., 2010).

Altogether, the findings presented in this chapter enabled the identification of Stg/Cdc25 as a novel Tau phosphatase that plays a neuroprotective role. Moreover, we disclose the potential of using the *elavG4; GMR-hTau Drosophila* model to uncover the mechanisms and molecular players involved in Tau spreading.

CHAPTER IV

Analysis of Tau PTMs modified by Stg/Cdc25

4. Analysis of Tau PTMs modified by Stg/Cdc25

Tau protein is the target of many PTMs that can alter its solubility and render it prone to aggregation. Our previous analysis shows that Stg/Cdc25 activity reduces hTau phosphorylation levels at residues associated with tauopathies, most likely through direct interaction. Importantly, Tau dephosphorylation mediated by Stg/Cdc25 activity contributes to reducing protein oligomerization. These features correlate with amelioration of brain vacuolization and climbing ability, hallmarks of neurodegeneration in flies.

Altogether, these findings support the hypothesis that the activity of Stg/Cdc25 towards Tau has potential therapeutic value. Following our observations that Stg/Cdc25 modifies Tau phosphorylation at Ser202/Thr205 and Thr181, AD - associated residues, we explored the unique potential of Tau; Stg - expressing flies to uncover the pattern of Tau PTMs implicated in toxicity and neurodegeneration progression. Herein, using a biochemistry approach we characterized protein extracts from Tau and Tau; Stg - expressing flies in order to determine Tau PTMs potentially modified, directly or indirectly, by Stg/Cdc25 activity. Our goal is the identification of Tau PTMs with potential diagnostic value and highlight epitopes for targeted therapy approaches.

4.1. Results

4.1.1. Analysis of Tau PTMs by LC-MS/MS

To identify Tau PTMs that could potentially be targeted by Stg activity we compared the proteomic profile of fly brains expressing the full-length Flag-tagged Tau2N4R under the control of the neuronal driver *elav-Gal4* (*elavG4; UAS-TauFlag; UAS-GFP*) with flies co-expressing Stg (*elavG4; UAS-TauFlag; UAS-Stg*) or the Stg phosphatase death allele (*elavG4; UAS-TauFlag; UAS-Stg^{C379S}*). The addition of UAS-GFP in the genetic background *elavG4; UAS-Tau:FLAG* flies guarantees equivalent UAS dosage between samples, (Tau; GFP vs Tau; Stg vs Tau; Stg^{C379S}). Anti-Flag M2 antibody coupled to magnetic beads was used to purify hTau from protein fly head extracts. The use of these beads provided a robust method to capture Tau tagged with FLAG peptide sequence, which is confirmed by western blot analysis using an antibody against total Tau (5A6) (Figure 20).

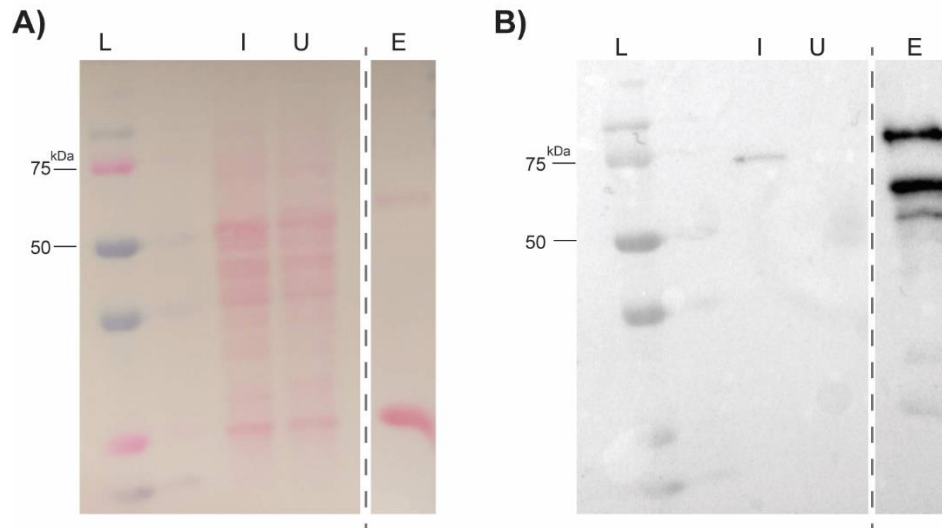


Figure 20. Western blot analysis of protein extracts from *elavG4; Tau-Flag; GFP* flies to validate immunoprecipitation (IP) of Flag-tagged hTau protein. (A)- Ponceau staining and (B) Western blot against total Tau. L-ladder, I-input protein, U-unbound protein (protein that does not bind to the magnetic beads), E- eluted protein.

The eluted protein was digested by trypsin followed by analysis by tandem mass-spectrometry (Orbitrap) (Figure 21).

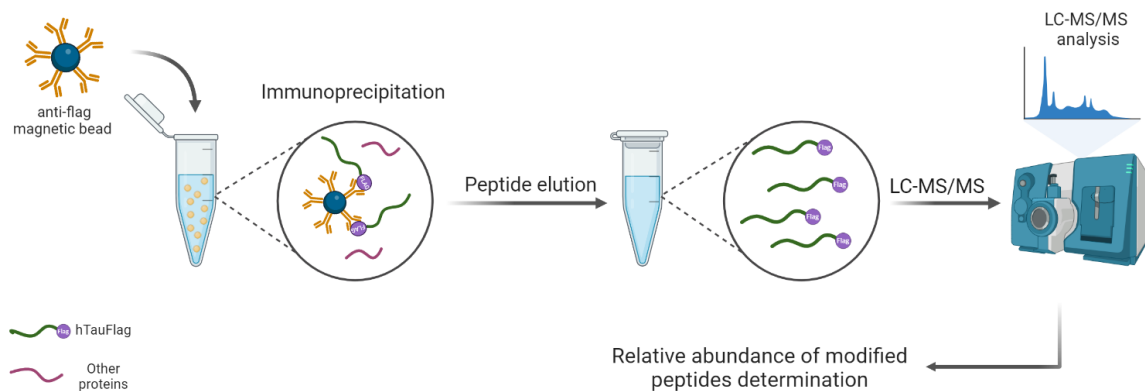


Figure 21. Schematic representation of immunoprecipitation followed by Mass-spectrometry workflow using anti-FLAG magnetic beads. Anti-FLAG beads are mixed with protein extracts. The beads bind only to proteins tagged with the FLAG sequence. After isolation of the beads, using a magnet, the bound protein is eluted, followed by digestion with trypsin and analysis by LC-MS/MS.

A total of 22 hTau peptides were identified in all samples (Table 7), which allowed a sequence coverage of 62,35 % for the 2N4R Tau isoform (Figure 22). hTau

was found in the three genotypes under analysis, with different abundances. The values of the protein abundance ratios (PAR) given by proteome discoverer showed that hTau was more abundant in samples from Tau;Stg individuals compared to samples Tau;GFP and in Tau; Stg^{C379S} individuals when compared to samples from TauFlag; GFP or TauFlag; Stg^{C379S} individuals (Table 8).

MAEPRQEFVMEHDHAGTYGLGDRKDQGGYTMHQDQEGD TDAGLKESPLQTPTEDGSEEPG	60
SETSDAKSTPTAEDVTAPLVDEGAPGKQAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAG	120
HVTQARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPQKQGQANATRIPAKTPPAPK	180
TPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREP KKVAVVRTPPKSPSSAK	240
SRLQTAPVPM PDLKNVKSIGSTENLKHQPGGGKVQIINKLDLSNVQSKCGSKDNIKHV	300
PGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNI	360
THVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTS PRHLSNV SSTGSIDMV	420
DSPQLATLADEV SASLAKQGL	

Figure 22. Sequence coverage map of 2N4R hTau isoform. LC- MS/MS covered 62% of hTau (2N4R, UniprotKB P10636-8). Peptide sequences detected in the analysis are highlighted in grey.

Table 7. Amino acid sequences obtained by mass spectrometry analysis of the longest hTau isoform (2N4R) and its associated PTMs.

Positions in Protein	Annotated Sequence	Modifications
[6-24]	[R].QEFEVMEDHAGTYGLGDRK.[D]	
[6-24]	[R].QEFEVMEDHAGTYGLGDRK.[D]	1xOxidation [M6]
[6-24]	[R].QEFEVMEDHAGTYGLGDRK.[D]	1xGln->pyro-Glu [N-Term]; 1xOxidation [M6]
[6-24]	[R].QEFEVMEDHAGTYGLGDRK.[D]	1xGln->pyro-Glu [N-Term]
[6-23]	[R].QEFEVMEDHAGTYGLGDR.[K]	1xOxidation [M6]
[195-209]	[R].SGYSSPGSPGTPGSR.[S]	
[195-209]	[R].SGYSSPGSPGTPGSR.[S]	1xPhospho [S8(100)]
[243-254]	[R].LQTAPVPMPDLK.[N]	
[243-254]	[R].LQTAPVPMPDLK.[N]	1xOxidation [M8]
[68-87]	[K].STPTAEDVTAPLVDEGAPGK.[Q]	
[68-87]	[K].STPTAEDVTAPLVDEGAPGK.[Q]	1xPhospho [S/T]
[396-406]	[K].SPVVSQDTSR.[H]	1xPhospho [T/S]
[241-254]	[K].SRLQTAPVPMPDLK.[N]	
[241-254]	[K].SRLQTAPVPMPDLK.[N]	1xOxidation [M10]
[210-221]	[R].SRTPSLPTPPTTR.[E]	
[226-240]	[K].VAVVRTPPKSPSSAK.[S]	1xPhospho [T6(100)]
[226-240]	[K].VAVVRTPPKSPSSAK.[S]	2xPhospho [T6(100); S12(99.6)]
[386-406]	[K].TDHGAEIVYKSPVVSQDTSR.[H]	2xPhospho [S11(100); S15(100)]
[175-190]	[K].TPPAPKTPSSGEPPK.[S]	1xPhospho [T7(100)]
[212-224]	[R].TPSLPTPPTREP.[K]	1xPhospho [T6(100)]
[25-44]	[K].DQGGYTMHQDQEGDTDAGLK.[E]	1xOxidation [M7]
[45-67]	[K].ESPLQTPTEDGSEEPGSETSDAK.[S]	
[322-340]	[K].CGSLGNIHHKPGGGQVEVK.[S]	1xCarbamidomethyl [N-Term]; 1xCarbamidomethyl [C1]
[354-369]	[K].IGSLDNITHVPGGGNK.[K]	
[354-369]	[K].IGSLDNITHVPGGGNK.[K]	1xDeamidated [N]
[354-369]	[K].IGSLDNITHVPGGGNK.[K]	1xPhospho [S3(100)]
[354-370]	[K].IGSLDNITHVPGGGNKK.[I]	
[260-267]	[K].IGSTENLK.[H]	1xPhospho [S3(100)]
[164-174]	[K].GQANATRIPAK.[T]	1xDeamidated [N4]
[281-290]	[K].KLDLSNVQSK.[C]	1xDeamidated [N6]
[299-317]	[K].HVPGGGSVQIVYKPVDSLK.[V]	
[151-163]	[K].IATPRGAAPPGQK.[G]	

Table 8. Abundance ratios of hTau2N4R isoform in samples from TauFlag; GFP, TauFlag; Stg and Tau; StgC379S – expressing flies according to proteome discoverer

	Tau; Stg / Tau;GFP	Tau; Stg ^{C379S} / Tau;GFP	Tau; Stg /Tau; Stg ^{C379S}
PAR	1,125	1,782	0,630

PAR- Protein abundance ratio

4.1.2. Phosphorylation sites identified on Tau

Maximum sensitivity of the Quadrupole-Orbitrap instrument during the screening is essential to detect minor ion fragments and facilitates the identification of phosphorylated (p) peptides together with the localization of phosphorylated sites. In the three genotypes under analysis, 8 phosphopeptides, common to all samples, including two double-phosphorylations, on Ser and Thr residues, were identified on hTau with a localization probability higher than 0.99. These are Thr181; Ser202; Ser217; Thr231; Thr231/Ser237; Ser262; Ser356; Ser396/ Ser400 (Table 7, 8). Most of the p-sites detected are presented in the proline-rich domain (Figure 23). Phosphorylation on tyrosines was not detected.

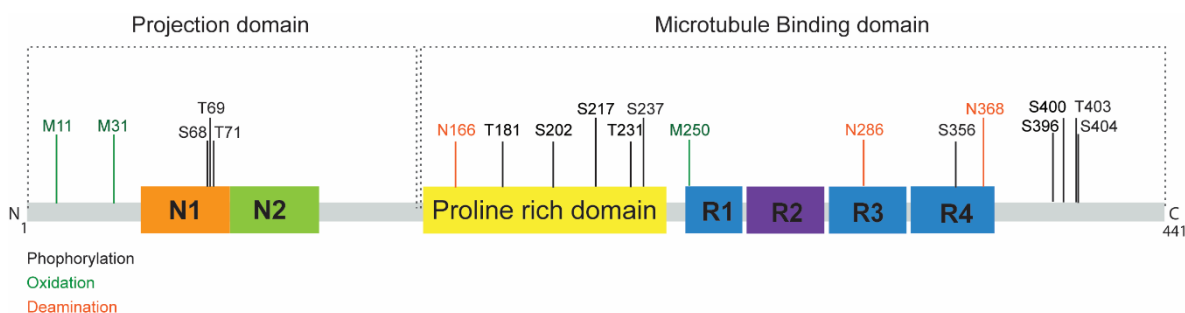


Figure 23. Schematic representation of Tau 2N4R protein and the PTMs identified in our analysis. The four protein domains: N-terminal, proline-rich, microtubule binding repeats (MBR) -containing the repeats R1, R2, R3 and R4- and the C-terminal domain are highlighted. Phosphorylated sites detected in our analysis are represented in black, oxidations in green and deaminations in orange. N- asparagine, M- methionine S- serine, T- threonine.

In order to pinpoint changes in the phosphorylation status of Tau residues mediated by Stg/Cdc25 phosphatase activity we compared the phosphopeptides identified in extracts of flies expressing hTau (*elavG4; hTauFlag; GFP*), with flies co-

expressing Stg (*elavG4; hTauFlag; Stg*) or Stg^{C379S} (*elavG4; hTauFlag; Stg^{C379S}*). According to our hypothesis, residues modified by stg/Cdc25 phosphatase activity are expected to show reduced phosphorylation levels specifically in hTauFlag; Stg samples when compared to samples from hTauFlag; GFP and hTauFlag; Stg^{C379S} individuals.

Analysis of LC-MS/MS results using proteome discover allows the direct comparison of the abundance of each phosphopeptide identified between samples. However, it should be noted that differential phosphopeptide abundances can also result from changes in phosphorylation status and/or changes in protein expression (Wu et al., 2011). Therefore, it is crucial to normalize the phosphopeptide ratios identified in order to infer phosphorylation changes between genotypes. Accordingly, we used the protein abundance ratios (PAR- hTauFlag;Stg / hTauFlag;GFP and hTauFlag;Stg^{C379S}/ hTauFlag;GFP) to normalize the values obtained by the software for each phosphopeptide detected. After normalization, the peptide ratio values of each phosphopeptide in the different samples (hTauFlag;Stg or hTauFlag;Stg^{C379S}) can be used to establish if its phosphorylation status is downregulated (peptide ratio after calibration $\leq 0,8$) or up-regulated (peptide ratio after calibration $\geq 1,2$) relative to hTauFlag- expressing flies (Table 9, 10).

Our analysis allows the conclusion that variations in phosphorylation levels detected in hTauFlag; Stg individuals occur in consequence of changes in phosphorylation status rather than changes in protein levels, since the values before and after calibration are similar (RBC vs RAC values). Comparing the phosphorylation status between hTauFlag; GFP and hTauFlag; Stg samples we detected up-regulation of phosphorylation status in 3 phosphopeptides, corresponding to residues at positions Ser202, Thr217, Ser356, and the double phosphorylation Ser396, Ser400. In contrast, phosphorylation at Thr181, Thr231 and Ser262 residues appears downregulated in hTauFlag; Stg samples (Table 9).

Analysis of phosphoresidues from hTauFlag; Stg^{C379S} samples showed that prior to normalization all phosphopeptides appeared to be up-regulated compared to hTauFlag; GFP samples (RBC > 1). However, upon normalization we observed that only the phosphopeptides Thr217, Ser262 and Ser 396/ Ser400 (Table 10) show increased phosphorylation levels. Therefore, the phosphorylation changes detected in the remaining residues ($0,8 \geq RBA \geq 1,2$) result from changes in protein expression, suggesting that Stg/Cdc25 phosphatase activity does not impact the other phosphopeptides identified.

Table 9. List of the eight phosphopeptides identified and their corresponding protein ratio in hTauFlag; Stg/ hTauFlag; GFP samples.

Position	Phosphopeptides	Modifications	R	RBC	PAR	RAC
[175-190]	[K].TPPAPKTPSSGEPPK.[S]	1xPhospho [T7(100)]	T181	0,876	1,125	0,779
[195-209]	[R].SGYSSPGSPGTPGSR.[S]	1xPhospho [S8(100)]	S202	1,401	1,125	1,245
[212-224]	[R].TPSLPTPPTREPK.[K]	1xPhospho [T6(100)]	T217	2,185	1,125	1,942
[226-240]	[K].VAVVRTPPKSPSSAK.[S]	1xPhospho [T6(100)]	T231	0,485	1,125	0,431
[226-240]	[K].VAVVRTPPKSPSSAK.[S]	2xPhospho [T6(100); S12(99.6)]	T231, S237	1,051	1,125	0,934
[260-267]	[K].IGSTENLK.[H]	1xPhospho [S3(100)]	S262	0,730	1,125	0,649
[354-369]	[K].IGSLDNITHVPGGGNK.[K]	1xPhospho [S3(100)]	S356	2,043	1,125	1,816
[386-406]	[K].TDHGAEIVYKSPVVS GDTSPR.[H]	2xPhospho [S11(100); S15(100)]	S396, S400	1,521	1,125	1,352

R- residue; RBC- Ratio before calibration; PAR – Protein abundance ratio (hTau; Stg / hTau; GFP); RAC- Ratio after calibration; T- threonine; S-serine; Orange-downregulated p-site. Green – Upregulated p-site. Residues whose phosphorylation status is up-regulated are highlighted in green. Residues whose phosphorylation status is downregulated are highlighted in orange.

Table 10. List of the phosphopeptides identified and the corresponding protein ratio in hTauFlag; Stg^{C379S}/ hTauFlag;GFP samples.

Position	Phosphopeptides	Modifications	R	RBC	PAR	RAC
[175-190]	[K].TPPAPKTPSSGEPPK.[S]	1xPhospho [T7(100)]	T181	1,596	1,782	0,896
[195-209]	[R].SGYSSPGSPGTPGSR.[S]	1xPhospho [S8(100)]	S202	2,253	1,782	1,264
[212-224]	[R].TPSLPTPPTREPK.[K]	1xPhospho [T6(100)]	T217	2,81	1,782	1,577
[226-240]	[K].VAVVRTPPKSPSSAK.[S]	1xPhospho [T6(100)]	T231	1,437	1,782	0,806
[226-240]	[K].VAVVRTPPKSPSSAK.[S]	2xPhospho [T6(100); S12(99.6)]	T231, S237	1,671	1,782	0,938
[260-267]	[K].IGSTENLK.[H]	1xPhospho [S3(100)]	S262	2,167	1,782	1,216
[354-369]	[K].IGSLDNITHVPGGGNK.[K]	1xPhospho [S3(100)]	S356	1,783	1,782	1,001
[386-406]	[K].TDHGAEIVYKSPVVS GDTSPR.[H]	2xPhospho [S11(100); S15(100)]	S396, S400	2,14	1,782	1,203

R- residue; RBC- Ratio before calibration; PAR – Protein abundance ratio (hTau; Stg^{C379S} / hTau; GFP); RAC- Ratio after calibration; T- threonine; S-serine; Orange-downregulated p-site. Green – Upregulated p-site. Residues whose phosphorylation status is up-regulated are highlighted in green.

These results show that Stg/Cdc25 activity promotes changes in hTau phosphorylation status. These changes can result from direct dephosphorylation of hTau or as a consequence of dephosphorylation and modulation of the activity of

upstream hTau kinases. Additionally, Stg/ Cdc25 could also act on distinct pathways affecting Tau conformational structure, leading to changes in the availability of the residues suitable to be phosphorylated.

Nonetheless, and taking advantage of our model, an efficient approach to identify hTau residues dephosphorylated, directly or indirectly by Stg/Cdc25, is the comparison of the phosphopeptide ratios in samples from hTauFlag; Stg and hTauFlag; Stg^{C379S} individuals. Accordingly, we compared phosphorylation changes detected in hTauFlag; Stg and hTauFlag; Stg^{C379S} expressing flies. Our results revealed two up-regulated p-sites, namely Thr217 and Ser356 and two downregulated p-sites, Thr231 and Ser262 (Table 11). Taking together, our analysis revealed that Thr231 and Ser262 may be potential targets of Stg/Cdc25 phosphatase activity.

Table 11. List of phosphopeptides identified and the corresponding protein ratio in hTauFlag; Stg/ hTauFlag; Stg^{C379S} samples.

Position	Phosphopeptides	Modifications	R	RBC	PAR	RAC
[175-190]	[K].TPPAPKTPPSSGEPPK.[S]	1xPhospho [T7(100)]	T181	0,549	0,630	0,871
[195-209]	[R].SGYSSPGSPGTPGSR.[S]	1xPhospho [S8(100)]	S202	0,622	0,630	0,987
[212-224]	[R].TPSLPTPPTREPK.[K]	1xPhospho [T6(100)]	T217	0,778	0,630	1,235
[226-240]	[K].VAVVRTPPKSPSSAK.[S]	1xPhospho [T6(100)]	T231	0,338	0,630	0,537
[226-240]	[K].VAVVRTPPKSPSSAK.[S]	2xPhospho [T6(100); S12(99.6)]	T231, S237	0,629	0,630	0,998
[260-267]	[K].IGSTENLK.[H]	1xPhospho [S3(100)]	S262	0,462	0,630	0,733
[354-369]	[K].IGSLDNITHVPGGGNK.[K]	1xPhospho [S3(100)]	S356	1,146	0,630	1,819
[386-406]	[K].TDHGAEIVYKSPVVS GDTSPR.[H]	2xPhospho [S11(100); S15(100)]	S396, S400	0,71	0,630	1,127

R- residue; RBC- Ratio before calibration; PAR – Protein abundance ratio (hTau; Stg / hTau; Stg^{C379S}); RAC- Ratio after calibration; T- threonine; S-serine; Orange-downregulated p-site. Green – Upregulated p-site. Residues whose phosphorylation status is up-regulated are highlighted in green. Residues whose phosphorylation status is downregulated are highlighted in orange.

Table 12 summarizes all the p-sites detected in our analysis and their phosphorylation status in the different genotypes under study (downregulation $\leq 0,8$; up-regulation $\geq 1,2$).

Table 12. Summary of the phosphoresidues identified in the three genotypes under analysis and their phosphorylation ratios comparisons.

Residue	hTau; Stg / hTau; GFP	hTau; Stg ^{C379S} / hTau; GFP	hTau; Stg / hTau; Stg ^{C379S}
T181	↓	nd	nd
S202	↑	↑	nd
T217	↑	↑	↑
T231	↓	nd	↓
T231, S237	nd	nd	nd
S262	↓	↑	↓
S356	↑	nd	↑
S396, S400	↑	↑	nd

↑ Upregulation; ↓ Downregulation; nd- no differences.

Additional to the phosphopeptides described above, two more phosphopeptides were identified with multiple potential phosphorylation sites (Table 13, 14). However, we could not clearly define the p-site within the peptide. A way to predict the most probable p-site is to look for the PSMs values that stand for “the total number of peptide-spectrum matches identified from all searches”, meaning the likelihood of each p-site be recorded in the experimental mass spectrum. This approach, called spectral counting, is based on the observation that more abundant peptides will be selected for fragmentation and produce a higher abundance of MS/MS spectra (Neilson et al., 2011). This method also allows qualitative information about possible phosphorylation changes between samples. To perform this analysis, for each genotype under study, we counted the number of PSMs for each potential p-site for the phosphopeptide identified divided by the total number of PSMs.

For the phosphopeptide in sequence [68-87], three potential mono-phosphorylation sites were predicted in all genotypes under analysis, namely Ser68, Thr69 and Thr71. For all samples, the results showed that this phosphopeptide could be phosphorylated with equal probability at Ser68 or Thr69 and both with higher probability compared to phosphorylation at Thr71. Although without a quantitative analysis, the total PSM values indicate that this phosphopeptide is phosphorylated in a similar ratio in hTauFlag; GFP (PSM = 85) and hTauFlag; Stg (PSM =84) individuals, and at reduced levels in hTau; Stg^{C379S} – expressing flies (PSM=66) (Table 13).

As for phosphopeptide in sequence [396-406], three potential mono-phosphorylation sites were predicted, Ser400, Thr403 and Ser404. However, Ser400 is only detected in hTauFlag; Stg^{C379S} samples and just one PSM was registered, suggesting that this event could be an artefact. The number of PSM in hTauFlag; GFP samples was analogous for the residues Thr403 (PSM= 23) and Ser404 (PSM= 26),

indicating equal phosphorylation probability for these residues. In contrast, in samples from hTauFlag; Stg and hTauFlag;Stg^{C379S} individuals the p-site Ser404 was recorded two times (PSMs = 42 and 40, respectively), compared with p-site Thr403 (PSMs = 26 and 19, respectively). Therefore, for these samples Thr403 residue is likely to be more phosphorylated (Table 14).

4.1.3. Other PTMs identified in Tau

We were expecting to detect other PTMs, in addition to phosphorylation, including lysine acetylation and/ or ubiquitination which are linked to neurodegeneration in AD, and O-GlcNAcylation, a modification of serine and threonine, which is reported to prevent Tau phosphorylation and aggregation (reviewed in (Alquezar et al., 2021). However, in our analysis we were not able to detect these modifications. Three oxidations at Met11, Met31 and Met250, and three deamination at Asn166, Asn286 and Asn368 were identified (Figure 23). These were observed sporadically and in low intensity. Moreover, it should be noted that PTMs like deamination and oxidation are often considered artefacts that result from protocol procedures in sample preparation or proteolytic digestion time (Soldi et al., 2016).

Table 13. PSM values for each possible p-site identified for the phosphopeptide in sequence [68-87] and quantification of the ratio between the PSM of each p-site and the total PSM determined per sample.

Position	Phosphopeptides	Modifications	p-sites	PSMs per sample			PSM per site / PSM per sample		
[68-87]	[K].STPTAEDVTAPLVDEGAPGK.[Q]	1xPhospho [S/T]		hTauFlag; GFP	hTauFlag: Stg	hTauFlag; Stg ^{C398S}	hTauFlag; GFP	hTauFlag: Stg	hTauFlag; Stg ^{C398S}
	Potential p-sites	S1	Ser68	32	32	27	0,39	0,38	0,41
		T2	Thr69	32	32	28	0,39	0,38	0,42
		T4	Thr71	19	20	11	0,23	0,24	0,217
			Total	83	84	66	1	1	1

Table 14. PSM values for each possible p-site identified for the phosphopeptide in sequence [396-406] and quantification of the ratio between the PSM of each p-site and the total PSM determined per sample.

Position	Phosphopeptides	Modifications	p-sites	PSMs per sample			PSM per site / PSM per sample		
[396-406]	[K].SPVVSQDTSPR.[H]	1xPhospho [S/T]		hTauFlag; GFP	hTauFlag: Stg	hTauFlag; Stg ^{C398S}	hTauFlag; GFP	hTauFlag: Stg	hTauFlag; Stg ^{C398S}
	Potential p-sites	S5	Ser400	0	0	1	-	-	0,02
		T8	Thr403	23	26	19	0,47	0,38	0,31
		S9	Ser404	26	42	40	0,53	0,62	0,67
			Total	49	68	60	1	1	1

4.2. Discussion

Tau exhibits a unique combination of properties that render it suitable for undergoing PTMs. Its hydrophilic character and high solubility, coupled with an intrinsically disordered structure, provide a molecular environment that promotes interactions with a variety of modifying enzymes (Zabik et al., 2017). This inherent flexibility and the nature of Tau amino acid residues offer numerous potential sites for PTMs, including phosphorylation, acetylation, ubiquitination, and glycation. These modifications are important to regulate Tau physiological functions and subcellular localization. Additionally, the susceptibility of Tau to PTMs contributes to its involvement in neurodegenerative disorders, highlighting the significance of understanding the intricate interplay between its structural properties and the dynamic landscape of post-translational modifications.

Phosphorylation is the most frequent Tau PTM, and it is important in physiologic conditions to control the interaction with cellular components, including microtubules. In physiological conditions, Tau is highly phosphorylated during fetal development compared to adult stages. However, in pathological conditions, Tau phosphorylation is highly associated with abnormal conformation and aggregation (Duquette et al., 2021). Several studies have been conducted aiming to identify kinases and phosphatases involved in the regulation of pathology-associated Tau phosphorylation status. More recently, work has been focused on the identification of the specific p-sites involved in pathology (Xia et al., 2021). The main objective is the identification of residues, preferably involved in the early stages of disease and amenable for targeted therapeutic approaches.

In Chapter III, using phospho-specific antibodies, we showed that Stg/Cdc25 overexpression reduces hTau phosphorylation levels at disease-associated residues. Our results also show that hTau dephosphorylation may result from direct Stg-Tau interaction, suggesting that Stg/Cdc25 is a Tau phosphatase. Importantly, we showed that Stg/Cdc25 phosphatase activity is required to suppress hTau-induced phenotypes in flies, suggesting that Stg/Cdc25 modifies residues linked with hTau toxicity. Therefore, we used our working model to identify hTau residues modulated by Stg/Cdc25 activity potentially relevant for future therapeutic approaches. With this purpose, we performed LC-MS/MS analysis of protein extracts derived from heads of hTau flies co-expressing Stg or Stg^{C379S}, the phosphatase death allele. According to our hypothesis residues modified in hTau; Stg expressing flies may correlate with reduced hTau toxicity.

All p-sites detected in our analysis were previously described in the literature. Phosphorylation at Thr181, Ser202, Thr217, Thr231, Thr237, Ser396 and Ser400 are described in normal and also in AD brains. In contrast, phosphorylation at Ser262 and Ser356 is only found in disease brains (reviewed in (Šimić et al., 2016).

Our analysis reveals that Thr231 and Ser262 appear less phosphorylated in hTauFlag; Stg- expressing flies when compared with hTauFlag; Stg^{C379S} or hTau; GFP- expressing flies. Therefore, we considered these as promising p-sites targeted by Stg/Cdc25 phosphatase. Moreover, we observed that phosphorylation at Thr181 is reduced in samples from hTau; Stg, compared with hTauFlag; GFP, suggesting that Stg/Cdc25 could also impact the phosphorylation of Thr181. However, comparing hTau; Stg samples with hTauFlag; Stg^{C379S}, no differences were found regarding Tau phosphorylation at Thr181, suggesting that a possible regulation of this residue by Stg/Cdc25 would be an indirect consequence of the phosphatase activity.

The MS/MS analysis corroborates the western blot results presented in Chapter III, regarding phosphorylation status of the residue Thr231. In both experimental approaches we observed a reduction in Tau phosphorylation levels at Thr231 in flies that overexpress Stg (*GMR-hTau; Stg* and *elavG4; hTauFlag; Stg*), emphasizing the idea that this residue could be a direct target of CDC25. We also used AT8 antibody, which detects phosphorylation at Ser202, Thr205 residues, and observed a reduction in phosphorylation levels upon Stg overexpression (*GMR-hTau; Stg* and *GMR-hTau/ Stg; GAL80^{ts}*). However, in LS-MS/MS analysis we detected up-regulated phosphorylation of Ser202 in samples from *elavG4; hTauFlag; Stg* flies, when compared to *elavG4; hTauFlag; GFP*. Although we failed to detect PTMs on Thr205 we put forward the hypothesis that in the western blot analysis, we are detecting reduced phosphorylation at Thr205, suggesting that Thr205 could be a target of Cdc25 modification. This hypothesis could be tested with the use of a phospho-specific antibody that uniquely recognizes Thr205 phosphorylation (as Phospho-Tau (Ser205)).

In our analysis we identified Thr231 as a potential target of Stg/Cdc25 phosphatase. Thr231 is localized in the proline-rich domain, just before the R1-R4 repeat units of MBD (Rani et al., 2020). Tau interacts with MTs mainly by the amino acid sequence within the second proline-rich region and the two hexapeptide motifs in repeats R2 and R3 of the MBD. Studies demonstrated that phosphorylation of Thr231 plus phosphorylation at Ser235 does not allow tubulin to polymerize into MTs. Schwabole et al (2015) showed that phosphorylation at Thr231 engages a salt bridge with Lys230 that can compete with the formation of intermolecular salt bridges to tubulin (Schwalbe et al., 2015). Moreover, phosphorylation at Thr231 is established as a valid blood/ cerebrospinal fluid (CSF) biomarker for AD diagnosis. Recently, it was found that Thr231 is detected in the cerebrospinal fluid (CSF) of early stages of AD, even when only subtle changes of amyloid- β (A β) pathology are detected (Ashton et al., 2021; Kidemet-Piskač et al., 2018; Milà-Alomà et al., 2022). Therefore, p-Tau at Thr231 could be considered a critical step in the initial stages of pathology, and a valuable target for therapy.

The residue Ser262 is within the MBD and its phosphorylation was shown to reduce drastically Tau binding to microtubules (Biernat et al., 1993; Iijima et al., 2010). Fischer et al (2009) demonstrated that pseudophosphorylation at Ser262 results in a conformational change of the protein, specifically the formation of a turn that promotes the contact of R1 and R2, and which interferes with MT binding (Fischer et al., 2009). Tau is hyperphosphorylated at Ser262 in tauopathies, including AD (Song et al., 2022). In a *Drosophila* AD model, Tau phosphorylation at Ser262 was shown to be crucial for A β -induced Tau toxicity *in vivo* (Iijima et al., 2010). The authors observed that co-expression of A β 42 and Tau carrying the non-phosphorylatable Ser262Ala mutation did not lead to neurodegeneration, in contrast to the expression of the normal Tau isoform. The role of Tau phosphorylation at Ser262 in disease progression is still controversial. Lauckner et al (2003) demonstrated that Ser262 was phosphorylated at lower levels in pre-tangles, compared with NFTs and suggested that phosphorylation at Ser262 may accelerate the conversion of pre-tangles into PHFs (Lauckner et al., 2003). In contrast, other studies revealed the presence of high levels of this phosphoresidue in pre-tangles (Augustinack et al., 2002).

Both residues, Thr231 and Ser262, were consistently hyperphosphorylated in neurofibrillary tangles and widely believed to be important players in pathological Tau activity. Therefore, both represent good targets for therapeutic approaches, either the use of specific antibodies to neutralize these epitopes or specific molecules that will inhibit or activate, respectively, the kinases or phosphatases that act on these residues.

There are a number of kinases that act on both epitopes, namely, GSK3 β , CDK5, AMPK, the MAPKs p38, p42 and JNK, DYRK1A and PKA (Billingsley and Kincaid, 1997; Gong et al., 2005; Gong et al., 2011; Hanger et al., 2009; Wang et al., 2007; Yoshida et al., 2004). Our results demonstrate that Thr231 and Ser262 can be targeted by CDC25 phosphatases. This can be a consequence of direct dephosphorylation or be mediated indirectly via the interaction of Stg/Cdc25 with kinases that act on these residues.

Besides phosphorylation, other PTMs are described as having an impact on tauopathies, including lysine acetylation and ubiquitination, both associated with neurodegeneration in AD and serine and threonine O-GlcNAcylation, which was reported to prevent phosphorylation (Alquezar et al., 2021). Unfortunately, in our analysis, we were not able to detect with a high degree of confidence other PTMs besides phosphorylation. In our approach, we used trypsin to digest Tau into peptides because it has advantages for MS/MS analysis. Trypsin is a serine protease that cleaves proteins into peptides with an ideal size range for MS. Moreover, it is highly specific cutting at the carboxyl side of arginine and lysine residues. The C-terminal arginine and lysine peptides are charged, making them detectable by MS. However, other proteases such as AspN could be used in

combination with trypsin to allow a better coverage of Tau and so provide a different set of Tau fragments (Sato et al., 2018).

CHAPTER V

Conclusions and future perspectives

5. Conclusions and future perspectives

Tau phosphorylation is considered an early and critical step in the development of tauopathies, including AD. Abnormal Tau phosphorylation leads to its detachment from microtubules and subsequent aggregation into neurofibrillary tangles. This process can disrupt normal cellular functions and ultimately lead to neuronal dysfunction and degeneration, contributing to the onset and progression of neurodegenerative diseases. The balance between Tau phosphorylation and dephosphorylation determines its ability to bind to microtubules. Alterations in this balance lead to abnormal accumulation of phosphorylated Tau. Therefore, the identification of Tau kinases and phosphatases is crucial to understanding the underlying mechanisms that lead to the formation of toxic Tau aggregates and neurofibrillary tangles.

In this study we took advantage of the well-characterized fly retina to evaluate the nature of Stg/Cdc25 -Tau interaction. The results presented in the current thesis allow the conclusion that Stg/Cdc25 acts as a Tau phosphatase, reducing Tau phosphorylation levels at disease-associated residues. The reduced Tau phosphorylation, in Stg overexpression conditions, is accompanied by an improvement of flies' neurodegenerative phenotypes, including retina degeneration, motor deficits (climbing ability), retina and brain vacuolization, and neuronal cell death.

Tau dephosphorylation by Stg/Cdc25 can result from direct or indirect interaction. Through proximity ligation assay we observed that the two proteins are in close proximity, strongly supporting the hypothesis for a direct interaction. However, accessory experiments should be conducted to clearly support direct interaction between the two proteins. *In vitro* dephosphorylation assays could be performed with Stg/Cdc25 or the mammalian CDC25 counterparts. Nonetheless, the main objective of this work was to explore whether decreasing Tau phosphorylation levels by CDC25 phosphatases, irrespective of the specific mechanism, yields positive outcomes in mitigating neurodegeneration-associated phenotypes. To mimetize disease conditions, we designed an experimental model to investigate the impact of Stg/Cdc25 on pre-established Tau aggregation. In this model, Tau is expressed during developmental stages, in the fly retina, where it accumulates and undergoes hyperphosphorylation, while Stg/Cdc25 expression is induced only in adult stages, simulating a disease-therapeutic strategy. Our findings demonstrate that Stg/Cdc25 activity significantly reduces Tau phosphorylation levels and ameliorates flies' neurodegenerative phenotypes, providing robust support for the potential therapeutic efficacy of CDC25 phosphatase-based approaches.

The findings that Stg/Cdc25 overexpression reduces brain vacuolization and oligomerization lead us to propose that Stg/Cdc25 could specifically counteract the build-up and propagation of Tau toxic species. In this work, we were able to detect phosphorylated Tau in flies' optic lobe and central brain, regions, where *GMR-hTau* is not expressed, showing that aggregated Tau spreads to other cell types and brain regions. However, we are not able to conclude if Stg/Cdc25 is able to reduce the spreading of Tau toxic species. To solve this paradigm specific PHF-Tau antibodies should be used, as is the case of PHF-1 which recognizes Tau phosphorylated at residues Ser396 and Ser404 or AT100, which recognizes Tau phosphorylation at Thr212 and Ser214. Moreover, electron microscopy (EM) techniques could be applied to analyze brain slices from *GMR-hTau* and *GMR-hTau; Stg* individuals. EM, in particular, cryo-EM emerged as an important technique for direct visualization of Tau oligomers with precision and clarity.

The successful demonstration that Stg/Cdc25 dephosphorylates Tau and consequently ameliorates neurodegenerative phenotypes, lead us to question the nature of the residues and PTMs, modified by Stg/Cdc25 phosphatase activity, that render hTau less toxic. Using MS/MS spectrometry analysis we concluded that dephosphorylation of Thr231 and Ser262 is one of the modifications that is regulated by Stg/Cdc25 activity. Importantly, hyperphosphorylation of Thr231 and Ser262 is only detected in tauopathies, suggesting that these are key residues and potentially good targets for target therapies, such as immunotherapy.

Overall, in this study we disclosure a role for Stg/Cdc25 phosphatases as regulators of Tau biology, that extends beyond their well- characterized function as cell-cycle regulators during cell proliferation, and point-out CDC25 based approaches as promising entry points to target abnormal Tau phosphorylation. Hopefully, this work will capture the attention of the scientific community, inspiring the translation of our findings into vertebrate models and novel therapeutic approaches.

CHAPTER VI

References

6. References

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