Understanding Prion-like Mechanisms of Tauopathy in Traumatic Brain Injury Using Novel *in vivo* Models

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Abstract:

Traumatic brain injury (TBI) is broadly acknowledged as a source of mortality and disability worldwide. It is one of the key risk factors resulting in neurodegeneration and the development of dementia. TBI has been shown to be associated with diseases such as chronic traumatic encephalopathy (CTE) and Alzheimer's disease (AD). Both diseases are classified as tauopathies, characterized by a distinct accumulation of abnormally phosphorylated tau protein. In TBI and other tauopathies, the pathology in patients seems to progress to connected brain regions at later stages of the disease, mimicking the spreading mechanisms seen in prion diseases. These prion-like characteristics, specifically the seeding and transmission of tau protein, in CTE and TBI have begun to be elucidated recently by a limited number of *in vitro* and *in vivo* studies. However, there is still a lack of information regarding the exact mechanisms of prion-like spreading of tau and the factors influencing it, especially how cells take up the tau seeds in TBI. Also, there is a lack of access to *in vivo* models to analyze prion-like mechanisms, especially ones which could also be used for high-throughput screening. Our aim is to understand the prion-like mechanism of the pathology of tau protein spreading in TBI patients by establishing a novel TBI paradigm to use in a zebrafish larvae model, as well as engineering a novel tau protein biosensor in transparent zebrafish that can be used to report tau seeding and spreading *in vivo*. We successfully isolated a stable transgenic zebrafish line that expresses a tauopathy biosensor reporter protein and validated that our transgenic zebrafish can report various forms of tau seeds via intraventricular injections. The biosensor output reports tau aggregation as GFP+ve puncta. Then, we developed an elegantly simple system to induce traumatic brain injury to the tau biosensor larvae and validated the presence of various markers associated with traumatic brain injury including seizure, axonal damage, cell death, hemorrhage, and vasospasm. Additionally,

larvae subjected to the blast injury formed GFP+ve puncta, indicative of tau aggregation in their brains and spinal cord at various time points. Further, we uncovered a link between the presence of post-traumatic seizure (PTS) and increased formation of tau aggregates. We also evaluated the impact of seizure activity on tau protein pathology in our TBI model via the use of convulsant and anti-convulsant drugs. We found that both the anticonvulsant drug retigabine and the convulsant drug 4-AP inhibited tau protein accumulation in our TBI model. Lastly, the pharmacological inhibition of dynamin-dependent endocytosis significantly reduced tau protein aggregation in our TBI model, hence demonstrating the important role of dynamin in the uptake of tau seeds and spreading of tau protein in TBI. The engineering of our novel tau protein biosensor and establishment of the TBI model in larval zebrafish will not only uncover more information about the prion-like spreading of tau protein pathology in tauopathies and TBI, but will also provide a valuable model for drug screening and intervention.

Preface

This thesis is an original work by Hadeel E. Alyenbaawi. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Animal Policy and Welfare Committee. The author has met the mandatory training requirements for animal users set out by the Canadian Council on Animal Care (CCAC) on the Care and Use of Animals in Research, Teaching and Testing.

The entirety of **chapter 2** (with some modifications) has been prepared for submission under the tittle:

"Tauopathy progression following traumatic brain injury is impacted by neural activity and requires endocytic mechanisms" Authors: Hadeel Alyenbaawi, Richard Kanyo, Razieh Kamali, Michele G. DuVal, Holger Wille, Edward A. Burton, W. Ted Allison.

Hadeel Alyenbaawi preformed the experiments, collected and analyzed data, composed and wrote the manuscript. Richard Kanyo assisted with the behavioural experiment, developed CaMPARI transgenic line, collected and analyzed data for CaMPARI experiments (Figure 2.9B-D). Razieh Kamali and Holger Wille obtained the electron microscope images of PTA (Figure 2.2E). Edward A. Burton engineered and provided human Tau Tg zebrafish that were used in figure (Figure 2.16). Michèle G. DuVal engineered SOD1:GFP Tg Zebrafish that were used in (Figure 2.10). Ted Allison was the supervisory author, was involved in concept formation and edited the manuscript.

The Appendix (chapter 4) is original work by Hadeel Alyenbaawi.

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List of common abbreviations

Aβ Amyloid-beta

AD Alzheimer disease

AGD agyrophilic grain disease

APP Amyloid Precursor Protein

ALS Amyotrophic Lateral Sclerosis

BBB blood-brain barrier

bp base pair

CaMPARI Calcium-Modulated Photoactivatable Ratiometric Integrator

CBD corticobasal degeneration

CCAC Canadian Council on Animal Care

CNS central nervous system

CSF Cerebrospinal fluid

CRISPR Clustered regularly interspaced short palindromic repeats

CTE chronic traumatic encephalopathy

DAPI 4′,6-diamidino-2-phenylindole

DPBS Dulbecco's Phosphate Buffered Saline

DMSO Dimethyl sulfoxide

dpf days post-fertilization

dpi days post-injection

dpti days post traumatic injury

E2 Exon two

E3 Exon three

EC entorhinal cortex

EDTA Ethylenediaminetetraacetic acid

ER endoplasmic reticulum

EPR Electron paramagnetic resonance

EM Electron Microsopy

EVs extracellular vesicles

eno2 neuronal enolase 2

F0 Parental generation

F1 First filial generation

FTD familial frontotemporal dementia

FTDP-17 familial frontotemporal dementia with Parkinsonism linked to chromosome-17

FRET Fluorescence resonance energy transfer

GGT globular glial tauopathy

GFP Green Flourescent Protein

GSK-3β glycogen synthase kinase–3β

HEK human embryonic kidney cells

Hsc70 Heat Shock cognate 70

HSPGs heparan sulfate proteoglycans

iPSC Induced pluripotent stem cell

ISF Interstitial fluid

KDa Kilodalton

MAPT Microtubule Associated Protein Tau

MAP6 Microtubule Associated Protein 6

MAP1A Microtubule Associated Protein 1A

MARKs Microtubule affinity regulating kinases

mAChRs muscarinic acetylcholine receptors

mTBI mild Traumatic Brain Injury

MTs Microtubules

MS-222 Tricaine methanesulphonate

NMDARs N- methyl-d-aspartate receptors

P7 Pyrimidyn-7

PD Parkinson Disease

PBS Phosphate buffered saline

PBS^{3+} Phosphate buffered saline containing 1% dimethyl sulfoxide, 1% Tween20 and 1% Triton

PFA Paraformaldehyde

PHFs Paired helical filaments

PiD Pick's disease

PrP^{Sc} Scrapie prion protein

PrP^{C} Cellular prion protein

PSP progressive supranuclear palsy

PTA phosphotungstate anion

p-tau phosphorylated tau

PTMs post-translation modifications

PTS Post-traumatic seizure

PW-TBI pressure wave induced traumatic injury

rDNA ribosomal DNA

RTG Retigabine

SDS-PAGE Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

Ser Serine

SNPs Single nucleotide polymorphisms

T7E1 T7 enndonuclease I assay

Tau (3R) isoforms of tau with three repeats

Tau (4R) isoforms of tau with four repeats

TBI Traumatic Brain Injury

TBST Tris buffered saline with 0.1% Tween

TD Tangle-only dementia

Tg transgenic

Thr Threonine

TNT tunnelling nanotube

Tyr Tyrosine

WT Wild type

4-AP 4-aminopyridine

1. Chapter 1: Introduction and Literature Review

1.1 Tau protein

1.1.1 Introduction

Tau pathology is one of the commonly seen proteinopathies in a subset of neurodegenerative disorders collectively called tauopathies. In tauopathies, the microtubule associated protein tau undergoes a plethora of changes at both the molecular and structural level leading to the production of toxic aggregates. These toxic aggregates, or oligomers, eventually develop into filaments that assemble into neurofibrillary or glial fibrillary tangles (Ferrer et al., 2014; Kosik et al., 1986; Nizynski et al., 2017). Since the identification of abnormally phosphorylated tau as one of the primary constituents of the filaments of neurofibrillary tangles in Alzheimer's disease (AD) (Grundke-Iqbal et al., 1986; Kosik et al., 1986), substantial progress has been made in the understanding of the physiological and pathological roles of tau proteins. Despite all the advances, no effective treatments have yet been developed for all tauopathies. Additionally, many aspects of the physiological and pathological roles of tau protein are still not fully understood, especially regarding the increasing evidence that supports tau protein pathology can be spread between neurons (Holmes and Diamond, 2014). In this chapter, an overview will be provided of tau genes, protein, structure, expression and physiological functions as well as their role in disease. An emphasis will be placed on the understanding of the spreading of tau protein pathology in tauopathies in general and more specifically in traumatic brain injury (TBI) and chronic traumatic encephalopathy (CTE).

1.1.2 Tauopathies

The term "tauopathy" was first introduced in 1997 (Spillantini et al., 1997) and is often used to describe a heterogeneous group of incurable neurodegenerative diseases with diverse

clinical presentations, yet they share a common pathological hallmark, the progressive deposit of filamentous tau inclusions (Table 1.1). Tau aggregates vary in tauopathies in terms of their isoform composition (3R vs 4R isoforms) (more details about tau isoforms mentioned in section 1.1.4), the affected cells where the aggregates are found (e.g. neurons or astrocytes) and their morphologies and ultrastructure (e.g. spherical Pick bodies vs. neuro-filamentous tangles in neurons; paired helical filaments vs. straight helical filaments) in addition to the regions they affect in the brain (Gotz et al., 2019). Most tauopathies are sporadic, but familial cases have also been identified (Bugiani et al., 1999; Hutton et al., 1998; Kovacs, 2017; Stanford et al., 2000).

Tauopathies can be divided into either primary tauopathies, in which tau inclusions are the main pathological marker, or secondary tauopathies, in which other protein aggregates (such as Amyloid-beta (Aβ) in AD) are also found (Table 1.1) (Irwin, 2016). Currently, some sporadic primary tauopathies comprising Pick's disease (PiD), progressive supranuclear palsy (PSP), and Corticobasal degeneration (CBD), globular glial tauopathy (GGT), argyrophilic grain disease (AGD) are referred to as frontotemporal lobar degeneration-tau (FTLD-tau), based on the latest tauopathy classification scheme (Gotz et al., 2019). On the other hand, familial cases, in which a mutation in the *MAPT* gene was identified are classified as familial frontotemporal dementia with Parkinsonism linked to chromosome-17 (FTDP-17) (Foster et al., 1997; Ghetti et al., 2015; Gotz et al., 2019; Kovacs, 2017).

Notably, a recent study by Forrest et al. (2018) suggests eliminating the use of the term FTDP-17 for familial cases with mutations in the *MAPT* gene, as mutations in another gene on the same chromosome were found to cause tau-negative FTD (Baker et al., 2006), as well as the identification of some mutations in *MAPT* that can cause the disease without Parkinsonism (Forrest et al., 2018). Besides, the analysis of the pathology showed no major differences

between those familial cases compared to the sporadic cases except age of onset, as genetic forms occurred earlier; hence, it was recommended to consider referring to these as familial forms of FTLD-tau (Forrest et al., 2018). Thus, practicing caution when using the term FTLD-17 with familial cases associated with mutations in the *MAPT* gene is highly recommended.

PiD is the least prevalent tauopathy among the FTLD-tau in which tau fibrils form round, interneuronal inclusions called Pick bodies that are composed only of 3R isoforms of tau and straight filaments (Gotz et al., 2019). In contrast, inclusions composed of 4R tau isoforms are commonly observed in the remaining FTLD-tau, including PSP, CBD, GGT, and AGD (Goedert et al., 2017a). In AD, tangle-only dementia (TD) and CTE, tangles are formed from both 3 and 4R isoforms (Kovacs, 2017). Whether sporadic and familial cases share similar pathomechanisms has not been fully investigated.

Table 1 1: Pathological features of major tauopathies

Primary Tauopathies	Isoforms composition	Affected cell types	Pathology
Pick's disease	3R tau isoforms	Neurons and glia	Pick bodies, neuropil threads, Ramified astrocytes, and round aggregates
Corticobasal degeneration	4R tau isoforms	Neurons and glia	Ballooned neurons, pretangles, astrocytic plaques, coiled bodies, and neuritic threads
Progressive supranuclear palsy	4R tau isoforms	Neurons and glia	Neurofibrillary tangles, globose tangles, tufted astrocytes, coiled bodies
Globular glial tauopathy	4Rtau isoforms	Neurons and glia	Globose oligodendrocyte inclusions
Argyrophilic grain disease	4R tau isoforms	Neurons and glia	Argyrophilic grains, oligodendritic coiled bodies, neuronal pretangles
Chronic traumatic encephalopathy	3R + 4R tau isoforms	Neurons and glia	Neurofibrillary tangles, dot-like or grain like neurites, astrocytic tangles
Secondary Tauopathy	**Isoforms composition**	**Affected cell types**	**Pathology**
Alzheimer's disease	3R + 4R tau isoforms	Neurons	Neurofibrillary tangles, neuropil threads

Table information obtained from (Gotz et al., 2019; McKee et al., 2018)

1.1.3 Genetics of Tau protein

Understanding tau protein genetics can provide mechanistic insights of tau toxicity in inherited forms of tauopathy, which could influence the diagnosis and treatment of patients. Indeed, many genetic studies on the human tau gene, some of which are discussed below, have elucidated the consequence of genomic changes such as mutations and tau haplotypes on tau functions (Chen et al., 2017; Pittman et al., 2005; Rademakers et al., 2005). These studies established the association of tau dysfunction with neurodegeneration in tauopathies (Pittman et al., 2006). The human tau gene (*MAPT*) was identified by (Neve et al., 1986), who used cDNA clones for *MAPT* and mapped it to chromosome 17. The *MAPT* gene, located at chromosome 17q21.31, comprises 16 exons, spanning ~150 kb. There are two main tau haplotypes, denoted as H1 and H2, occurring because of an ancient inversion of a 900 kb region in *MAPT*. Compared to the H2 haplotype, which is found almost exclusively in persons with European ancestry, the H1 haplotype is observed in more diverse ethnicity (Wolfe, 2012). There is no difference between H1 and H2 at the level of the amino acid sequence, but they do have a different set of single nucleotide polymorphisms (SNPs), and there is a 238 bp deletion in intron 9 in H2 (Baker et al., 1999).

Genetic studies have highlighted the significance of haplotype-specific polymorphism in tauopathies, as the H1 haplotype has an association with increased risk of PSP and CBD, while H2 was strongly negatively associated with these (Baker et al., 1999; Houlden et al., 2001). Regarding the H2 haplotype, allele-specific gene expression analysis has shown a two-fold increase of *MAPT* transcripts with the 2N splice variant (splice variants and tau isoforms are explained in section 1.1.4, Figure 1.1 A) in the gray matter area, which may indicate that exon 3 may contribute to protection against neurodegeneration (Caffrey et al., 2008). Because there are

no differences between the two haplotypes in terms of the protein sequences they are able to produce, it has been suggested that the pathogenic effects observed with particular haplotype may result from changes in splicing, transcription or post-translation modifications (Wolfe, 2012). This is consistent with the observation that silent and intronic mutations can cause familial frontotemporal dementia (FTD) by shifting the alternative splicing of tau pre-mRNA (Jiang et al., 2000; Wolfe, 2009).

MAPT mutations account for approximately 5% of familial frontotemporal dementia cases (Goedert et al., 2012). By 2016, around 59 pathogenic mutations had been identified that were linked to the development of familial frontotemporal dementia with Parkinsonism associated with chromosome 17 (FTDP-17) (Goedert et al., 2017a). Some mutations are associated with other tauopathies such as CBD (mutations found on exon 13) and PSP, but those are rare (Iyer et al., 2013; Kouri et al., 2014; Spillantini and Goedert, 2013). The mutations are either missense, deletion or silent in nature, affecting exons and intron regions mostly with the protein's repeat domain region (Dubourg et al., 2011; Goedert et al., 2017a; Shaw-Smith et al., 2006). *MAPT* mutations can affect the binding functions of tau to microtubules, increase its propensity to be phosphorylated and aggregate, and/or alter the splicing of *MAPT* pre-mRNA leading to an imbalance in the ratio of tau isoforms (Goedert et al., 2012; Gotz et al., 2019). Additionally, mutations in exons 9–13 have been shown to increase mRNA splicing and associations with abundant tau inclusions in neuronal cells, while mutations in exon 1, 10, intron 9 and 10 have been linked to tau protein aggregation in both glial and neuronal cells (Ghetti et al., 2015). The fact that mutations in *MAPT* were linked to progressive neurodegenerative diseases could support the potential effect of tau dysfunction in neurodegeneration (Ke et al., 2012; Poorkaj et al., 1998).

1.1.4 Tau isoforms, expression and structure

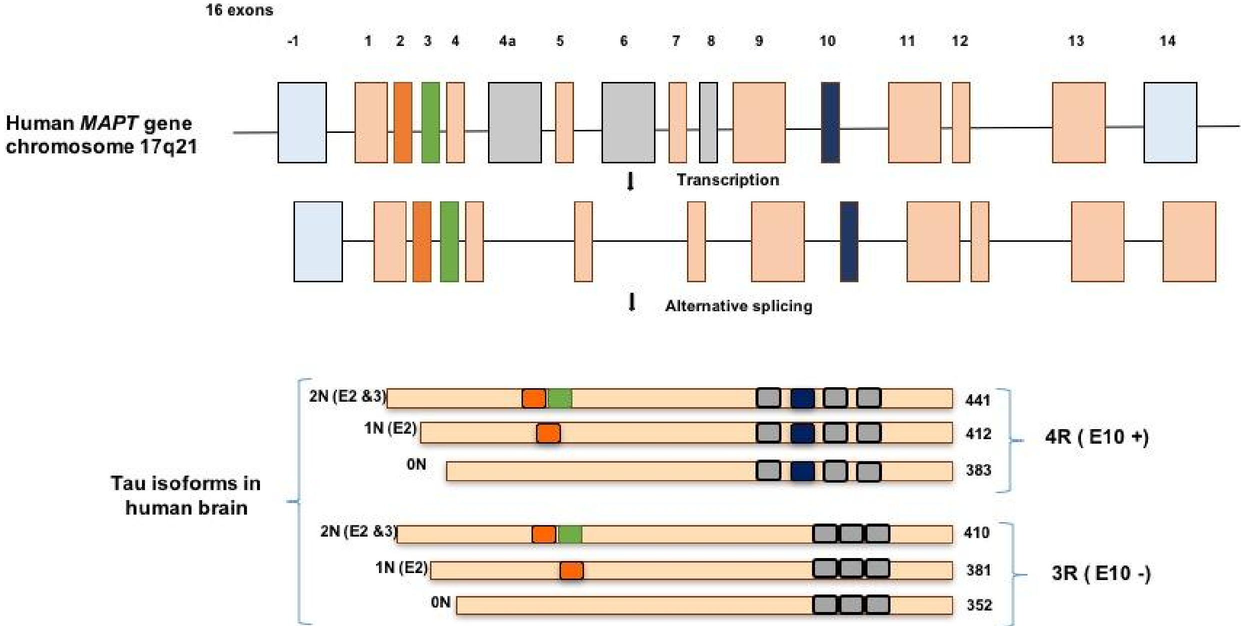
Tau isoforms

Tau protein which is encoded by the *MAPT* gene is a microtubule associated protein (Neve et al., 1986). In adult human brain, there are six main isoforms of tau that are generated from alternative splicing of exon 2 (E2), E3, and E10 (Figure 1.1 A). The splicing of E2 and E3 generates isoforms containing either zero, one, or two amino-terminal inserts of 29 amino acid residues known as 0N, 1N, and 2N, respectively. The six isoforms can be categorized based on the presence or absence of the second microtubule binding repeat domain, which is encoded by exon 10 into three (3R) or four repeats (4R) (Goedert et al., 1989; Neve et al., 1986). The expression of tau isoforms are developmentally regulated, with the ratio of the expression of 3R and 4R isoforms approximately equal in the human adult brain, while the 2N, 1N and 0N tau isoforms encompass ~9%, 54% and 37% of the total tau protein fraction respectively (Goedert and Jakes, 1990; Goedert et al., 1989). However, in the human fetus, the 3R isoform is predominantly expressed (Goedert and Jakes, 1990).

In tauopathies, mis-regulation of tau alternative splicing and the imbalance in the expression of 3R vs 4R isoforms is of importance as it is linked with distinct diseases as mentioned previously (Kovacs, 2017).

Figure 1.1

A.



B.

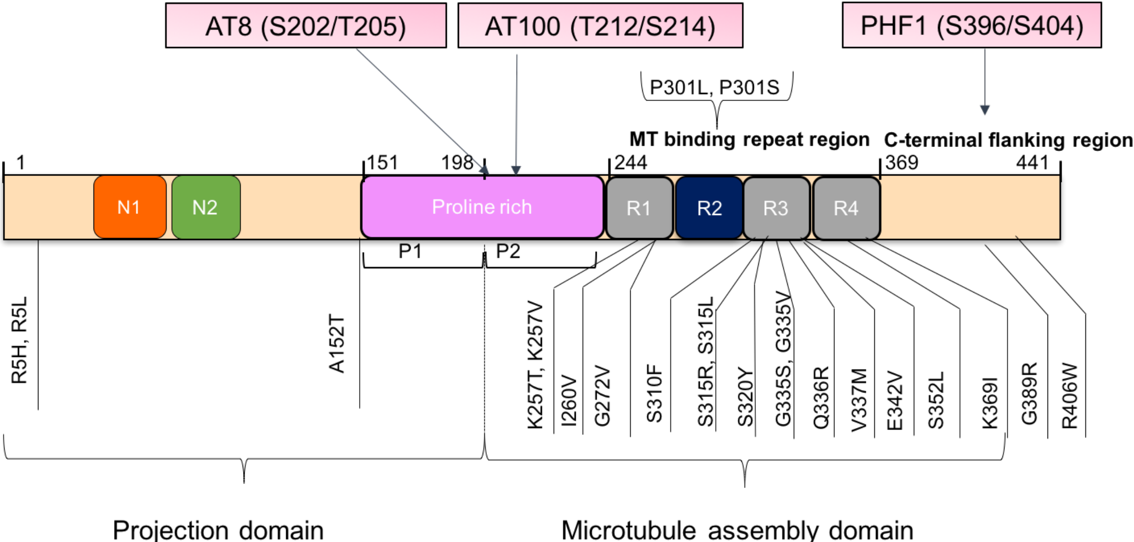


Figure 1. 1: The human *MAPT* gene, tau isoforms in human brain, structure and mutations.

A) Schematic of The *MAPT* gene on chromosome 17q21.31 which compromises 16 exons (Neve et al., 1986). There are six main isoforms of tau that are generated from alternative splicing of E2, E3 and E10. The splicing of E2 and E3 generates isoforms containing either 0,1 or 2 amino-terminal inserts of 29 amino acids known as 0N, 1N and 2N respectively. The presence or absence of the second repeat R2 domain which is encoded by exon 10 categorize the isoforms with 3R or 4R (Wang and Mandelkow, 2016). B) Tau major domains are divided into the projection domain and the assembly repeat domain in the carboxy-terminal sections separated by proline-rich region (Wang and Mandelkow, 2016). The projection domain comprised residues 1-197 and is not involved in microtubule (MT) binding. The proline-rich region subdivided into P1 and P2 separated by the chymotryptic cleavage site at residue 198 that divide the assembly and projection domain (Mukrasch et al., 2009; Mukrasch et al., 2007). The C-terminal assembly domain is important for MT binding and assembly. The assembly domain contains the MT binding repeat region followed by a flanking region that shows weak sequence similarity to the repeat domain (Mukrasch et al., 2007). The four repeats, around 30-31 residues each, are labeled R1-R4. Both P2 and the flanking regions contribute to MT assembly and binding. Major disease-associated missense mutations that alter the sequence are shown with arrows, and some well-known motifs that are hyper-phosphorylated in diseases and recognized by well-characterized tau phosphorylation antibodies are highlighted in pink. Figure 1.1B is a re-creation version of Figure 2b from (Wang and Mandelkow, 2016) with additional details.

Expression

Tau protein is expressed in various mammalian tissues like heart, kidney, and skeletal muscles (Uhlen et al., 2015; Wolfe, 2012)(https://www.proteinatlas.org). However, it is extremely enriched in the central nervous system (CNS), predominantly in neurons, but can also be found in glia at low amounts, and it can be released outside cells and found in extracellular fluid space such as in brain interstitial fluid (ISF) (LoPresti et al., 1995; Magnoni et al., 2012; Yamada et al., 2014). In mature neurons, tau protein is found primarily in axons, where it binds microtubules, but it can be found in small amounts in soma and dendrites (Dotti et al., 1987; Ittner et al., 2010; Kempf et al., 1996). However, in immature neurons tau is ubiquitously expressed equally in both neurites and the cell body (Sotiropoulos et al., 2017). In the brain, tau expression displays intriguing regional differences, with both mRNA and protein levels reaching two-fold higher levels in the neocortex compared to the white matter and cerebellum (Boutajangout et al., 2004; Trabzuni et al., 2012). The splicing of the MAPT gene has some regional variation as exemplified by the smallest isoform (0N3R), which has lower expression in the cerebellum compared to higher expression in the temporal cortex (Boutajangout et al., 2004). The differences in tau expression in various brain regions may contribute to deferential vulnerability of certain areas to tau pathology (Feany et al., 1996).

Tau domains and Structure

Normally folded tau is a highly water-soluble (hydrophilic) and overall basic protein (Wang and Mandelkow, 2016). Structural studies have revealed that tau protein is natively an unfolded protein with a small number of secondary and tertiary structure elements and little tendency to aggregate (Mukrasch et al., 2009). However, tau is a flexible protein that can form a

global conformation upon interactions with other proteins or microtubules (Avila et al., 2016). Indeed, data obtained from analysis of global folding of tau molecule in solution using electron paramagnetic resonance (EPR) and fluorescence resonance energy transfer (FRET) indicated that tau has a preference to change its conformation to “a paper clip–like” form in which the C-terminal end folds over the repeat domain and approaches the N-terminal end (Jeganathan et al., 2006).

Tau protein comprises two main major domains based on its interactions with microtubules and its amino acid characteristics (details about tau structure outlined in the legend of Figure 1.1B). The C-terminal assembly domain and the N-terminal projection domain are separated by a Proline-rich region. The C-terminal assembly domain contains the repeat domain and flanking region and contributes to binding to the microtubules and tau protein aggregation. In contrast, the N-terminal domain, also termed the projection domain, projects away from the microtubule domains (Avila et al., 2016; Wang and Mandelkow, 2016), and is responsible for the interactions with plasma membrane (Buee et al., 2000).

The middle region or the proline-rich domain consists of seven PXXP motifs that serve as binding sites for proline-directed kinases such as Fyn (Lee et al., 1998). Many of the motifs in the middle regions become hyper-phosphorylated in disease conditions and therefore can be recognized by various phosphorylation-dependent antibodies such as AT8 (Wang and Mandelkow, 2016). Phosphorylation of several sites in the Proline-rich domain have recently been found also to play a key role in the localization of tau protein in axons (Iwata et al., 2019).

Interestingly, sequence comparison data of tau proteins from various species showed considerable sequence similarity in C-terminal domain especially the microtubule repeat region, but more variability in the N-terminal domain, isoforms and the alternative splicing (Chen et al.,

2009; Himmler et al., 1989; Lee et al., 1988; Nelson et al., 1996; Olesen et al., 2002; Yoshida and Goedert, 2002). This suggests that some of the conserved residues are under relatively strict selection pressure and important for tau functions.

Compared to normally folded tau, phosphorylated tau (p-tau) found in tangles are extremely insoluble (Jeganathan et al., 2008; Kopeikina et al., 2012). The repeat domain of tau molecules forms the core when tau protein aggregates into paired helical filaments (PHFs) with both the N-terminal and C-terminal forming a "fuzzy coat" that surrounds the core of the filaments (Avila et al., 2016; Wischik et al., 1988). The core of PHFs consists of a β-sheet structure, which is characteristic of many amyloid-like structures (Wang and Mandelkow, 2016). The arrangements of these β-sheet structures in PHFs has not been fully explored, as imaging of the "fuzzy coat" is considered difficult due to its high flexibility. Thus, the fuzzy coat structure is mostly obscure, but it has been described as a two-layered 'polyelectrolyte brush', a structure that may be responsible for the stabilization of tau filaments (Wegmann et al., 2013). Interestingly, recent advances in analyzing the structure of tau filaments from AD and PiD using electron cryo-microscopy denoted the ability of tau protein to adopt distinct folds and disease-specific conformation that may contribute to the neuropathological diversity observed in tauopathies (Falcon et al., 2018; Fitzpatrick et al., 2017).

1.1.5 What is known about Tau physiological functions?

The perturbation of tau physiological functions in disease may also contribute to the pathogenesis of tauopathies (Lee and Leugers, 2012). Considering the increased interest in the development of therapeutic strategies aimed at either reducing tau mRNA levels or increasing the stabilization of microtubules, it is essential to have a deeper understanding of tau functions under physiological conditions and the role of loss of tau function on cognitive and neurodegeneration.

Microtubule assembly

Tau is a multifunctional protein that was known initially for its roles in microtubule assembly and polymerization (Cleveland et al., 1977). Microtubules (MTs) are essential structural elements in axons that play an central role in various important processes such as neurite growth, differentiation as well as axonal transport of motor proteins (Goodson and Jonasson, 2018). Tau interacts with the MTs through the repeat domain and flanking regions to regulate MT assembly, dynamic behaviors and spatial organization (Castellani and Perry, 2019; Hirokawa et al., 1988; Prezel et al., 2018; Samsonov et al., 2004). The process in which tau binds and promotes the polymerization of microtubules is a highly dynamic and implicates conformational changes within tau protein itself (Fischer et al., 2009; Melkova et al., 2019). The microtubule-related function of tau protein is tightly regulated by various factors including post-translation modifications (PTMs), especially phosphorylation (Lindwall and Cole, 1984; Mandelkow et al., 1995; Ramkumar et al., 2018). However, the exact mechanism for MT assembly remains challenging to explore due to the disordered nature of tau and the highly dynamic process (Barbier et al., 2019). Besides regulating MT dynamics, tau protein also

regulates axonal transport by influencing the functions of motor proteins such as dynein and kinesin, which transport cargo towards the cell body and axons termini, respectively (Stamer et al., 2002).

Microtubule stabilization

One of the broadly accepted physiological functions of axonal tau protein with regard to microtubules is its ability to stabilize microtubules and stiffen them to support the long axons and drive neurite growth (Castellani and Perry, 2019; Kadavath et al., 2015; Wang and Mandelkow, 2016). Thus, mis-localization and loss of tau from axonal microtubules in disease is assumed to be responsible for microtubule destabilization, hence, many therapeutic approaches aim to develop microtubule-stabilizing therapies as treatments options for AD and other tauopathies (Baas and Qiang, 2019; Zhang et al., 2012). Despite the wide acceptance of tau's stabilization functions, the mechanisms by which it stabilizes microtubule assembly has not been well investigated, which has raised skepticism and led to a re-evaluation its role as a genuine stabilizer (Baas and Qiang, 2019). Each microtubule is comprised of a stable and labile domain (Baas and Qiang, 2019).

A very recent work on the stabilization function of tau used cultured neurons to unravel interesting findings (Qiang et al., 2018). In the study, tau has been found to be enriched at the labile MT domain, contrary to the previous view of tau as a stabilizer. The data also suggested that tau aids in the assembly of the labile domain and making it achieve more length without being stabilized (Qiang et al., 2018). When tau was depleted experimentally from the cultured neurons, the labile domain mass decreased. However, the stability of the labile domain was not affected as the genuine stabilizer microtubule-associated protein 6 (MAP6) was found to bind to

the stable domain and promote microtubule growth and stabilize it. Only when the true stabilizer MAP6 was depleted did the labile domain become less stable (Qiang et al., 2018). This finding and others (Baas and Qiang, 2019) should be taken into consideration when it comes to investigating options of treatments that rely on the stabilization of microtubules in tauopathies.

Synaptic functions and role in nucleus, and other functions of Tau

Tau is found in various subcellular compartments other than axons such as the nucleus, dendrites and dendritic spines and the plasma membrane (Sotiropoulos et al., 2017). The localization of tau in other subcellular compartments indicates that it has other functions in addition to microtubules polymerization, such as signal transduction (Buee et al., 2000; Bukar Maina et al., 2016; Lee, 2005). The physiological phosphorylation of tau and its structure are imperative for regulating the subcellular localization of tau, which could trigger signaling cascades and have functional implications (Morris et al., 2015; Wang and Mandelkow, 2016). Aside from phosphorylation, other post-translational modifications such as O-glycosylation, SUMOylation, prolyl-isomerization, ubiquitination, nitration, truncation, and acetylation are shown to influence some functions of tau protein (Arnold et al., 1996; Cohen et al., 2011; Dorval and Fraser, 2006; Horiguchi et al., 2003; Mori et al., 1987; Nakamura et al., 2012; Wang and Mandelkow, 2016).

Tau's role in dendrites and synaptic functions have also been broadly investigated recently (Regan et al., 2017). Tau is found in both pre- and post-synaptic compartments in healthy neurons and found to also accumulate at synaptic sites in disease conditions, particularly AD, in its hyper-phosphorylated form (Fein et al., 2008; Tai et al., 2012). Several pieces of circumstantial evidence highlight that tau can regulate synaptic functions (Regan et al., 2017).

For instance, tau can modulate in a direct or indirect way the signaling of synaptic receptor such as muscarinic acetylcholine receptors (mAChRs) and N- methyl-d-aspartate receptors (NMDARs), which are known for their normal physiological roles in cognition and synapse function and excitotoxicity (Gomez-Ramos et al., 2009; Ittner et al., 2010). Various findings support the role of tau as an important regulator of synaptic plasticity (Regan et al., 2015; Regan et al., 2017). For instance, tau can be phosphorylated by glycogen synthase kinase–3β (GSK-3β), an enzyme found in post-synaptic compartments that is involved in long-term synaptic plasticity (Goedert et al., 1997; Peineau et al., 2007). In addition, analyses of neuronal functions of the hippocampus (this area is employed considerably for studies on synaptic plasticity events) in *MAPT* knockout mice, showed deficits in synaptic plasticity particularly in long-term depression; this further supports the post-synaptic role of tau in synaptic plasticity (Kimura et al., 2014). In dendritic spines, tau has been found to bind filamentous actin, supporting its role in cytoskeletal integrity (Matsuo et al., 1992; Moraga et al., 1993).

Aside from synaptic functions, various evidence supports the presence of tau in the nucleus of both neuronal and non-neuronal cells (Bukar Maina et al., 2016). Although the full range of tau's physiological functions in the nucleus remains to be elucidated, evidence suggests a role of nuclear tau in maintaining the stability of DNA, as well as cytoplasmic and nuclear RNAs (Rossi et al., 2013; Sotiropoulos et al., 2017). Also, new findings suggest a role for nuclear Tau in regulation of heterochromatin stability and ribosomal DNA (rDNA) transcription repression and nucleolar stress response (Maina et al., 2018b; Mansuroglu et al., 2016). Nuclear tau may also play a role in protein synthesis machinery (Maina et al., 2018a). There is still a lack of understanding of the full functions of nuclear tau, its post-translation modifications and the isoforms present within the nucleus (Bukar Maina et al., 2016).

In addition to the role of tau in neurons in the CNS, tau is found in the peripheral nervous system, where its physiological role has started to become understood only recently (Dugger et al., 2016). Tau was recently found to be expressed in the Schwann cells, in which is tau shown to modulate their proliferation and migration, especially after peripheral nerve injury (Yi et al., 2019). In that study, the reduction of tau expression using *MAPT* siRNA *in vivo* as well as the use of *MAPT* knockout mice both showed reduced migration of Schwann cells post-injury, as well as impairment in myelin and lipid debris clearance (Yi et al., 2019). These interesting findings expand on the existing knowledge in understanding of the biological functions of tau protein. Our understanding of tau's diverse functions is continuing to be refined and supports the argument that caution is warranted when considering therapeutics that are designed to reduce tau abundance.

1.1.6 Physiological Tau phosphorylation and pathological hyper-phosphorylation

The phosphorylation of tau protein is an important dynamic process in the brain that controls many of its biological functions such as axonal transport and organelle delivery to the somatodendritic compartment (Ebneth et al., 1998; LaPointe et al., 2009). The regulation of tau phosphorylation is tightly controlled and can developmentally vary, as fetal tau protein carries more phosphatase (seven per molecule) compared to adult tau (two per molecule) (Kanemaru et al., 1992). Tau has many phosphorylation sites, around 85 potential sites including 80 serine (Ser) or threonine (Thr), and 5 tyrosine (Tyr), in its longest isoform (2N4R), of which most are accessible because of its unfolded nature (Wang and Mandelkow, 2016; Wolfe, 2012). Among these, at least 45 sites have been examined experimentally (Hanger et al., 2009). However, the functions associated with many of the phosphorylation sites and their tendencies to be

phosphorylated *in vivo* and by which kinases is still unclear (Castellani and Perry, 2019; Wolfe, 2012). Many phosphorylation sites are clustered in the flanking regions of tau (Figure 1.1B), while other are near or in the repeat region.

Tau can be phosphorylated by various kinases including microtubule affinity regulating kinases (MARKs), which control the affinity of tau binding to the microtubule, glycogen synthase kinase 3β, Ca^{2+} or calmodulin-depedent protein kinase II (CaMKII) and cyclin AMP–dependent protein kinase (PKA) (Hanger et al., 2009; Wagner et al., 1996; Yoshimura et al., 2003). Tyrosine kinases such as FYN from the SRC family also can phosphorylate tau at tyr-18 (Lee et al., 2004). Tau can be de-phosphorylated by various phosphatases particularly PP2A, which accounts for ~70% of the phosphorylation activity of tau in the human brain; however, its activity is decreased in AD and TBI (Gong et al., 1993; Hanger et al., 2009; Yang et al., 2017). The regulation of phosphatase activity could result from post-translation modification of its catalytic domains or through increase or decrease of its endogenous inhibitor, denoting the complexity of tau phosphorylation (Chen et al., 2008).

Abnormal phosphorylation of tau can contribute to pathogenesis of many neurodegenerative diseases. Abnormal phosphorylation of tau can be caused by an imbalance between phosphatase activity and kinases, which could be crucial to the formation of pathological aggregates (Alavi Naini and Soussi-Yanicostas, 2015). In tauopathies such as AD, tau becomes hyper-phosphorylated, which is considered a hallmark for tau aggregation in these diseases (Simic et al., 2016). In AD, around 39 potential phosphorylation residues are reported to be phosphorylated, with an increase of up to eight phosphates per molecule (Hanger et al., 2007; Kopke et al., 1993; Morishima-Kawashima et al., 1995). It is worth mentioning that when it comes to studies using brain tissue obtained by autopsy (post-mortem) showing an increase in

the total number of phosphorylation sites in AD and tauopathies compared to normal brain tissue, post-mortem processing or time intervals before such may lead to the de-phosphorylation of soluble tau, creating an artefact in which the true level of phosphorylation may be underrated (Gartner et al., 1998; Oka et al., 2011; Song et al., 1997; Wang and Mandelkow, 2016; Wang et al., 2015). Findings from biopsy-derived human tau and transgenic mice have shown that most of the phosphorylation sites in PHF also to be phosphorylated in normal brain (Matsuo et al., 1994; Morris et al., 2015). These findings suggest that the increase in phosphorylation observed in disease state results from excess of phosphorylation of tau at certain sites rather than disorganized increase in the total number of the phosphorylation sites (Castellani and Perry, 2019).

Interestingly, the increase of tau phosphorylation may not always be pathological, as it has been observed in animals going through hibernation and anaesthesia-induced hypothermia, in which tau was phosphorylated in PHF-related epitopes (Arendt et al., 2003; Planel et al., 2007). The major differences, however, in such cases is that the hyper-phosphorylation did not lead to fibril formation and was reversible after arousal (Arendt et al., 2003). This hyper-phosphorylation is likely caused by changes in phosphatase activity, as phosphatases have been shown to be impacted by temperature changes; particularly, hypothermia has been shown to inhibit phosphatases such as PP2A exponentially (Planel et al., 2004; Planel et al., 2007)

Various antibodies are available to use for p-tau analysis *in vivo* and *in vitro* that recognize selective epitopes with functional and pathological implications (Simic et al., 2016). For instance, AT8 is a widely-used antibody to identify tau phosphorylation at Ser 202, Thr 205, and Ser 208 and detect a wide range of tau aggregates including pre-tangles in brain autopsies (Bancher et al., 1989). Another antibody used is the monoclonal antibody AT100, which is less

sensitive than AT8 but highly specific for pathological aggregates and can identify phosphorylation at Thr 212 and Ser 214 (Allen et al., 2002). The sensitivity and specificity of an antibody should be considered carefully, as the appropriate phosphorylation sites may vary depending on the tauopathy studied (Castellani and Perry, 2019).

1.1.7 Tau aggregation and toxic species

As described earlier, the formation of tau inclusions and aggregates is characteristic of tauopathies. It is assumed that hyper-phosphorylation of tau can impact aggregation, as tau aggregates from tauopathy patients and transgenic animals display hyper-phosphorylation, which seemed to proceed tau aggregation and tau filament formation (Braak et al., 1994). Whether the hyper-phosphorylation of tau is the main driver to filament formation is still a matter of debate due partly to the heterogeneity and number of phosphorylation sites (Wang and Mandelkow, 2016). The formation of tau aggregates is presumed to be a multi-step process in which post-translation modifications, specifically hyper-phosphorylation, structural changes, and the detachment of tau from microtubules are part of the initial steps of this process (Gendron and Petrucelli, 2009; Kuret et al., 2005; Mandelkow and Mandelkow, 2012). *In vitro* studies have suggested that some cofactors can contribute to the formation of tau fibrils and filaments, such as heparin and nucleic acid, which have been shown to induce the dimerization of tau (Goedert et al., 1996; Kampers et al., 1996). In addition, some mutations such as P301L, which is associated with familial FTD, have been shown to accelerate tau aggregation *in vitro* and *in vivo* (Khlistunova et al., 2006). However, the mechanism resulting in filament formation in sporadic tauopathy patients remains unknown.

Tau aggregates can exert toxicity from gain of function, such as interfering with cell signaling pathways or other intercellular functions (Wang and Mandelkow, 2016). Although NFTs are the histopathological marker for many tauopathies, it is not understood whether filamentous tau assembly is accountable for cytotoxicity observed in these diseases or it is a productive response to cellular stress or the process of aging (Cowan and Mudher, 2013; Shafiei et al., 2017). Recent studies seem to support the latter, as NFTs not only can be detected in the brain of some aged people with no noticeable cognitive deficits, but also neurons with NFTs have been shown to be functionally intact *in vivo* (Kuchibhotla et al., 2014; Shafiei et al., 2017). Suppression of mutant tau in repressible transgenic mice that showed recovery of memory functions and decreased neuronal loss despite the continuation of NFT accumulation also highlights the fact that NFTs may not be sufficient to cause cognitive deficits and neuronal death (Santacruz et al., 2005).

On the other hand, several pieces of evidence indicate that smaller and "pre-tangle" assemblies of tau aggregate, specifically oligomers, are the prime culprit behind toxicity (Berger et al., 2007; Cowan et al., 2010; Maeda et al., 2006; Wittmann et al., 2001; Yoshiyama et al., 2007). When hyper-phosphorylated, tau becomes separated from microtubules and its affinity and binding to other tau monomers increase, leading to the formation of detergent-soluble oligomer aggregates that can potentiate neuronal damage and synaptic dysfunctions in tauopathies and traumatic brain injury (Gerson et al., 2016; Gerson et al., 2014; Hawkins et al., 2013; Shafiei et al., 2017). When the length of oligomers grows, they start adapting a β-structure and become insoluble, eventually forming fibrils (Cowan and Mudher, 2013; Nizynski et al., 2017; Ren and Sahara, 2013).

Many studies in animal models have highlighted the toxicity of tau oligomers (Berger et al., 2007; Spires et al., 2006; Yoshiyama et al., 2007). For instance, neuronal loss was observed before the formation of NFTs in *Drosophila* model of Tauopathy (Wittmann et al., 2001). Additionally, synaptic, cognitive and mitochondrial abnormalities was noted when oligomers rather than monomers of fibrils were injected in normal mice (Castillo-Carranza et al., 2014; Lasagna-Reeves et al., 2011). Beside those seen in AD and PSP patients, the onset of clinical symptoms was associated with the higher level of tau oligomers (Gerson et al., 2014; Lasagna-Reeves et al., 2012b; Maeda et al., 2006; Patterson et al., 2011). Importantly, tau oligomers seem to induce the misfolding and propagation of endogenous tau when injected into mice, which is not observed with fibrils (Lasagna-Reeves et al., 2012a; Lasagna-Reeves et al., 2012b; Swanson et al., 2017; Wu et al., 2013). Although this evidence and more supports the toxic proprieties of Tau oligomers (Ward et al., 2012), their role in the neurodegeneration in tauopathies is still under debate. This is likely due to the lack of characterization of tau oligomers, as the assays used for oligomers preparation vary and thus may give contrasting findings, with oligomers inducing dramatic toxicity in one study compared to mild effects in another (Flach et al., 2012; Tepper et al., 2014; Tian et al., 2013).

1.1.8 Toxicity from loss or reduction of tau functions

In tauopathies, neurodegeneration and neuronal dysfunction can be attributed to pathological gain of function from tau aggregations and/or loss of physiological functions (Wang and Mandelkow, 2016). To understand the toxicity generated by loss or reduction of tau functions, if any, many tau knockout mice have been generated (Ke et al., 2012; Tan et al., 2018). Interestingly, all the tau knockout mice presented no overt phenotype, which could be due

to partial compensation of some of tau's physiological functions by other microtubule-associated proteins such as MAP1A (Harada et al., 1994). Indeed, the level of MAP1A increased in some of these knockout mice especially during birth, but dropped to normal levels during brain maturation in adult mice (Dawson et al., 2001). This fact supports that although microtubule associated proteins such as MAP1A may compensate the loss of tau during birth, tau is needed for neuronal and brain functions during brain maturation as well (Dawson et al., 2001; Harada et al., 1994; Ke et al., 2012). In agreement with this conclusion is the fact that tau knockout mice, although they seemed normal at birth, exhibited behavioral and cognitive impairments when as they aged (~12 months old) (Ke et al., 2012; Mori et al., 1987).

As mentioned in a previous section, tau is involved in synaptic functions, LTD, and neuronal DNA homeostasis, and hence impairments of any of these processes due to loss of tau function may contribute to neurodegeneration (Wang and Mandelkow, 2016). In disease state, loss of functions may be attributed instead to tau aggregation, as hyperphosporylation of tau and the aggregation process may reduce the number of soluble tau, which may affect microtubule binding and assembly (Wang and Mandelkow, 2016). How much loss of function contributes to the neurodegeneration vs. the gain of functions toxicity associated with the formation of tau aggregates, especially oligomers, remains under study, but what is important is that both mechanisms contribute to pathogenesis in not only tauopathies but in other neurodegenerative diseases (Leighton and Allison, 2016; Medina et al., 2016).

1.2 "Prion Like" proprieties of Tau

In tauopathies like AD, the progressive accumulation of tau aggregates is observed in certain regions of the brain, in which the tau inclusions are found restricted to a small area (the

transentorhinal cortex for AD) during the early stages of the disease, but as the disease progress, the pathology seems to progress and affect other regions that are anatomically or synaptically connected (Braak and Braak, 1991; Goedert et al., 2017b). For a long time, cell-autonomous mechanisms and the concept of selective neuronal vulnerability were assumed to explain neurodegenerative diseases (Mattson and Magnus, 2006; Saxena and Caroni, 2011). The cell-autonomous mechanisms suggest that the same events, such as protein aggregation, can occur in brain cells independently while the concept of neuronal variability imply that subpopulations of neurons, varying depending on the disease, are intrinsically more vulnerable and get affected earlier than other neurons, which may be due to their gene expression profile and the aging process (Mattson and Magnus, 2006).

Although the cell-autonomous mechanisms may account for familial cases as the mutant proteins are broadly expressed, it is challenging to explain how these mechanisms could be involved in sporadic diseases. However, an alternative hypothesis, which has recently arisen, indicates that the first inclusions in a small number of cells which can act as a seed for aggregation and capable of being released and spread in non-cell autonomous mechanisms to other cells resulting in degeneration (Goedert et al., 2017b). This view not only provided an alternative explanation that fit sporadic diseases, but also it could work on familial cases and disease such as amyotrophic lateral sclerosis (ALS) or Huntington's disease (Ilieva et al., 2009) (Cicchetti et al., 2014). Since the spreading of protein aggregates occur in a manner similar to those of infectious misfolded prion protein (PrP^{Sc}), the propagation and spreading of pathology is referred to as "prion-like" (Clavaguera et al., 2015). Interestingly, due to the large amount of studies that support the propagation and spreading proprieties of tau, the use of stronger terms such as "tau prions" has been noted (Johnson et al., 2017; Woerman et al., 2016). However,

since the term “prions” has been long used to describe “proteinaceous infectious particles” in which inter-individual transmissibility has been well-demonstrated (Prusiner, 1982), this does not fully apply to tau aggregates as no evidence states that tauopathies are infectious or can transfer between humans. Thus, the use of the terms “prion-like” or “prionoids” (Aguzzi, 2009) seems more appropriate.

1.2.1 What is the concept of seeding, propagation and spreading of protein aggregates?

Seeded aggregation, also called the nucleation-elongation mechanism, has long been studied in prion diseases as a feature and a cause for neurodegeneration (Scheckel and Aguzzi, 2018). In prion diseases, the process of seeding or nucleation starts when the misfolded infectious prion protein replicate by interacting with the physiological form of prion protein (PrP^C) and mediating its conformational change from its native endogenous confirmation into the pathological conformation (Jarrett and Lansbury, 1993; Prusiner, 1982; Scheckel and Aguzzi, 2018). The misfolded form of the protein in this case is referred to as a “seed” due to its ability to induce (i.e. “seed”) aggregation (Falcon et al., 2015; Lewis and Dickson, 2016). How a sole protein can act similar to infectious agents and mediate the misfolding of natively folded protein is still not fully comprehended even in the prion field. When the template seeding or nucleation occurs in a cell, some seeds in the affected cells can escape and transfer to adjacent naïve cells or tissues by ill-defined mechanisms (Frost et al., 2009). The entire process from template seeding to transfer of the seeds between cells is termed “propagation” (Lewis and Dickson, 2016). Although seeding and propagation were long assumed to be exclusive properties of infectious prions, accumulating evidence in the past decade revealed that other proteins such as tau, Aβ, and alpha synuclein (α-Syn), follow similar mechanisms of self-perpetuating, seeded

aggregation and cellular spreading *in vitro* and when introduced to animals *in vivo* (Polymenidou and Cleveland, 2012). Regarding tau, the "spreading" refers to the macro- or/and microenvironment of tau pathologically spreading from region to region, which could be mediated by interconnected axonal projections as suggested by Braak et al. (Braak and Del Tredici, 2011), or microglia (Asai et al., 2015), or cell-to-cell (transcellular) (Lewis and Dickson, 2016).

1.2.2 What evidence support the seeding and spreading of Tau *in vitro* and *in vivo* ?

The ability of tau seeds to initiate seeding and accelerate aggregation into filaments was first observed *in vitro* in 2009 and was achieved by the addition of external seeds of synthetic tau filaments (Frost et al., 2009). The study also demonstrated the capacity of tau aggregates to transfer or be transferred between co-culture cells. Around the same time, the prion-like mechanisms of tau were also reinforced *in vivo* following experimental approaches commonly used in the research of prion diseases (Clavaguera et al., 2009). In the prion field, spreading and transmissibility are usually studied *in vivo* via the introduction of tissue or extracts from affected animals to naïve ones (Prusiner, 1998). Accordingly, brain extract from a tauopathy mouse that expressed mutant P301S tau and showed features of disease including neurodegeneration and filament formation, was injected into the brains of mice expressing human wild-type tau but do not develop tau filaments or slow the rate neurodegeneration. Remarkably, the injections of the brain extract induced seeding of wild-type tau into filaments and spreading of pathology far from the injection site to other brain regions (Clavaguera et al., 2009).

Afterward, various groups studied the prion-like propagation of tau using the same approach by inoculation of brain extract intracerebrally from brains of patients with AD or

tauopathies as well as synthetic tau filaments into both transgenic mice and wildtype mice and the results were similar, with tau aggregates regardless of the source inducing aggregation and spreading in most cases (Clavaguera et al., 2015; Goedert et al., 2017b; Guo et al., 2016). Transmission of tau in the CNS was also observed after peripheral injections of tau aggregates (Clavaguera et al., 2014). Recently, the source of tau seeds in those types of experiments has seemed to cause a bit of a debate with regard to which source of tau aggregates (recombinant tau fibrils, or fibrils from patients or transgenic mice in form of brain extract) may be more relevant to human tauopathies. This debate may be influenced by the revelation that the confirmations of PHFs from recombinant tau proteins that were different than the ones isolated from humans AD were also more capable of seeding recombinant tau to its confirmation (Morozova et al., 2013). While tau aggregates from human patients may have more relevance to human diseases, there is no doubt that the data obtained from recombinant tau fibrils, in addition to the data from injections of brain extract with tau fibrils, are still critical, as it further supports the notion that prion-like seeding and propagation is a protein-only phenomenon.

Some studies employed different approaches to observe prion-like propagation such as expressing mutant tau in a confined area and then observing the spreading of the pathology (de Calignon et al., 2012; Harris et al., 2012; Liu et al., 2012). In de Calignon et al. (2012), a transgenic mice model engineered to have restricted expression of human P301L tau in the entorhinal cortex (EC) was found to develop tau pathology beyond the EC and spread to synaptically connected regions in which the transgene was not expressed, by seeding endogenous mouse tau protein (de Calignon et al., 2012). This was followed also by neurodegeneration and neuronal loss, suggesting the spreading precedes neurodegeneration and synaptic dysfunction. Interestingly, the spreading of tau did not only involve neurons but other

cells such as microglia, which were shown to participate in the spreading, as depleting microglia seemed to halt the spreading of tau in a tauopathy model (Asai et al., 2015).

Despite the support of the evidence provided regarding the ability of tau to propagate in a prion-like manner, there is still lack of information about which tau species are involved in seeding and spreading of the pathology, especially when taking into consideration the role of oligomers vs fibrils. There is also a debate on the size and structures of tau aggregates that seed and are transmitted in tauopathies. According to Michel et al. (2014), monomeric tau can act as an efficient seed *in vitro* (Michel et al., 2014). However, considering what was mentioned earlier regarding the use of recombinant tau alone (which is the case in the mentioned study) and how relevant it is to tau from human brains, more work may be required to support this conclusion, including work addressing whether monomeric tau can efficiently seed aggregation *in vivo* as well. In contrast, Falcon et al. (2015) state that only competent tau species can seed aggregation, a characteristic that is absent from monomeric tau (Falcon et al., 2015). Mirbaha et al. (2015) proposed that trimer is the smallest size of tau aggregates that can induce seeding (Mirbaha et al., 2015). In the former study in cell culture, the group utilized both recombinant tau as well as tau fibrils isolated from AD and control brains, which contain no fibrils as a source for tau monomer. While all the previously mentioned studies provided valuable information regarding which tau species may be involved in prion-like propagation, further validation *in vivo* is essential.

1.2.3 Can tau have strains?

One of the characteristics for real prions (PrP^{Sc}) is the capability to propagate as distinct and stable "strains," which refers to protein aggregates that have the same primary protein

sequence yet assume distinct conformations and can stably maintain their conformations when isolated from either cells or the host then reintroduced to naïve cells or organisms (Collinge and Clarke, 2007). In addition to conformational differences, prion strains have different biochemical proprieties, different morphology of protein aggregates, different effects on disease duration, trigger different pathology and clinical courses that affect distinct regions or specific tissues (Morales, 2017). Considering that tau inclusions formed in each tauopathy are morphologically and structurally distinct, prompts the hypothesis that similar to prions, different tau strains may exist that can induce distinct tauopathies. This notion initially was supported by observations from various *in vitro* and *in vivo* studies (Audouard et al., 2016; Clavaguera et al., 2013; Guo et al., 2016; Narasimhan et al., 2017; Sanders et al., 2014). In the earliest studies, brain homogenate from tauopathy patients (including PiD, AGD, PSP, and CBD) or intracerebral fluid was injected into mice expressing the longest isoform of human tau (2N4R) and resulted in the formation of inclusions that were reminiscent of what was observed in patients, with exception of PiD (Clavaguera et al., 2013). Indeed, the injections of CBD brain extract caused the formation of silver-positive inclusions similar to astrocytic plaques noted in CBD, while the aggregates from PSP injections were similar in morphology to tufted astrocytes (hallmark lesions of PSP) (Clavaguera et al., 2013; Goedert and Spillantini, 2017).

Similar findings were observed when the homogenate from the tauopathies cases was injected into non-transgenic mice (Narasimhan et al., 2017). When the brain homogenate from injected mice that developed pathologies was re-introduced to naïve mice, similar pathologies were observed supporting the presence of tau strains (Goedert and Spillantini, 2017). To characterize tau strains better and provide further validation to the seeding and strain proprieties of tau, various groups developed tau reporter cell lines that express the core-repeat region of tau

encompassing pro-aggregation mutations fused to fluorescent reporter protein (Sanders et al., 2014; Woerman et al., 2016). The first model, which was developed by Sanders et al. (2014), not only helped to detect the seeding activity of tau, in which tau seeds that transduced to the cells were able to seed the reporter protein and form GFP positive inclusions indicative of tau seeds, they showed two distinct tau strains. One of the tau strains was efficient in seeding the reporter into small internuclear aggregates while the other led to the formation of a large juxtanuclear inclusion (Sanders et al., 2014). When the tau strains from these cell lysate were introduced to animals they induced unique pathologies that were stable even after successive inoculation and formed inclusions when re-introduced to naïve cells that resembles the original inclusions (Sanders et al., 2014).

Afterwards, similar *in vitro* models were used to isolate and characterized tau strains from various diseases (Kaufman et al., 2016; Woerman et al., 2016). *In vitro* models have provided an important tool for understanding tau strains, as shown by the study by Kaufman et al. (2016) in which the model was used for the analysis of 18 tau strains isolated from patients. Their work revealed that strains can be distinguished based on their biological and biochemical proprieties, the brain region they affect, and the rate of the propagation of pathology, suggesting that tau strains alone may account for the diversity of human tauopathies (Kaufman et al., 2016). Moreover, structural analysis of tau filaments from AD and PiD via Cryo-EM also supports the presence of tau strains, as tau seemed to adapt distinct conformations in each of these diseases (Falcon et al., 2018; Fitzpatrick et al., 2017). Further characterization of tau strains may be needed, as various aspects of strain phenomena such as whether more than one strain can co-exist, what factors may influence the dominance of one strain versus another, and do all strains share similar mechanisms of spreading or do they vary depending on the disease remains

unknown. Understanding more about tau strains may help in the improvement or development of diagnostic tools for early detection of toxic strains in living patients, which may lead to accurate diagnoses as well as therapeutic approaches.

1.2.4 Mechanisms and factors underlying Tau transmissions remain mysterious

The exact mechanisms of how tau aggregates spread between neurons, or how other cells, oligodendrocytes, astrocytes, and microglia are involved in the spreading as well as what factors influence or impede prion-like propagation are still not fully investigated. Various mechanisms of transcellular spreading of tau have been proposed. The cell-to-cell transmission of tau can occur either transcellularly or by secretion and be released to the extracellular space followed by cellular uptake (Tardivel et al., 2016; Zhou et al., 2017) (Figure 2). However, most of the suggested mechanisms that will be mentioned below on tau transmission are supported largely by cell culture and remain to be examined/validated *in vivo*. Studying the mechanisms of prion-like spreading *in vivo* is essential for better understanding of this phenomena. *In vivo* models can help to validate some of the suggested in vitro mechanisms and address questions regarding the prion-like spreading of tau in the complex environment of the living brain. For example, can tau seeds spread via cerebral spinal fluid (CSF), blood, or immune cells such as microglia? How do factors like seizure, sleep/wake, neuronal activity, and the glymphatic system influence the prion-like spreading?

<u>How does tau spread intercellularly?</u>

Tau can spread intercellularly via tunnelling nanotubes (TNT) (Tardivel et al., 2016). TNT are thin filamentous actin-containing extensions of the plasma membrane that connect

remote cells, have a diameter of 50–200 nm, and allow the intercellular transport of various cargo including proteins such as prion proteins from neuron to neuron (Gousset et al., 2009; Rustom et al., 2004). A study by Tardivel et al. (2016) showed that TNT may partly account for Tau spreading, since their data showed that both soluble and fibrillar tau transferred from neuronal cell to another through TNT (Tardivel et al., 2016). However, more information regarding the role of TNT in the spreading is required, such as the status of these tau fibrils transferred and whether they are free-form or in vesicles. Also, validation *in vivo* is needed, but considering how thin TNT are, it might make this challenging.

How does tau get released to extracellular space during prion-like spreading?

Tau can be released outside cells under physiological conditions without neuropathy and cell death (Chai et al., 2012; Karch et al., 2012). Work by Pooler et al. (Pooler et al., 2013) showed that physiological tau can be secreted into the media during neuronal stimulation. Monomeric tau was found in the brain interstitial fluid (ISF) in wild-type mice, supporting the release of tau under physiological conditions (Yamada et al., 2011). Additionally, tau was found in normal cerebral spinal fluid (CSF) and hyper-phosphorylated tau in the CSF of AD and TBI patients where its level was higher than normal (Buch et al., 1998; Rubenstein et al., 2015). It is reasonable to assume that the presence of tau externally in pathological conditions is one of the initial steps involved in the propagation of tau, especially if the tau secreted in pathological conditions is capable of seeding. While the exact mechanism of pathogenic tau release is unknown, data from *in vitro* studies supports the proposition that tau could be released extracellularly either in free form or via vesicular-mediated secretory pathways (Demaegd et al., 2018).

Tau has been suggested to be released as free soluble forms, through either unconventional mechanisms including direct plasma membrane crossing dislocation and release by secretory lysosomes, or conventional mechanisms, in which the release occurs by incorporation of protein content in secretory vesicles that pass through the endoplasmic reticulum (ER) and Golgi apparatus then is released via SNARE-mediated exocytosis (Demaegd et al., 2018; Viotti, 2016). Evidence of SNARE-mediated exocytosis (the conventional mechanism) was demonstrated first and highlighted that the release of tau depends on the dynamics of Heat Shock cognate 70 (Hsc70) and DnaJ co-chaperone complex as overexpression of DnaJC5, a DnaJ well-known for its ability to stimulate exocytosis, induced the secretion of wild-type and mutant tau (Fontaine et al., 2016).

The possibility of pathogenic tau being released via unconventional mechanisms was raised later after considering the fact that blocking the ER/Golgi-mediated secretory pathway did not block the physiological secretion of tau (Chai et al., 2012; Karch et al., 2012). Taking into consideration the role of the previously mentioned mechanisms of tau release in pathological conditions, it is tempting to think that pathogenic tau could employ the same mechanisms of physiological release of monomer tau to spread pathology. Indeed, a recent study by Merezhko et al. (2018) has shown that phosphorylated oligomeric forms of tau can be secreted via plasma membrane translocation, and this secretion is mediated by heparin sulfate proteoglycans (Merezhko et al., 2018). Interestingly, the tau species secreted via the unconventional mechanisms transcellularly spread to adjoining cells and seeded aggregation. Additionally, the hyper-phosphorylation of tau was shown to increase its secretion extracellularly via membrane translocation (Katsinelos et al., 2018). Tau can be released via endo-lysosomal pathways and

such secretion is mediated by Rab7, a GTPases that is involved in endosomes, autophagosomes, and lysosomes trafficking (Rodriguez et al., 2017).

Recent evidence has emphasized the role of extracellular vesicles (EVs) in the transfer of pathogenic protein such as tau in diseases like AD (Perez et al., 2019; Polanco et al., 2016). There are two types of extracellular vesicles secreted by mammalian cells that are associated with tau: 1) exosomes which are small single membrane vesicles that are derived from multivesicular bodies with a diameter of 50–100 nm and 2) ectosomes or microvesicles that are shed from plasma membrane with a diameter of 100–1000 nm (Choi et al., 2013). Exosomes are an important elements of cell communication as they transfer molecules trans-synaptically and intercellularly (Fevrier and Raposo, 2004; Korkut et al., 2013). Phosphorylated tau was revealed to associate with exosomes *in vitro* in experiments that overexpress tau (Saman et al., 2012; Simon et al., 2012). Moreover, the analysis of exosomes isolated from CSF of Alzheimer's patients contained tau protein (Saman et al., 2012). A recent study by Wang et al. (2017) provided evidence that Tau, whether phosphorylated, dephosphorylated, monomer or in oligomeric form, can be released extracellularly via exosomes by cultured neurons, N2a cells and in human brains *in vivo* (Wang et al., 2017). The role of microglia and exosomes in the transmission of tau was demonstrated *in vivo*, in which tau is phagocytosed by microglia and then released via exosomes (Asai et al., 2015). Pharmacological inhibition of exosome synthesis in microglia reduced tau propagation. These studies have supported the potential role of exosomes in transmission of tau. However, whether all tau species or strains spread through exosomes is still unknown.

Unlike exosomes, there are a limited number of studies regarding the role of ectosomes in the propagation of tau. Dujardin et al. (2014) showed tau to be released in ectosomes under

physiological conditions and in the absence of cell damage (Dujardin et al., 2014a). Based on the *in vitro* and *in vivo* experiments in this study, tau seems to be predominantly secreted via ectosomes under normal conditions; however, during disease conditions, the accumulation of pathogenic tau may cause a shift towards secretion via exosomes or lysosomal pathways (Dujardin et al., 2014a). Whether pathogenic tau can be secreted via ectosomes in human CSF, and whether they are involved in the transmission of tau pathology with exosomes are still unanswered questions.

<u>What mechanisms underline the cell uptake of tau aggregates?</u>

When it comes to cellular uptake of tau, various mechanisms have been proposed, supported by a few, mostly *in vitro* studies (Demaegd et al., 2018). In general, once tau is released, it is assumed to enter the cell through the following: a) fluid-phase endocytosis, which is a bulk uptake of solutes and is a type of macropinocytosis; b) adsorptive endocytosis that occurs when molecules are binding and concentrating on the cell surface before being internalized; c) receptor-mediated endocytosis (also known as clathrin-mediated endocytosis); d) uptake of tau when it is in vesicles (Amyere et al., 2002; Demaegd et al., 2018). Tau aggregates were initially suggested to internalize into the cells via fluid-phase endocytosis (macropinocytosis), an active process of invagination of the plasma membrane that leads to internalization of extracellular fluid, as tau aggregate not the monomer was taken up and localized with dextran, a marker of fluid endocytosis (Frost et al., 2009; Santa-Maria et al., 2012; Wu et al., 2013). Interestingly, work by Holmes et al (2013) indicated that tau aggregates can trigger their internalization by binding to heparan sulfate proteoglycans (HSPGs) on the cell

surface. The blocking of heparin both *in vitro* using human embryonic kidney cells (HEK) and *in vivo*, a mechanism shown to be used by prion protein (Holmes et al., 2013).

In contrast, a recent work by Evans et al. (2018) employing neurons derived from human iPSC, showed that both monomeric and aggregated tau can internalize into the neurons but they use different modes of endocytosis. Monomeric tau can be internalized efficiently by bulk endocytosis while tau aggregates utilized dynamin-dependent endocytosis, a GTPase involved in various fission events including clathrin-mediated endocytosis and synaptic vesicle endocytosis (Evans et al., 2018; Singh et al., 2017). The role of dynamin-dependent endocytosis was supported by the fact that inhibiting dynamin via the inhibitor dynasore significantly reduced tau aggregate entry by 95% compared to monomeric tau (Evans et al., 2018). While this finding is in agreement with the work done on TauP301L, in which inhibition of dynamin decreased tau propagation in neuron culture, it was opposite to the findings of Holmes et al., mentioned earlier in this section, in which the same inhibitor did not affect the uptake of tau fibrils (Calafate et al., 2016; Holmes et al., 2013).

There are various technical issues that may account for these contradictory results that include the cells and type of tau species used in each study. In the Holmes et al. (2013) study, human embryonic kidney cells (HEK) were used, which are non-neuronal cells and may function differently from human neuronal cells (Parton and Dotti, 1993). In addition, Holmes et al. (2013) also used Tau RD fibrils, composed of amino acids 243 to 375 only, while the other two studies used oligomeric forms of tau and low molecular weight aggregates (Calafate et al., 2016; Evans et al., 2018). Regardless of these inconsistent findings, there is little doubt that dynamin plays an important role in tau pathology; for example, the loss of Bin1, a negative regulator of dynamin, showed enhanced tau pathology (Calafate et al., 2016).

Regarding the uptake of vesicles, in normal conditions exosomes are shown to surf above filopodia before entering cells and being sorted into endosomal trafficking (Heusermann et al., 2016). Whether exosomes carrying tau aggregates use the same uptake mechanism or not remain unknown. Further work employing fibrils or aggregates isolated from human samples may provide valuable insights towards mechanisms of cellular uptake.

<u>What are the role of trans-synaptic connections and neuronal activity in the spreading of tau?</u>

Early *in vivo* evidence of tau propagation, in which tau propagation affected areas that are anatomically connected, has supported the role of synaptic connectivity and activity in the spreading of tau seeds (de Calignon et al., 2012; Dujardin et al., 2014b; Liu et al., 2012). Synaptic connectivity appears to determine the pattern of spreading more than proximity (Ahmed et al., 2014; Calafate et al., 2015). The importance of synaptic connectivity was supported by the recent work of Wang et al. (2017). The study, which utilized microfluidic device to prevent all mechanisms of tau transfer except synaptic transmission, showed that depolarization of neurons stimulated the release of tau-containing exosomes and release depended on synaptic connectivity (Wang et al., 2017). Additionally, the presence of misfolded tau in both pre- and post-synaptic terminals in AD patients is consistent with the suggestions that tau can transfer over synapses and exerts a pathological role at synapses that may lead to neurodegeneration (Fein et al., 2008; Sokolow et al., 2015; Tai et al., 2012). Indeed, pathogenic tau was shown to be able to bind to synaptic vesicles and interfere with their functions leading to synaptic dysfunction (Zhou et al., 2017).

Whether tau can utilize synaptic vesicles to get released and spread to other neurons during synapses or not is still unknown. However, the trans-synaptic spread of tau via synapses

seems to occur before synaptic loss and axonal terminal degeneration as indicated by human P30L tau (Pickett et al., 2017). The role of neuronal activity in the spreading of tau pathology was supported both *in vivo* and *in vitro*, in which stimulation of neuronal activity led to the release of tau extracellularly *in vitro* as well as enhanced the tau pathology *in vivo* (Pooler et al., 2013; Wu et al., 2016). Further investigation of this factor may help in understanding the mechanisms underlining tau propagation in tauopathies and could inspire the development of new therapeutic approaches or diagnostic tools.

Figure 1.2

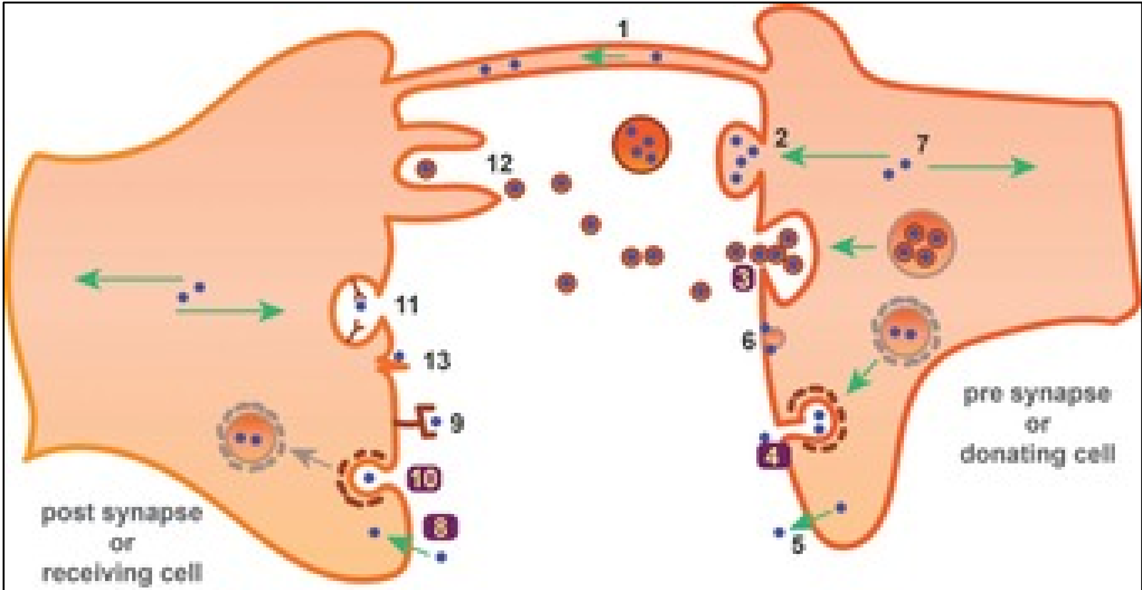


Figure 1. 2: Schematic overview of the different spreading mechanisms of tau between two neurons.

Presynaptic: 1) tunnelling nanotube, 2) formation and budding off of ectosomes, 3) fusion of multivesicular body (MVB) with plasma membrane, releasing exosomes, 4) SNARE-based exocytosis, 5) tau release through plasma-membrane translocation, 6) presynaptic cytotoxic effects of tau: restriction of vesicle mobilisation, and 7) axonal antero- and retrograde transport of tau. Postsynaptic: 8) fluid-phase translocation, 9) postsynaptic cytotoxic effects of tau through binding of NMDA receptors, 10) clathrin-mediated endocytosis, 11) HSPG-guided macropinocytosis, 12) exosome uptake with filopodia and 13) postsynaptic cytotoxic effects of tau through M1/M3 receptors, leading to calcium-ion influx.

Figure adapted from (Demaegd et al., 2018). The source of this figure is available under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License CC BY-NC-ND and permits non-commercial use, distribution and reproduction in any medium, without alteration

1.3 Prion-like properties of Tau seeds in TBI

1.3.1 Overview of TBI

Traumatic brain injury is one of the leading causes of mortality and disability with around 69 million individuals estimated to sustain TBI each year worldwide (Dewan et al., 2018; Masel and DeWitt, 2010). Additionally, it is a well-accepted risk factor for development of various neuropathological disorders, some of which are associated with dementia, including both AD and chronic traumatic encephalopathy (CTE) tauopathies, the latter of which is most commonly associated with repetitive mild traumatic brain injury and more frequent among athletes and military personal (Bieniek et al., 2020; Washington et al., 2016). Also, TBI is considered a risk factor for neurodegenerative diseases that are not typically linked to dementia, such as Parkinson Disease (PD) (Jafari et al., 2013). The risk of developing any of these diseases depends on multiple factors such as the severity of the injury, type of injury (e.g. concussive injury, skull fracture), the age of patient during the injury (e.g. increased risk with age) and genotype (e.g. *APOE4* gene) (Dhandapani et al., 2012; Graves et al., 1990; Jordan et al., 1997; Mannix and Meehan, 2015; Plassman et al., 2000; Washington et al., 2016). The correlation between the TBI severity and increased risk of developing dementia has been supported by many retrospective and observational studies (Guo et al., 2000; Plassman et al., 2000; Schofield et al., 1997). Current estimates suggest that at least 5-15% of dementia cases could be associated with TBI (Shively et al., 2012). Despite ample evidence supporting the link between TBI and the development of neurodegenerative diseases (primarily AD and CTE), the mechanisms by which head trauma induces neurodegeneration are still poorly understood. Additionally, the data supporting the aforementioned link come from retrospective studies, which are susceptible to

bias. Therefore, prospective studies may provide more reliable evidence of the risks associated with TBI and improve our understanding of the long-term effects of TBI.

TBI is mostly caused by traumatic events such as blows to the head from falls, accidents, sport-related injury, or in some cases from domestic abuse (Corrigan et al., 2003; Fu et al., 2016; Jordan, 2013). It can also result from explosive blasts (Cernak and Noble-Haeusslein, 2010). The severity of the injury and diagnosis is usually determined by clinical observations, such as the occurrence of seizure, hemorrhage, and history such as duration of loss of consciousness and post-traumatic amnesia (Forde et al., 2014; McKee and Daneshvar, 2015). Based on these symptoms, and imaging of pathology, TBIs can be classified as mild, moderate or severe (McKee and Daneshvar, 2015). Mild TBIs (mTBIs), a term sometime associated with concussion, are among the most common type of injury observed (Blennow et al., 2012; Cruz-Haces et al., 2017). TBI falls into two categories: acute brain injury and chronic brain injury. Acute brain injury comprises mTBI or concussions, including the short-term sequelae of these injuries, and catastrophic brain injuries that may result in death (Blennow et al., 2012). Chronic brain injury refers to late-term effects, including neurodegeneration and the development of chronic syndromes and diseases such as CTE (DeKosky et al., 2013). Of note, there are currently no therapies for the long-term effects of TBI.

Types of brain damage caused by different traumatic insults

TBI can cause a primary injury that directly results in damage to nervous tissues as well as perturbation of brain functions (McKee and Daneshvar, 2015). The primary injury is thought to occur either from the rapid acceleration and de-acceleration of linear forces on the head, direct blunt impact, or by forces (shockwaves) generated by blast wind associated with blast injury

(McKee and Daneshvar, 2015; Meaney and Smith, 2011). When head trauma occurs, the brain can sustain either focal or diffuse injury, depending on the type of impact (Hemphill et al., 2015). Focal injuries are associated with severe direct impact generated from localized forces. They generally present with contusion and hemorrhage near the impact site caused by the impact of the brain against the skull. Diffuse injuries are caused by distributed forces associated with inertial impact (due to rapid acceleration and deceleration) or by increased pressure resulting from blast shockwaves. The distributed forces can produce shear force within the brain tissue, causing diffuse axonal injury, petechial hemorrhage in the white matter, and neuronal injury at various sites throughout the brain (Shaw, 2002). While the lesions associated with focal injuries can be radiographically detected using computerized tomography (CT) or traditional magnetic resonance imaging (MRI), damage from diffuse injuries cannot be imaged using both technology (Borg et al., 2004; Hemphill et al., 2015). Interestingly, when focal lesions were detected, the pathology did not correlated with the clinical outcomes (Lee et al., 2008). The damage from diffuse injury does not require any fracture or crush injury to the brain surface to occur; thus, it is often seen even in patients with mTBI (Inglese et al., 2005). Currently, it is challenging to find a reliable diagnostic tool to detect this type of injury; hence, most diffuse pathology, such as swollen and damaged axons, is detected post-mortem (Strich, 1956).

The primary insults caused by head trauma can lead to multiple biochemical, cellular and molecular alterations perturbing the CNS environments and resulting in a secondary injury that can take place days, weeks or years after TBI (Cruz-Haces et al., 2017). Major alterations include loss of ionic homeostasis combined with the release of excitatory neurotransmitters, vasculature abnormalities and disruption of the blood–brain barrier (BBB), neuroinflammation, and alterations affecting the cytoskeleton (Giza and Hovda, 2014; Salehi et al., 2017). The

involvement of tau pathology was included among the many hypotheses behind this ongoing injury in TBI (Cheng et al., 2014; Huber et al., 2013; Tran et al., 2011).

1.3.2 Prion-like proprieties of tau in TBI and CTE

Tau pathology is one of the neuropathological features found post-mortem in CTE and TBI patients (Blennow et al., 2012; McKee et al., 2018). In CTE, tau pathology can be observed as NTF, as clusters in the depth of cortical sulci, and as inclusions in astrocytes around blood vesicles (McKee et al., 2018). The mechanistic relationship between TBI and tauopathy-associated neurodegeneration is poorly understood. However, *in vivo* experiments on mice have shown that tau phosphorylation increases after TBI events as early as one to six hours post-TBI events and depends on the severity of the brain trauma (Liliang et al., 2010). In 1973, Tau aggregates and filaments were first observed and reported in the post-mortem analysis of boxers' brains (Corsellis et al., 1973), and since then the pathological association of tau deposits in CTE and after repetitive mTBI has started to become well characterized (McKee et al., 2016; McKee et al., 2013). However, new evidence from investigations of tau pathology in long term survivors of single moderate to severe TBI showed the presence of a wide and abundant distribution of NFTs with a similar distribution of tau aggregates with those associated with repetitive mTBI (Zanier et al., 2018). A similar recent study by Gorgoraptis et al. (2019) has shown using tau PET technology the presence of tau pathology in TBI patients years after being subjected to a single brain trauma and also revealed a correlation between tau pathology and long-term neuronal damage associated with TBI compared to healthy control (Gorgoraptis et al., 2019). These studies further supported a link between tauopathy-associated neurodegeneration and TBI

as well as challenging the contention that CTE and tauopathy neurodegeneration is solely associated with repetitive mTBI.

Similar to other tauopathies, tau pathology in CTE seems to be confined in specific areas early in the disease progression as perivascular glia and neurons containing p-tau appear in the depth cortical sulci; the pathology spreads at later stages to cortical, medial temporal and subcortical grey matters (Geddes et al., 1999; McKee et al., 2016; McKee et al., 2015). Thus, it is reasonable to assume that tau species in TBI propagate in a prion-like manner. Indeed, a few recent studies have supported the capability of tau species formed after TBI and CTE to seed aggregation *in vitro* using tau reporter assays and transmit in prion-like manners *in vivo* (Woerman et al., 2016; Zanier et al., 2018). *In vivo* work by Gerson et al. (2016) showed that tau oligomers, isolated from rats subjected to two methods of TBI (blast-induced injury and fluid percussion injury) and injected into mice expressing hTau, induced seeding and aggregation in the injection site and other areas (Gerson et al., 2016). The study also showed that these tau species caused cognitive deficits and enhanced pathology, which not only supports the prion-like proprieties of tau species formed post-TBI but also their role in inducing toxicity (Gerson et al., 2016).

Other studies have shown the prion-like spreading of tau species after severe TBI, in both transgenic mice expressing P301S and wild-type human tau (Edwards et al., 2019; Zanier et al., 2018). In the first study, P301S mice were subjected to moderate to severe TBI and showed an increase of tau aggregation, and acceleration of the pathology compared to sham mice post-injury (Edwards et al., 2020). Further, the pathology spread to synaptically connected regions; the authors postulated that TBI could increase the risk of developing tauopathies through the induction of tau aggregation (Edwards et al., 2019). Zanier et al. (2018) found that a single

severe brain trauma can trigger a progressive and widespread tau pathology in humans and in mice; the introduction of brain homogenate from such mice to naïve mice induced similar pathology and was associated with synaptic loss and memory deficits (Zanier et al., 2018).

Despite a myriad of evidence supporting the prion-like proprieties of tau seeds formed after TBI, the knowledge gaps that were reviewed above still exist. In particular, the factors and mechanisms governing the spreading of tau pathology in TBI are not yet understood. Many changes that occur in the brain after TBI may be involved in the development and spreading of tau pathology. These include changes in the glymphatic system, which has been shown to be important for the clearance of extracellular tau (Iliff et al., 2014; Sullan et al., 2018). The glymphatic system can be functionally impaired after TBI, with a 60% reduction in glymphatic pathway function after TBI reported in a recent study (Iliff et al., 2014).

Given the prevalence of seizures and post-traumatic epilepsy in patients with blast-related TBI, the roles of neuronal excitability in the sequelae of TBI (including whether it is involved in tau pathology spreading or not) are also of interest (Kovacs et al., 2014). Post-traumatic seizures (PTS) and epilepsy (PTE) are among the major complications associated with TBI, particularly blast-related TBI, that lead to morbidity and mortality both during early stages and for several years after TBI (Asikainen et al., 1999; Rao and Parko, 2015). In a recent study in veterans, 57% of seizures observed were linked to TBI (Salinsky et al., 2015). Early post-traumatic seizures (occurring within the first week after the brain injury) are seen in the majority of cases of TBI (both adults and children), and their occurrence and frequency are associated with the severity of the injury (Asikainen et al., 1999; Temkin, 2003). Thus, prophylactic application of anti-convulsants is used to prevent acute post-traumatic seizures after TBI (Schierhout and Roberts, 1998). TBI can trigger various electrophysiological changes that can be detected by

electroencephalography (EEG) (Schmitt and Dichter, 2015). Some of the changes triggered by TBI at the cellular and the molecular levels, such as the disruption of microRNAs that promote the release of excitatory neurotransmitters (e.g., glutamate), could create microenvironments that are more favorable for developing seizures and epilepsy (Dorsett et al., 2017; Wang et al., 2014). Nonetheless, the exact mechanisms underlying the development of late PTS and PTE is poorly understood. Interestingly, tau phosphorylation and aggregation have been observed in patients with late-onset epilepsy several years post-injury (Zheng et al., 2014). Tau phosphorylation was also noted in animal models of chemically or electrically induced seizures (Crespo-Biel et al., 2007; Liang et al., 2009; Tian et al., 2010). Although these studies support a link between seizures and tau aggregations, more information is needed in the context of TBI. Future studies elucidating the roles of PTS in the spreading of tau pathology may be especially enlightening. A deeper understanding of the mechanisms and factors underlying the spreading of tau species in TBI may lead to the development of therapeutic strategies that mitigate the long-term adverse effects of TBI.

1.3.3 Can zebrafish provide a solution?

When it comes to understanding the mechanisms underlying prion-like spreading, most of the suggested mechanisms mentioned earlier have been investigated using *in vitro* models (Demaegd et al., 2018). Despite the insights provided by such models, we still lack full understanding of the mechanisms that govern the prion-like spreading of tau pathology. Besides, studying these mechanisms in traumatic brain injury specifically could be achieve mainly through *in vivo* models. An *in vivo* model with an intact central nervous system (CNS) that will complement *in vitro* studies well is needed to explore the etiology of various tau strains, and to understand the route of spread, or tissue tropisms. Engineering such an *in vivo* model would be

beneficial not only for investigating tau strains and understanding the kinetics of spreading, it could also uncover targets for therapeutic intervention and be adapted for high-throughput drug screening.

Zebrafish are becoming increasingly a popular animal model for studying neurodegenerative diseases (Newman et al., 2014) and can be promising models for studying tauopathies and prion-like mechanisms for the following reasons. The vertebrate zebrafish have an intact and vibrantly active CNS resembling that of mammals, and a thoroughly characterized genome amenable to genomic manipulation (Saleem and Kannan, 2018). They can produce a large number of embryos that are transparent and that develop quickly and externally (unlike mammals), making them accessible for interventions and daily monitoring. Furthermore, zebrafish can exhibit quantifiable neurobehavioral phenotypes (Saleem and Kannan, 2018). Additionally, zebrafish larvae are ideal for *in vivo* high-throughput screening, as they grow well in 96-well plates.

1.4 Thesis summary and hypotheses

The overall goal of this thesis is to gain deeper understanding of the mechanisms of tau uptake and factors involved in the prion-like spreading of tau pathology in TBI. Towards this end, it introduces several new tools to support investigation of prion-like tauopathy *in vivo*. This thesis comprises three chapters and an Appendix. Chapter 2 addresses the following hypotheses:

1) **post-traumatic seizure activity promotes tau aggregation.**

2) **Blocking cellular uptake, particularly dynamin endocytosis, minimizes or halts the spreading of tau seeds following TBI.**

To test these hypotheses in Chapter 2, I engineered a novel tauopathy biosensor model using zebrafish that could detect and report tau seeds from various sources. I also invented the first TBI method suitable for larval zebrafish, which had been lacking. This TBI method was used on the tauopathy biosensor zebrafish larvae to study tau aggregation specifically in the context of TBI. I investigated the role of seizure and neural activity on tau aggregation and spreading in a TBI model using convulsant and anti-convulsant drugs. Subsequently, I examined the role of dynamin-dependent endocytosis in the uptake of tau seeds after TBI via specific dynamin inhibitors. Chapter 3 is a final discussion chapter that focuses on future directions. Appendix 1 describes my efforts in generating amyloid precursor protein (*appb*) mutant zebrafish using the CRISPR/Cas9 system.

2. Chapter 2: Unravel role of endocytosis Tau seeds in Traumatic Brain Injury using zebrafish model for blast injury

As outlined by the preface, the entirety of **chapter 2** (with some modifications) has been prepared for submission under the tittle
"Tauopathy progression following traumatic brain injury is impacted by neural activity and requires endocytic mechanisms" Authors: Hadeel Alyenbaawi, Richard Kanyo, Razieh Kamali, Michele G. DuVal, Holger Wille, Edward A. Burton, W. Ted Allison.

2.1 Summary:

Traumatic brain injury (TBI) is a prominent risk factor leading to neurodegeneration and dementia, in particular chronic traumatic encephalopathy (CTE). TBI and CTE are tauopathies characterized by a distinct accumulation of hyperphosphorylated Tau. Similar to other tauopathies, pathology progresses to other brain regions in a prion-like manner. However, the mechanisms underlying the prion-like spreading and cell uptake of tau seeds in TBI is still not fully understood. Here, we test the *in vivo* roles of neuronal and seizure activity and dynamin-dependent endocytosis in the spreading of tau seeds. We engineered a novel transparent Tau biosensor zebrafish that reliably reports accumulation of tau species following seeding by intra-ventricular injections. Zebrafish larvae subjected to a novel pressure wave induced traumatic brain injury (PW-TBI) paradigm displayed various markers associated with blast traumatic brain injury, including cell death, hemorrhage, blood-flow impairments, post–traumatic seizures, and Tau inclusions. Interestingly, we found that reducing post-traumatic seizures in our TBI models using retigabin, an anti-convulsant drug, significantly reduced the abundance of Tau inclusions in our TBI models. We obtained similar findings with the treatment with 4-Aminopyridine, convulsant drug, and dynamin inhibitors. These data suggest a role for seizure activity and dynamin-dependent endocytosis in the prion-like seeding and spreading of Tauopathy following TBI. Moreover, the data highlight the utility of deploying *in vivo* Tau biosensor and TBI methods in larvae zebrafish, especially regarding drug screening and intervention. The models offer good potential to uncover information surrounding prion-like mechanisms of Tau pathology.

2.2 Introduction:

Traumatic brain injury (TBI) is one of the leading causes of mortality and disability worldwide (Hay et al., 2016; Nguyen et al., 2016; Rimel et al., 1981). It is also a well-known risk factor associated with neurodegeneration and dementia, such as CTE, which is linked to repetitive mild TBI (Chauhan, 2014; Gardner and Yaffe, 2015; Uryu et al., 2007). TBI can result from direct physical insults, from rapid acceleration and deceleration of the brain, or from shock wave impacts such as those produced by explosive blasts (Cruz-Haces et al., 2017). Regardless, the neuropathology in TBI and CTE patients includes the wide distribution of hyperphosphorylated tau pathology, neuronal loss, axonal degeneration, and disruption of the blood-brain barrier (BBB)—especially after blast injury and neuro-inflammation (Hay et al., 2016; Johnson et al., 2013; McKee et al., 2015; Ojo et al., 2016). The progressive deposition of hyperphosphorylated tau protein, a microtubule associate protein encoded by *MAPT* gene, in filamentous forms is a hallmark of a group of diseases collectively known as tauopathies (which both AD and CTE are part of). Each one of the tauopathies affect distinct regions and have unique clinical presentations (Kovacs, 2017; Orr et al., 2017). In CTE, hyperphosphorylated tau is accumulated in a cluster of perivascular neurons and glia in the depths of cortical sulci at earlier stages. Later, however, a wide spread of the tau pathology from these focal cortical deposits, spreading to the cortical and subcortical grey-matter areas is observed (Hay et al., 2016; Johnson et al., 2012; McKee et al., 2015). This broad spreading of tau pathology in CTE cases has also been reported in long-term TBI survival from single trauma cases (Johnson et al., 2012). A recent *in vivo* evidence from wild-type mice injected with brain homogenate from TBI mice that were subjected to severe TBI and presented with p-tau pathology similar to single severe TBI patients—developed a similar pathology that spread from injections sites to distant

regions. These patient observations alongside the evidence from *in vivo* works support the conclusion that tau species in TBI also act similarly to bona fide prions (Zanier et al., 2018).

Recently, numerous studies have supported the self-propagation and prion-like spread of tau aggregates in tauopathies (mostly AD) and pointed to the key role these prion-like abilities might play in the progression of these devastating diseases (Iba et al., 2013; Iba et al., 2015; Mudher et al., 2017; Narasimhan et al., 2017; Sanders et al., 2014). Despite the importance of these findings, a critical knowledge gap exists regarding mechanisms of prion-like transmission or the spread of tau seeds in TBI.

Studies on tau spreading (mostly performed *in vitro*) have recently suggested various mechanisms for the release and uptake of tau aggregates or seeds from one cell to adjusting cells, including tunnelling nanotubes and extracellular vesicles (EVs) such as exosomes and synaptic vesicles, as well as endocytosis as an uptake mechanism (Demaegd et al., 2018; Evans et al., 2018). In other tauopathies (AD), observations from patients and mice have highlighted the capacity of tau seeds to spread trans-synaptically (Goedert et al., 1989; Pickett et al., 2017). Moreover, it has been shown that neuronal activity serves an important role in the spread of tau pathology and general proteostasis (Pickett et al., 2017; Wu et al., 2016; Yamada et al., 2014). Indeed, the *in vitro* stimulation of neuronal activity increased the extracellular release of tau to media while enhancing tau pathology in Tg mice expressing a repressible form of human tau containing the P301L mutation that has been linked to familial frontotemporal dementia (Pickett et al., 2017; Wu et al., 2016; Yamada et al., 2014). However, whether similar mechanisms of tau release and spread occur in TBI cases remains unknown.

An intriguing and poorly studied aspect of TBI is the mechanisms of post-traumatic seizures and their potential role in exasperating the spread of tau pathology. Seizures are one of

the key consequences of all types of TBI, and they have been more commonly reported in patients who suffered from blast injuries, though the prevalence remains undetermined (Lucke-Wold et al., 2015; Salinsky et al., 2015). However, it is anticipated that over 50% of TBI patients with severe injuries will develop seizures and post-traumatic epilepsy (Kovacs et al., 2014). Studies of seizures in AD patients and animal models of AD have implicated a link between the occurrence of seizures and tau pathology (Sanchez et al., 2018; Yan et al., 2012). Whether the occurrence of seizures in TBI impacts the prion-like spread of tau pathology in TBI patients, or if reducing post –traumatic seizure can delay or minimize the progression of disease, has yet to be fully explored.

Notably, a lack of information exists regarding how tau seeds are taken up by cells *in vivo* and the role of endocytosis in the prion-like spreading of tau seeds in TBI. This knowledge gap may be due to a lack of access to *in vivo* models that could complement *in vitro* models in which we can visualize and examine the progression and spread of tauopathy in living brains with an intact CNS. Additionally, a model is needed that is accessible to modulating and measuring neural activity associated with TBI. To address these issues, we engineered a tauopathy biosensor zebrafish that develops GFP+ puncta when tau aggregates in the brain and spinal cord area. Additionally, we introduce a novel and simple method to induce TBI in zebrafish larvae. Combining these, we report the occurrence of post-traumatic seizures and their positive correlation with tau pathology. Manipulating seizure activity impacted tau aggregation and revealed an *in vivo* role for endocytosis in the prion-like spread of tau seeds in TBI. Our results highlight the importance of dynamin-dependent endocytosis in the spread of tau seeds in our novel *in vivo* TBI model.

2.3 Methods:

2.3.1 Animal Ethics and Zebrafish Husbandry

Zebrafish were raised and maintained following protocols approved by the Animal Care and Use Committee: Biosciences at the University of Alberta, operating under the guidelines of the Canadian Council of Animal Care. The fish were raised and maintained within the University of Alberta fish facility under a 14/10 light/dark cycle at 28ºC as previously described (Westerfield, 2000).

2.3.2 Generating Transgenic Tauopathy Reporter Zebrafish

To engineer the Tau4R-GFP reporter zebrafish, the human wild-type *MAPT* sequence of the four-microtubule binding repeat region (aa 244-372 of the full-length tau (2N4R)(NCBI NC_000017.11, protein id NP_005901) with a seven-amino acid C-terminal linker (RSIAGPA) was ordered as a gene block from IDT. The gene block was subcloned into a middle entry cloning vector (Multisite Gateway® technology, ThermoFisher) and recombination was carried out consistently according to the manufacturer's instructions using the p5E- enolase2 and p3E-GFP components of Tol2kit (Guo and Lee, 2011; Kwan et al., 2007) to generate the transgenic construct within a destination vector (pDestTol2CG2). The destination vector contained a reporter construct [encompassing EGFP driven by the cardiac myosin light chain (clmc) promoter that helps identify stable transgenic zebrafish]. The resulting plasmid pDestTol2CG2.eno2:Tau4R-GFP was delivered in an injection solution including 750ng/μl of the construct mixed with 250ng/μl Tol2 transposase mRNA, 1μl of 0.1M KCL, and 20% phenol red. The solution was injected into the single-cell embryos of Casper zebrafish line (transparent zebrafish line) (White et al., 2008). At two days post-fertilization (dpf), embryos were screened

for mosaic expression of the Tau4R-GFP fused protein using a Leica M165 FC dissecting microscope and raised to adulthood. F0 mosaic fish were outcrossed to wild-type Casper fish, and F1 embryos were identified by the abundant expression of Tau4R-GFP in the CNS and green heart marker. The stable transgenic line Tg[eno2:Tau4R-GFP] was assigned the allele number ua3171.

An equivalent transgenic zebrafish biosensor was engineered to detect human SOD1 aggregation. Subcloning from existing vectors (Pokrishevsky et al., 2018) produced pDestTol2CG2.eno2:SOD1-GFP and similar transgenesis methods engineered *Tg[eno2:SOD1-GFP]* that was assigned allele number ua3181.

2.3.3 Cell culture and Generation of Tauopathy Reporter Stable Cell Line

To move the Tau4R-GFP reporter into a vector appropriate for cell culture, BamHI and Xhol nuclease restriction enzymes were employed to remove the Tau4R-GFP fragment from pDest tol2CG2.eno2.Tau4R-GFP.pA. The Tau4R-GFP fragment was subcloned into the pCDNA3.1 vector using a T4 DNA ligase enzyme. Sequencing of the cloned vector with the following reverse primer for GFP (TCTCGTTGGGGTCTTTGCTC) confirmed the proper orientation. Purification of the plasmid was conducted with the Qiagen purification kit. HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (GibcoTM, ThermoFisher) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were maintained at 37°C in a humidified 5% CO2 incubator. For passaging cells, cells were washed with phosphate-buffered saline (PBS) before trypsinization with 0.05 Trypsin-EDTA (Sigma Aldrich, T4174).

HEK293T cells were plated at 1 x 10^6 cells/well in six-well plates. After 24 hours, cells were transfected with pCDNA3.1.Tau4R-GFP plasmid using lipofectamine 2000 reagents according to the manufacturer's guidelines. Briefly, 4µg of pCDNA3.1.Tau4R-GFP was diluted in 250µl of Opti-MEM media (GibcoTM, ThermoFisher). After 24 h, the expression of the fluorescent reporter was confirmed through microscopic analysis. To establish a stable cell line, the transfected cells were replated at a 1:10 dilution. Stable cell lines were selected in DMEM media containing 1200µg/ml geneticin (GibcoTM, ThermoFisher). Expression of the fused fluorescent proteins in the stable cell lines was confirmed using fluorescent microscopy. Polyclonal cells and monoclonal cells were grown to confluency in 10-cm dishes, then stored in liquid nitrogen until use.

2.3.4 Immunoblotting of Cell Lysate and Zebrafish Brain Lysate

For cell lysate preparation, cells were washed with cold PBS, then collected and incubated with cold lysis buffer (150mM NaCl, 50mM Tris-HCl (pH 8), 1 mM EDTA and 1% Nonidet P-40) and supplemented with protease inhibitor (Cocktail Set III; Millipore) for 10 mins on ice. Cells were lysated using a bio-vortexer homogenizer for 20 sec for two rounds. The lysate was centrifuged at 13000rpm for 10 mins at 4ºC. The supernatant was collected, and the protein concentration was determined using the Qubit™ Protein Assay Kit (Invitrogen).

For zebrafish brain lysate preparation, the brains of adult zebrafish were dissected. Brains were homogenized in cell lysis buffer (20mM HEPES, 0.2mM EDTA, 10mM NaCl, 1.5mM $MgCl_2$, 20% glycerol, 0.1% Triton-X) with protease inhibitor and phospSTOP (Sigma-Aldrich) in the case of pt-40 Tg. Brains were lysed using a bio-vortexer homogenizer and sonicated for 3 sec for one round. Samples were centrifuged with the same sitting mentioned previously. The

concentration of samples was assessed in a Qubit® fluorometer (Invitrogen). Later, 30-40 g of the total protein was combined with 2X sample buffer (Sigma-Aldrich) and boiled for 10 mins before loading in 11% SDS-PAGE. Electrophoresis was performed using the Bio-Rad Power PAC system in running buffer (25 mM Tris base, 192 mM glycine and 0.1% SDS). The gel was transferred to a PVDF membrane using a wet transfer system. All membranes were blocked for one hour in protein-free blocking buffer PBS (ThermoFisher) or TBST with 5% milk and then incubated with primary antibody overnight at 4ºC with gentle agitation. The primary antibodies used in this study include rabbit monoclonal GFP (abcam, EPR14104) at 1:3000 dilution, rabbit anti-β-actin (Sigma-Aldrich, A2066) at 1:10000. All membranes were washed three times with 1X TBST before incubation with secondary antibody (goat-anti-mouse HRP or HRP-conjugated anti-rabbit at 1:5000 dilution (Jackson ImmunoResearch) for one hour at room temperature. The membranes were washed for the final time before visualization using Pierce® ECL Western Blotting Substrate (ThermoFisher) on a ChemiDoc (Biorad). For stripping and re-probing, the membranes were stripped using mild stripping buffer (199.8 M Glycine, 0.1% SDS and 1% Tween 20 with a pH of 2.2) before blocking them and repeating the methods described before.

2.3.5 Immunohistochemistry

Larvae subjected to traumatic brain injury were fixed 5 hours or 1 day post TBI for immunostaining of neurofilaments and Activated-Caspase3, respectively, in 4% paraformaldehyde overnight, and whole-mount immunocytochemistry was carried out as previously described (DuVal et al., 2014). Larvae were washed with 0.1 M PO4/5% sucrose three times before washing with 1% Tween/H_2O (pH 7.4), and then −20°C acetone. Larvae were incubated in PBS3+ containing 10% normal goat serum (NGS) for one hour to reduce any non-

specific antibody binding and then incubated with 2% normal goat serum/PBS3+ and a primary antibody. Primary antibodies used were polyclonal Anti-Active-Caspase-3 (BD Pharmingen, 559569) and monoclonal NF160 (Sigma Aldrich, N2787) at 1:500 dilutions. Secondary antibodies and stains applied were Alexafluor 647 anti-rabbit at 1:200 dilution for Caspase 3 staining (Invitrogen) and Alexafluor 647 anti-mouse at 1:500 for NF160, and counterstained for 30 minutes with 4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher).

2.3.6 Preparations of Mouse Brain Homogenate (Crude and PTA Precipitated)

Brains from TgTau $^{+/P301L}$ and Wild-type (the genetic background 129/SvEvTac) mice were provided by Dr. David Westaway and Dr. Nathalie Daude. Crude brain homogenate was prepared by homogenizing the brains to 10% (wt/vol) in calcium- and magnesium-free DPBS that included a protease inhibitor and phosSTOP, using a glass homogenizer and power gen homogenizer (Fisher Scientific). Samples were then centrifuged at 13000 rpm for 15 mins at 4◦C. The clear supernatant was collected, aliquoted and stored in -80°C until use for experiments.

The phosphotungstate anion (PTA)-precipitated brain homogenate was prepared as described (Woerman et al., 2016). Briefly, 10% (wt/vol) brain homogenate was prepared as reported previously and mixed with a final concentration of 2% sarkosyl (Sigma Aldrich) and 0.5% benzonase (Sigma Aldrich, E1014), and then incubated at 37oC for two hours with constant agitation in an orbital shaker. Sodium PTA (Sigma Aldrich) was dissolved in ddH$_2$O, and the pH was adjusted to 7.0 before it was added to the samples at a final concentration of 2% (vol/vol). The samples were then incubated overnight under the previous conditions. The next day, the samples were centrifuged at 16,000xg for 30 mins at room temperature. The supernatant

was discarded, while the resulting pellet was resuspended in 2% (vol/vol) PTA in ddH_2O (pH 7.0) and 2% sarkosyl in DPBS. The samples were next incubated for one hour before the second centrifugation, as aforementioned. At this point, the supernatant was removed and the pellet was re-suspended in DPBS. An aliquot of 5µl of PTA purified brain homogenate was employed for electron microscopy (EM) analysis to confirm the presence of fibrils in each sample.

2.3.7 Tau Fibrillization and EM Analysis:

Synthetic human WT tau protein full-length monomers were purchased as a lyophilized powder (rPeptide, T-1001-2) and resuspended in ddH_2O at a concentration of 2mg/ml. The recombinant protein was fibrillized as described before (Guo and Lee, 2011). Recombinant tau was incubated with 40M low-molecular-weight heparin and 2mM DTT in 100 mM sodium acetate buffer (pH 7.0) at 37ºC, thereafter being agitated for seven days. The fibrillization mixture was centrifuged at 50,000 x g for 30 mins, and the resulted pellet was resuspended in 100 mM sodium acetate buffer (pH 7.0) without heparin or DTT. Successful fibrillization was verified by EM.

Negative staining for EM analysis of fibrils was conducted as described elsewhere (Eskandari-Sedighi et al., 2017). Briefly, 400 mesh carbon-coated copper grids (Electron Microscopy Sciences) were glow-discharged for 40 sec aliquots before adding the sample. (5µL) of PTA-purified brain homogenates or synthetic tau fibrils, were applied on the top of the grid for 1 min. These grids were washed using 50µL each of 0.1M and 0.01M ammonium acetate and negatively stained with 2 × 50µL of filtered 2% uranyl acetate. After removing excess staining and drying, the grids were examined with a Tecnai G20 transmission electron microscope (FEI Company) with an acceleration voltage of 200 kV. Electron micrographs were recorded with an Eagle 4k × 4k CCD camera (FEI Company).

2.3.8 Liposome-Mediated Transduction of Brain Homogenate into Tauopathy Reporter Cells

Polyclonal Tau4R-GFP cells were plated at 2 x 10^5 per well in 24-well plates. The next day, 40µl of 10% clarified brain homogenate was combined with Opti-MEM to a final volume of 50µl. Added was 48µl of Opti-MEM and 2µl of Lipofectamine-2000 (Invitrogen) to the previous Opti-MEM mixture to a total volume of 100µl. After 20 mins of incubation, the liposome mixture was added to the cells. Eighteen hours later, cells were washed with PBS, trypsinized, and re-plated on coated coverslips (ThermoFisher) for imaging and analysis.

For PTA-precipitated brain homogenate, 1:10 dilution of precipitated fibrils was used for the transfection. 5µl of PTA-purified fibrils was diluted in 45µl Opti-MEM to a final volume of 50µl. The previous Opti-MEM mixture was added to 47µl of Opti-MEM and 3µl of Lipofectamine-2000 and incubated in room temperature for 2 hours as described in (Woerman et al., 2016). The mixture was added to cells, washed after 18 hours and re-plated before analysis exactly as mentioned previously.

2.3.9 Quantification of the Percentage of Cells with Positive Inclusions

For imaging transfected cells, they were fixed using 2% PFA in PBS for 15 mins. Cells were washed twice with PBS then stained with DAPI (1:3000 from 1mg/ml stock) for six mins. The coverslips were mounted on Superfrost Plus slides (Fisher Scientific, catalog no. 12-550-15) with VECASHILD Antifade mounting media (VECTOR Laboratory). The edges of the coverslips were sealed with nail polisher and imaged using a Zeiss LSM 700 scanning confocal microscope featuring Zen 2010 software (Carl Zeiss, Oberkochen, Germany). For the

quantifications, a total of nine field images were taken, each with ~100 cells, were analyzed per conditions. DAPI-positive nuclei were utilized to determine the number of cells per field. The number of cells with inclusions (multiple nuclear inclusions or one cytoplasmic puncta) were counted and the percentage was calculated. Mean and standard error were determined and plotted.

2.3.10 Brain Ventricle Injections into Tauopathy Reporter Larvae

Injections were as described with few modifications (Gutzman and Sive, 2009). For the intraventricular brain injections, all embryos had their chorion removed at two dpf. Embryos were anesthetized with tricaine (%4) (MS-222, Sigma Aldrich). The embryos were placed in a 1% agarose-coated dish with small holes. Under a stereomicroscope, the embryos were immobilized and oriented so that the brain ventricles were visible and accessible for injections. The injection was carried out via pulled capillary tubes mounted in a micromanipulator. The injection volume was calibrated to 5nL by injection into mineral oil first to assess volume, and measured with an ocular micrometer prior to injections. Thereafter, the needle containing the injection solutions was placed through the roof plate of the hindbrain and 5-10nl of either 10% clarified brain homogenate (non-Tg or Tg hTau) or synthetic tau fibrils mixed with 20% dextran Texas Red fluorescent dye (Invitrogen) were injected into the ventricles. For all the brain injection experiments, an uninjected control group and control group injected only with 20 % red dextran fluorescent dye in PBS were included. After the injections, embryos were screened using a Leica M165 FC dissecting microscope and appropriately injected larvae were gathered for further analysis. The injections were considered appropriate if they had (Figure 2.1) sharp edges and non-diffuse dye in the ventricle. Incorrect injections in which the needle was inserted too

deep in the brain ventricles resulting in the dye being visible outside the ventricle space and/or in the yolk were excluded from analysis.

Figure 2.1

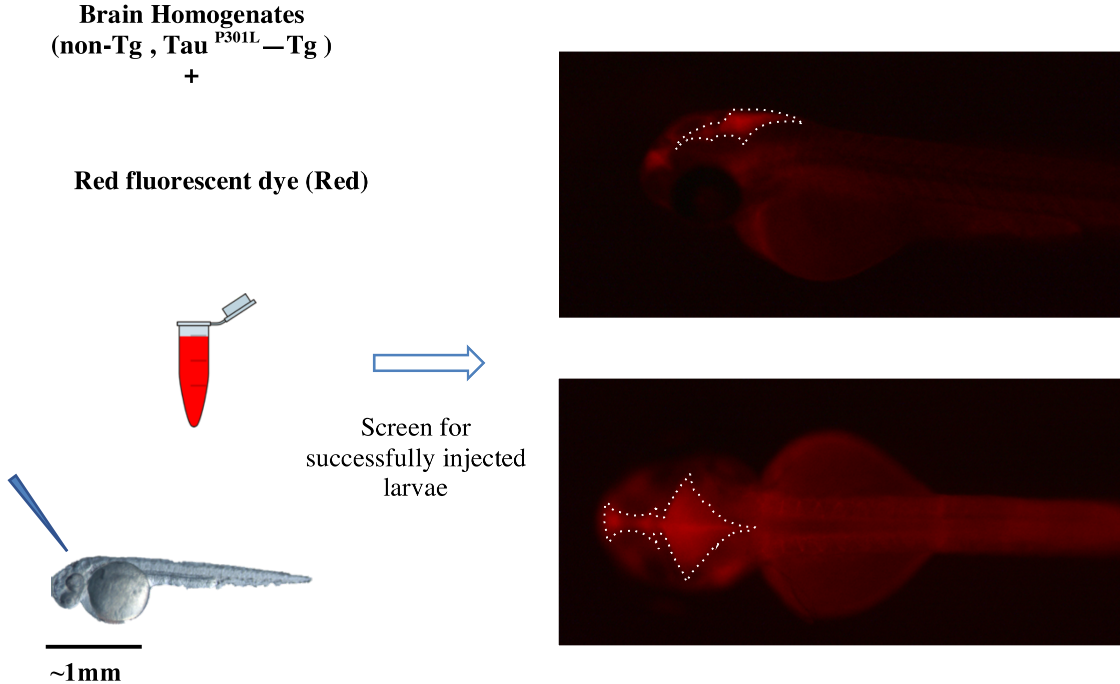


Figure 2. 1: Schematic showing the microinjections of brain homogenate into 2dpf larvae.

An example image of properly injected embryo with sharp edges and non-diffuse dye in the ventricle (area where the dye injected the dye is highlighted by white dotted line). Top image is lateral view; bottom image is dorsal view; anterior to the left.

2.3.11 Microscopy Analysis of GFP Positive Puncta in Tau Reporter Larvae

For the microscopic analysis of GFP-positive inclusions, larvae that were either injected or treated with traumatic brain injury, along with the control groups were anesthetized via tricaine at one of the indicated time points (two, three, four, or five days post-injection (dpi) or –post traumatic brain injury (dpti) depending on the experiment). Images for GFP-positive puncta on the brain area or lateral line above the spinal cord were taken using a Leica M165 FC dissecting microscope and the number of GFP-positive puncta were manually counted.

2.3.12 Pressure waves-Induced traumatic Brain Injury (PW-TBI) paradigm for Zebrafish Larvae

To induce (PW-TBI), 10-12 unanesthetized three-dpf larvae were loaded into a 10ml syringe with 1ml of E3 media. The syringe was blocked using a valve to ensure no larvae or media left the syringe upon compression of the plunger. The syringe was held vertical using a metal tube holder at the bottom end of a 48” tube apparatus. At the other end of the tube (top), a defined weight (30 to 300g) was dropped manually. The tube diameter was matched to (slightly greater than) the weight’s diameter to enhance repeatability. This was done once or repeated three times with both 65 and 300g weights. Once larvae were subjected to the PW-traumatic bain injury, they were moved back to a petri dish with fresh media and larvae were used for further analysis.

2.3.13 Recording blood flow following TBI

Abnormalities of blood flow and circulation resulted from TBI was detected 5 to 10 mins after larvae was subjected to TBI. The blood flow in the tail area of zebrafish larvae, that either subjected to TBI or uninjured control larvae, was recorded using Leica DM2500 LED optical microscope.

2.3.14 Measuring the Seizure-like Phenotype in TBI Larvae

The seizure-like behavior and activity of zebrafish larvae post traumatic brain injury experiment was quantified via behavioral tracking software as described in our recent publication (Leighton et al., 2018). Briefly, control 3dpf larvae or larvae subjected to traumatic brain injury using 65g weight, were placed individually in wells of 96-well plates. The locomotor and seizure activity were assessed 40 minutes after the traumatic brain injury through EthoVision® XT-11.5 software (Noldus, Wageningen, Netherlands).

2.3.15 Engineering CaMPARI transgenic zebrafish for integrative calcium imaging

The Tg[elavl3:CaMPARI (W391F+V398L)]ua3144 zebrafish line expressing the calcium sensor CaMPARI was generated using the Tol2 transgenesis system. We re-derived CaMPARI fish previously established due to a federal moratorium on importing zebrafish into Canada (Hanwell et al., 2016). The Tol2 vector, pDestTol2-elavl3:CaMPARI (W391F+V398L), was a gift from Eric Schreiter's lab and was published in (Fosque et al., 2015). The Tol2 vector was injected in embryos at the 1-2 cell stage as previously described (Fisher et al., 2006). Transient larvae were identified through green fluorescent cells in the central nervous system and were

outcrossed to obtain a stable Tg[elavl3:CaMPARI (W391F+V398L)] ua3144 F1 line. The F1 line was crossed into the transparent Casper background.

2.3.16 Measuring neuronal activity during TBI using CaMPARI

Bright green CaMPARI larvae were loaded into 20ml syringe containing 1ml E3 media (prepared as per Westerfield, 2007. but without ethylene blue) and were exposed to 405 nm LED array (Loctite), which illuminated the syringe entirely (Figure 2.9A). Larvae were exposed for 10 sec at a distance of 7.5 cm while subjected to traumatic brain injury using the 300g weight as previously explained in TBI methods. The weight hit the plunger of the syringe one time only. Following photoconversion and TBI, larvae were anesthetized in 0.24 mg/mL tricaine (MS-222, Sigma Aldrich) and embedded in 2 % low-gelling agarose (A4018, Sigma Aldrich) for analysis under confocal microscopy.

Maximum intensity projections were acquired from Z-stacks (8 μm steps) using a 20x/0.8 Objective and a laser-point scanning confocal microscope (Zeiss 700). The hindbrain area was analyzed as it was the most responsive brain region to traumatic brain injury. To specifically isolate the brain regions and obtain data points, a 3D area was isolated by creating a surface with Imaris® 7.6 (Bitman, Zuerich) and the fluorescence mean of the green and red channels intensities were calculated. Data points were presented as a red/green ratio for each individual fish and interpreted as relative neural activity, which is defined as red photoconverted CaMPARI in ratio to green CAMPARI (Fosque et al., 2015).

2.3.17 Bath Application of Drugs

For Proteasome inhibition experiments, tau biosensor larvae treated with 20 µM MG-132 at 2dpf following injections with brain homogenate from Tg human Tau mice. Controls included untreated group that was not injected, untreated group that was injected group and uninjected control that was treated with the same does of MG-132. The treatment was left for 48 hours before changing the media and evaluating the percentage of larvae developing GFP+ puncta in the brain region.

For 4-aminopyridine (4-AP), two doses of 4-AP (200, and 800µM) were added either six or 24 hours post traumatic brain injury. For Retigabine (RTG) treatment, 10µM was used to treat TBI larvae six hours post traumatic brain injury. The larvae were incubated with both 4-AP and/or RTG for 38 hours, then a fresh drug-free E3 media was added. The formation of GFP-positive puncta was analyzed at four to five days post injury. Control groups consisted of larvae that were not treated with any drug nor subjected to traumatic brain injury, and a group of larvae that were subjected to traumatic brain injury but not treated with 4-AP or RTG. Control groups were compared to the group that was subjected to TBI and treated with the aforementioned doses of 4-AP or RTG, or with both drugs combined.

Pyrimidyn-7™ (P7), the dynamin inhibitor, was purchased at a 50mM concentration supplied in DMSO (Abcam). Larvae that were subjected to TBI were treated within six hours following the injury with 3µM of P7. The dose was chosen based on the previous use of the P7 drug on zebrafish larvae (Verweij et al., 2019). The larvae were incubated with the drug for 20 hours, after which they were transferred to a fresh plate with drug-free media. Dayngo 4a, another dynamin inhibitor (McCluskey et al., 2013), was purchased from (Abcam) and 4 µM of Danygo4a was used to treat larvae as previously explained with P7. The formation and

abundance of GFP-positive puncta was evaluated as previously described at four dpti. All the drugs experiments were done on larvae subjected to traumatic brain injury using either 65g or 300g weight.

2.3.18 Statistics

All statistical analyses were performed using GraphPad Prism Software (Version 7, GraphPad, San Diego, CA). Unpaired t-test was used to compare between two groups. For comparison between three groups at three different time points or the same time point, 2-way and Ordinary One-Way ANOVA were used followed by post-hoc Mann-Witney U tests and Kruskal-Wallace multiple comparison tests.

2.4 Results:

2.4.1 Engineering and Validation of Tauopathy reporter lines

Previously engineered tools have enabled the assessment and quantification of tau inclusions in living cells (typically Human Embryonic Kidney cells), via measuring aggregation of fluorescent proteins fused to tau protein, providing sensitive detection of pathological tau species and strain variants (Kaufman et al., 2016; Sanders et al., 2014; Woerman et al., 2016). We reasoned that these biosensor tools would have good potential to reveal additional characters when expressed in vibrantly active neurons of the CNS, and that prion-like mechanisms of tauopathy spread (if any) are best modeled in an intact brain (e.g. vectored by blood and glymphatic circulation, ventricles, and immune systems). Therefore, we engineered a tauopathy biosensor transgenic zebrafish that expresses a fluorescent tau reporter protein. Our genetically encoded fluorescent reporter protein was composed of the sequence of the human tau core-repeat

region fused to GFP with a linker sequence and is referred to here as Tau4R-GFP (Figure 2.2A). Contrasting previous *in vitro* models, our biosensor did not feature any pro-aggregation mutations in tau; this was a deliberate design intended to minimize spontaneous aggregation events. The expression of the biosensor protein is under the control of the pan-neuronal promoter *neuronal enolase 2* (*eno2*), which drives expression throughout the CNS (Figure 2.2A). We deployed the transgene in a transparent zebrafish line ('Casper' background (White et al., 2008)) to facilitate analysis beyond the early larval development stages (when pigmentation would otherwise begin). We isolated a stable transgenic (Tg) line that expresses the Tau4R-GFP biosensor reporter robustly and clearly in the CNS (Figure 2.2C), *Tg[eno2:Tau4R-GFP]*, and assigned it allele number ua3171.

Simultaneously, we expressed the same biosensor *in vitro* to validate the construct we deployed *in vivo* (Figure 2.2 A and B). Both in HEK293T cells and Tg zebrafish brains, immunoblotting using anti-GFP antibody detected our Tau-4R-GFP reporter protein at the expected size of ~45 KDa, similar to a SOD1:GFP biosensor protein of similar predicted size, and an appropriately larger size relative to GFP protein alone (Figure 2.2 B' and C').

We assessed the capacity of our Tau4R-GFP biosensor to report the presence of tau pathology when tau-laden brain homogenates were transduced into cells. Brain homogenates from transgenic mice expressing mutant human tau (Tg human Tau ^{p301L}), were compared to non-Tg mouse homogenates as a negative control. Congruent with findings obtained in other similar cell assays (Sanders et al., 2014), GFP+ inclusions were detected only when clarified brain homogenate full of pathogenic tau fibrils (from Tg human P301LTau mice) were transduced to biosensor cells (Figure 2.2 D). The *in vitro* assay detection rate was approximately 5% of cells having GFP+ inclusions in total with 2% of cells forming multiple nuclear inclusions and ~3%

forming one cytoplasmic puncta, whereas various negative controls consistently displayed 0% of cells with inclusions (Figure 2.3). To verify that tau aggregates in the clarified brain homogenate caused the GFP+ puncta, we purified tau aggregates through PTA precipitations (Woerman et al., 2016). Purified tau fibrils were characterized via EM analysis (Figure 2.2 E) and produced GFP+ aggregation (Figure 2.2 F), confirming the ability of our Tau4R-GFP chimeric protein to report tau aggregation.

Figure 2.2

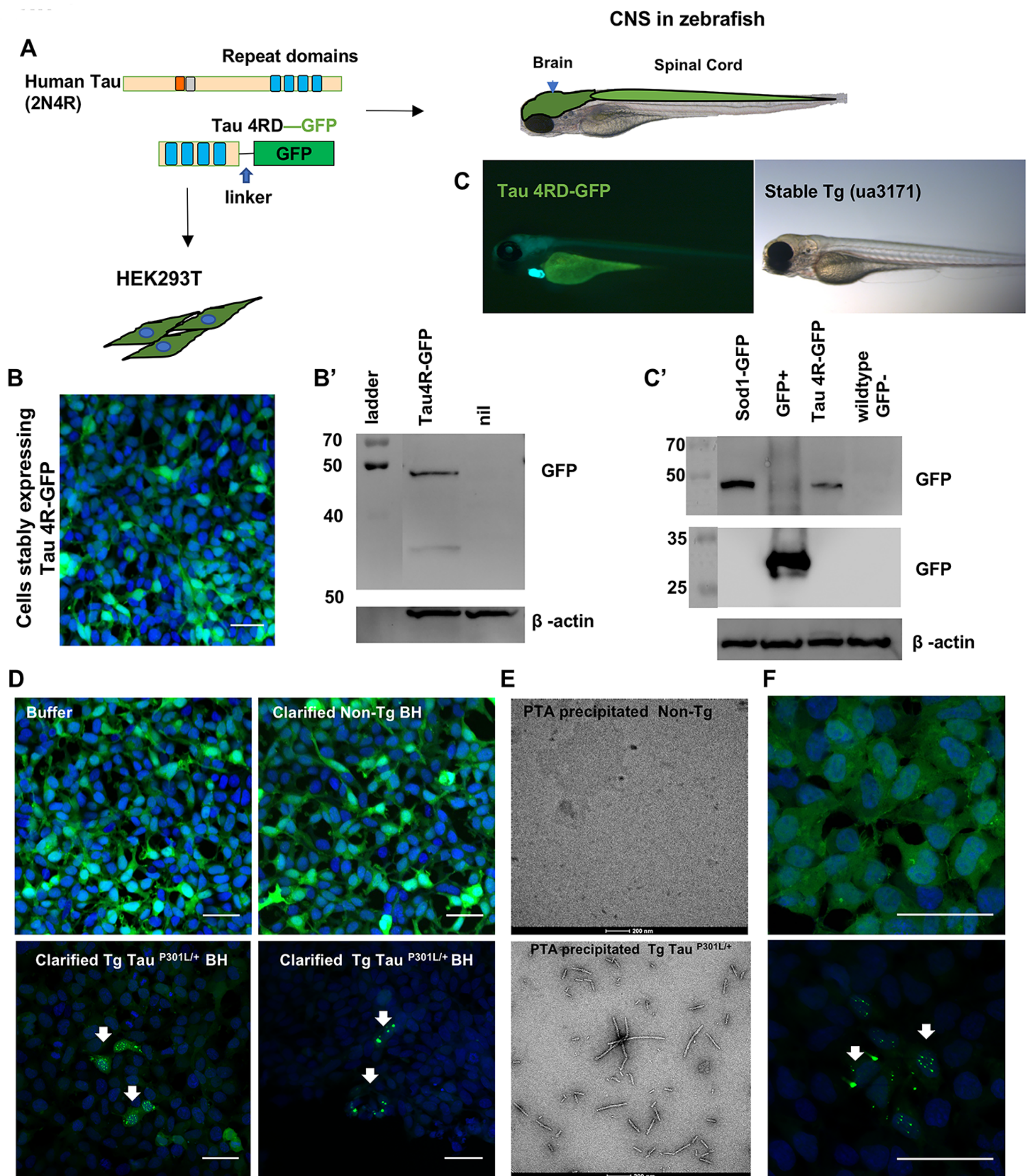


Figure 2. 2 : Validating tauopathy fluorescent biosensor *in vitro*.

Biosensor Tau4R-GFP was validated *in vitro* for its ability to detect tauopathy seeds (derived from Tg mouse brains) *in vitro*, and also expressed in zebrafish. (A) Schematic of Tau4R-GFP "Tau biosensor" that contains the four binding repeats (4R) region of wildtype human tau linked to green fluorescent reporter protein (GFP) and its expression in cells and CNS in zebrafish larvae. (B) HEK293T polyclonal cells stably expressing Tau4R-GFP reporter and (B') immunoblot vs. GFP suggests the fusion protein is intact and appropriate size. (C) Transgenic zebrafish engineered to express Tau4R-GFP biosensor throughout neurons of the CNS. Wildtype GFP is also abundant in the heart, which serves as a marker of the transgene being present but is otherwise irrelevant to our analyses. (C') Western Blot on zebrafish brain confirmed production of Tau4R-GFP at the expected size, similar to a SOD1-GFP biosensor and coordinately larger than GFP alone. (D) Tau4R-GFP biosensor cells display GFP+ inclusions only upon transduction of crude brain homogenate from Tg human Tau$^{+/P301L}$mouse, not from non-tg mouse. (E) PTA-purified brain homogenate assessed by EM confirms presence of fibrils (EM images obtained by RK). (F) Application of PTA-purified brain homogenate induced the formation of tau inclusions similar to clarified brain homogenate (scale bar 50μm).

Figure 2.3

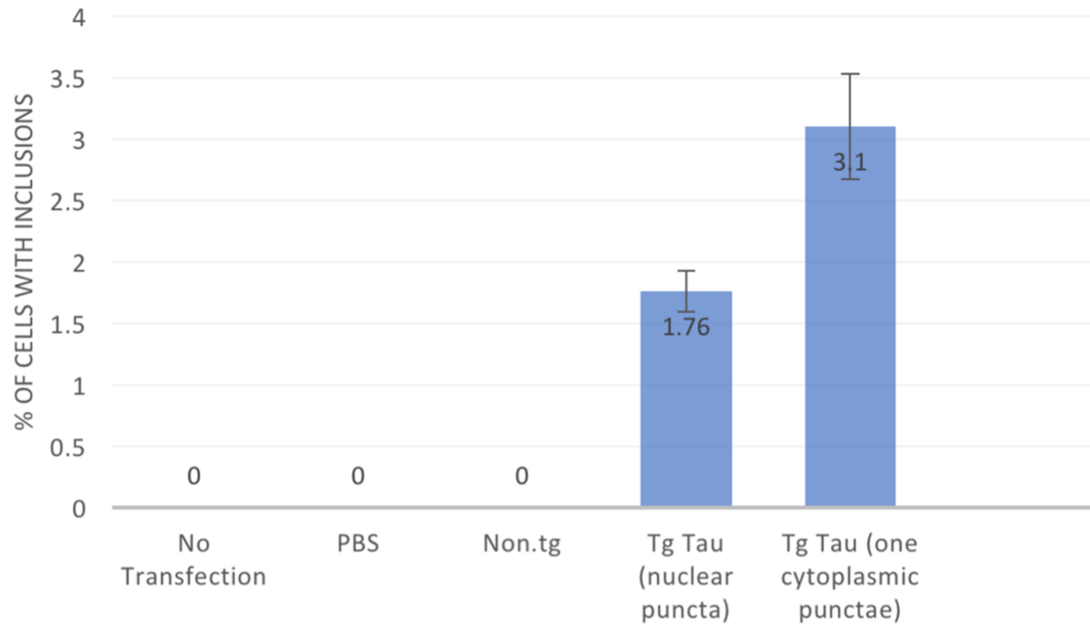


Figure 2. 3: Tau biosensor (Tau4R-GFP) expressed *in vitro* shows an increase in GFP+ puncta following application of pathological human tau.

Quantification of cells with inclusions after transfection of mouse brain homogenate that is laden with human tau pathology showing detection rate of approximately 5% of cells having inclusions with 0% in various negative controls. Negative controls include transfection with wildtype mouse brain homogenate, transfection with PBS, or no transfection. For the quantification, the number of cells with inclusions and the number of total cells from 9 field images were counted. Bars represents mean of three independent experiments ± standard error.

2.4.2 Validation of the *in vivo* biosensor model through intra-ventricular brain injections of Tau fibrils

Fluorescent tau biosensor proteins expressed *in vitro* have a strong track record of characterizing tau aggregates and revealing prion-like strain properties, whereas an *in vivo* model should additionally permit assessment of spreading of tau aggregation (and potential treatments) in the context of complex CNS neurophysiology and modelling neuropathology. To test if the Tau4R-GFP biosensor can report the *in vivo* progression of tauopathy, we emulated intracerebral injection methods that induce (prion-like) tau pathology in mice (Clavaguera et al., 2013; Guo et al., 2016; Peeraer et al., 2015). We injected clarified brain homogenate laden with human tau fibrils, prepared as above from Tg mice, into the hindbrain ventricle of two dpf tau biosensor zebrafish (Figure 2.1). The injected larvae and control groups were monitored daily for up to four days post-injection (dpi). Tau biosensor larvae developed GFP+ puncta, which is reflective of tau aggregation in the brain (Figure 2.4A). These tau inclusions were prominent near the ventricle wall as well as in sensory neurons along the spinal cord, when injected with brain homogenate from human-tau transgenic mouse (Figure 2.4A). These puncta appeared to have either a lone dot-like shape or were similar to the multiple nuclear puncta detected *in vitro*, in which three to four small puncta are clustered together. Repeated assessment of the location of tau aggregates on the spinal cord of the same individuals over multiple days, using somite numbers as landmarks, indicated a movement of some of these puncta over time (Figure 2.5 A and B).

Amongst larvae injected with human tau fibrils from Tg mouse brain homogenate, 35% of the fish developed puncta in the brain and 80% along the spinal cord (Figure 2.4 B and C). On the other hand, a lower proportion of tau biosensor larvae developed the inclusions post-injection

with the control brain homogenate from wild-type mice (Figure 2.4 B and C). Tau aggregates were detected on the spinal cord region as early as 2 dpi. Intriguingly, a small percentage of larvae developed sporadic GFP+ tau aggregates regardless of treatment. The abundance of the GFP+ spinal cord inclusions was progressive and significantly higher when pathogenic tau brain homogenate was injected compared to the non-injected control, $p<0.0001$ at 3 and 4 dpi, and other control groups, $p<0.001$ at 3 dpi and $p<0.01$ at 4 dpi (Figure 2.4 D). Few larvae in the control groups developed spontaneous inclusions but the number of the larvae and the abundance of those inclusions were minimal (Figure 2.4 D). Visualizing the data as distributions of larvae with particular abundances of GFP+ inclusions (Figure 2.4 E) highlights a trend where most larvae would not develop aggregates unless they were injected with brain homogenate full of pathogenic human tau species. In such a case, the biosensor larvae would develop an abundant number of aggregates. Overall, these data confirm the ability of our biosensor model to detect pathogenic tau species *in vivo*.

Figure 2.4

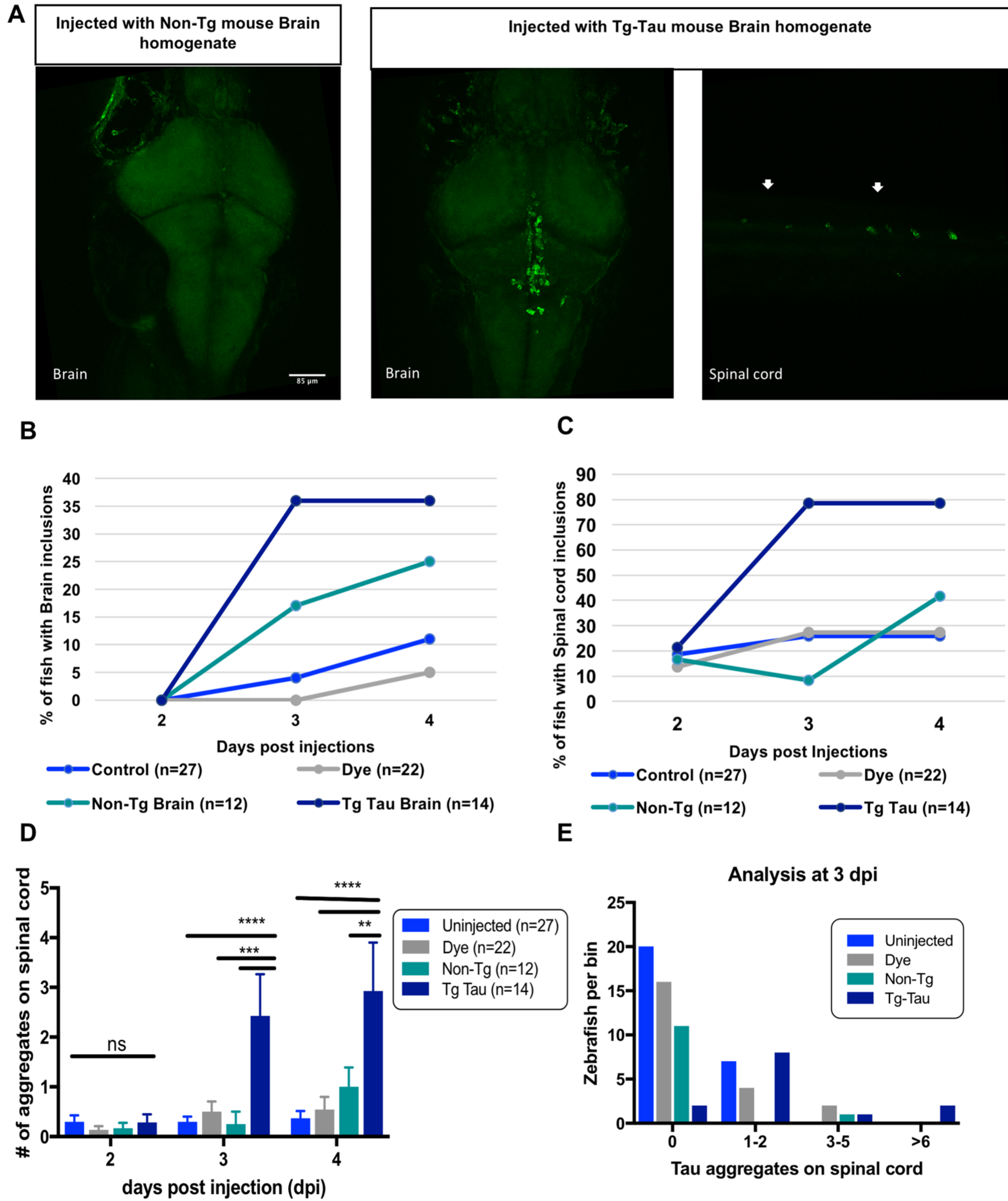
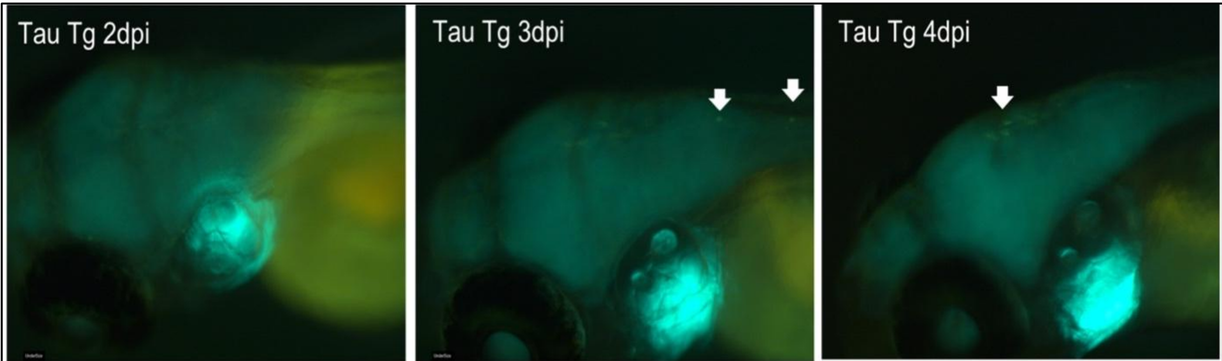


Figure 2. 4: Tau biosensor zebrafish detects disease associated human tau fibrils following intraventricular injection of brain homogenate.

Brain homogenates were microinjected into the zebrafish hindbrain ventricle at two days post-fertilization, and tau inclusions were analyzed at several time points. (A) Tau biosensor zebrafish larvae developed readily apparent GFP+ inclusions in the brain and spinal cord when injected with brain homogenate laden with human tau pathology (from Tg $hTau^{+/p301L}$ mouse; scale bar 85μm). (B) Injection of brain homogenates carrying tau pathology (from Tg-hTau mouse) into hindbrain of Tau4R-GFP zebrafish induced GFP+ puncta in brains and (C) spinal cord at a significantly higher percentage compared to control groups. (D) Tau biosensor zebrafish injected with Tg Tau Brain homogenate developed significantly more aggregates on the spinal cord compared to uninjected control and other control groups, including compared to wildtype mouse brain homogenate (ns for $p>0.05$, * for $p\leq0.05$, ** for $p\leq0.01$, *** for $p\leq0.001$) (E) The distribution of larvae displaying various quantities of aggregates in the spinal cord was quantified. Error bars represent standard error of the mean, n= number of larvae assessed.

Figure 2.5

A



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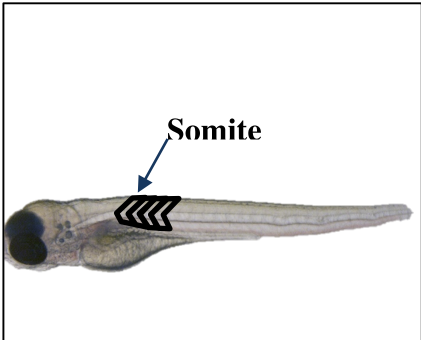

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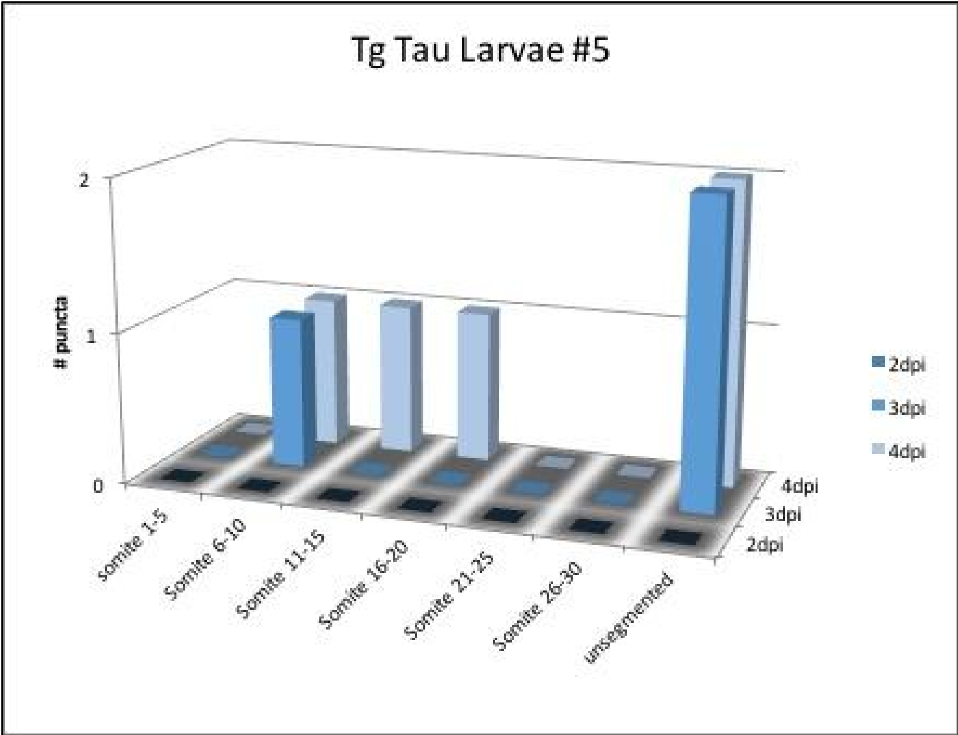

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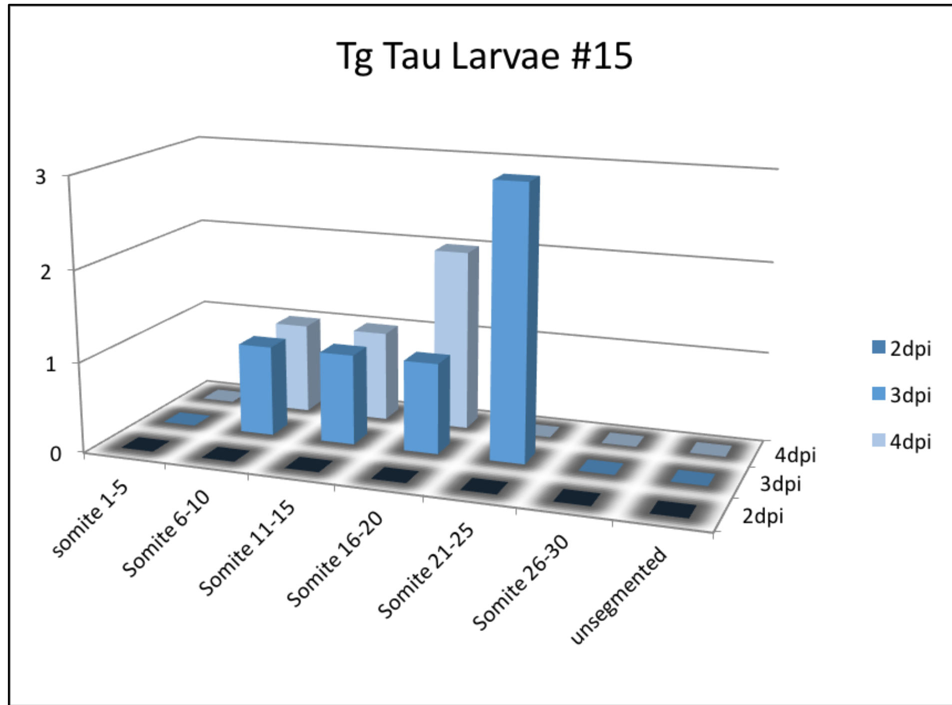


C''

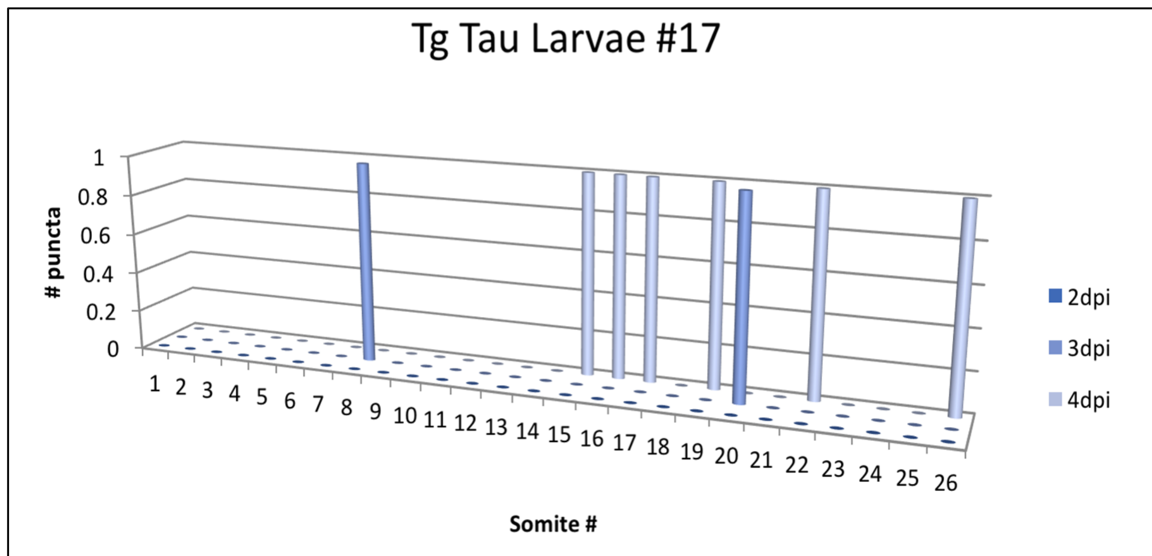


Figure 2. 5*:* **the movement of some of tau puncta overtime after injection of mouse brain homogenate that is laden with human tau pathological aggregates.**

(A) Images of the brain area, after injection with Tg hTau$^{+/p301L}$ mouse brain homogenate, for the same zebrafish larvae over three consecutive days post injection (dpi) showing the movement of one puncta over time. (B) schematic illustrate a somite in zebrafish larvae. (C, C' and C") The location of tau aggregates on the spinal cord of the same individuals over multiple days, using somite numbers as landmarks, denote a movement of some of these puncta over time.

Like other protein misfolding diseases, tauopathies reflect a proteostatic imbalance wherein the clearance of pathological tau species is insufficient relative to accumulation (Lim and Yue, 2015) (Chiti and Dobson, 2006). We reasoned that if the tau biosensor larvae are faithfully reflecting tau proteostasis concepts *in vivo*, then this could be revealed via inhibition of the proteasome. We treated larvae with the proteasome inhibitor MG-132 and observed an approximate expanding of spontaneous GFP+ inclusions (Figure 2.6 D). Following injection of mouse brain homogenate loaded with human tau fibrils, inhibiting the proteasome with MG-132 substantially enhanced the percentage of larvae bearing Tau4R-GFP+ inclusions in the brain (to ~70%, Figure 2.6 E), relative to equivalent larvae without MG-132 (~36%, Figure 2.6 E).

It was striking that the zebrafish tau biosensor was robustly able to discriminate brain homogenates that were loaded with human tau aggregates from those that were not. However, we considered an alternative explanation for the data: the difference may not depend directly on human tau in the brain homogenate, but instead reflect other disease-associated-components of the degenerating Tg mouse brain. To verify that the formation of GFP+ puncta in zebrafish can report tau protein only, we fibrillized and delivered synthetic human tau protein (2N4R). After confirming the synthetic tau proteins were appropriately fibrillized via EM (Figure 2.6A), we delivered them by intraventricular injections as described above. Similar to previous data with brain homogenate, the larvae that were injected with synthetic tau fibrils developed inclusions proximal to the brain ventricles as well as along the spinal cord at 3-6 dpi. The abundance of tau aggregates along the spinal cord was significantly higher in larvae injected with the synthetic tau fibrils compared to larvae injected with tau monomers or to the non-injected group ($p<0.05$) (Figure 2.6 B). The distribution of larvae based on the number of tau aggregates they accumulated also supported these findings (Figure 2.6 C). In sum, the Tau4R biosensor deployed

in the CNS of larval zebrafish was able to report tau species, and further revealed the prion-like induction of tauopathy via protein-only seeding *in vivo*.

Figure 2.6

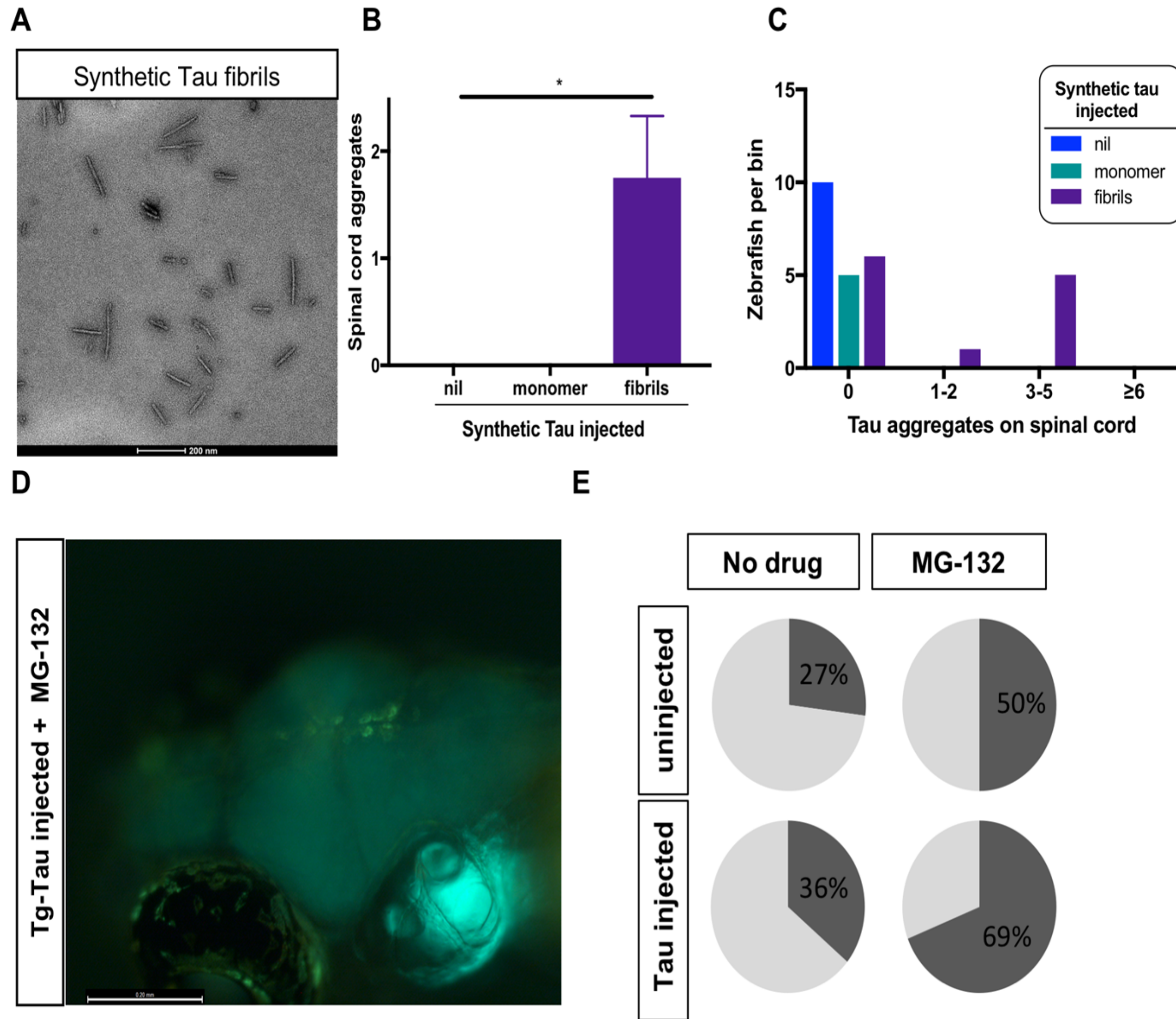


Figure 2. 6: Protein-only induction of puncta in *in vivo* tau biosensor.

Injections of synthetic tau fibrils into Tau4R-GFP zebrafish induced GFP+ puncta in brains and spinal cord. (A) Synthetic tau proteins were fibrilized as confirmed via EM analysis. Tau fibrils were microinjected into the larval hindbrain at two days post-fertilization, and tau inclusions were analyzed at three days post injections. (B) Tau aggregates were only observed after

injection of tau fibrils, not monomers (*$p<0.05$). (C) Tau aggregates were presented as distribution of larvae that displayed various amounts of GFP+ puncta only after injection of tau fibrils. (D, E) Inhibiting the proteosome with MG132 enhanced the percentage of larvae bearing GFP+ inclusions in the brain following injection of tau-laden brain homogenate.

2.4.3 Introduction of a novel traumatic brain injury method for larval zebrafish

We sought to deploy our tau biosensor as a detector of tauopathy following traumatic brain injury. Although a few methods have been reported to induce traumatic brain injury in adult zebrafish (Maheras et al., 2018; McCutcheon et al., 2017), no such methods were available to use on zebrafish larvae. Here we introduce a simple method to induce Pressure wave (PW) induced traumatic brain injury in zebrafish larvae. Measuring tauopathy in larvae offers substantial benefits regarding experimental throughput, economy, accessibility of drug and genetic interventions, as well as bioethics.

We devised a traumatic brain injury paradigm by loading zebrafish larvae (in their typical growth media) into a syringe with a closed stopper, and applying a hit on the plunger to produce a pressure wave through the fish body akin to pressure or shock waves experienced during human blast injury (Nakagawa et al., 2011) (Figure 2.7A). To ensure repeatability, and to permit manipulation of injury intensity, a series of defined masses were dropped on the syringe plunger. To assess if our method faithfully induced traumatic brain injury that mimic injury produced by blast wave, we examined multiple markers known to be associated with blast traumatic brain injury, including cell death, small or large hemorrhage, blood flow abnormalities and axonal injury and tauopathy (Bir et al., 2012; Kovacs et al., 2014; Nakagawa et al., 2011). Additionally, we evaluated the occurrence of post-traumatic seizure activity and the increased in neuronal activity during the trauma.

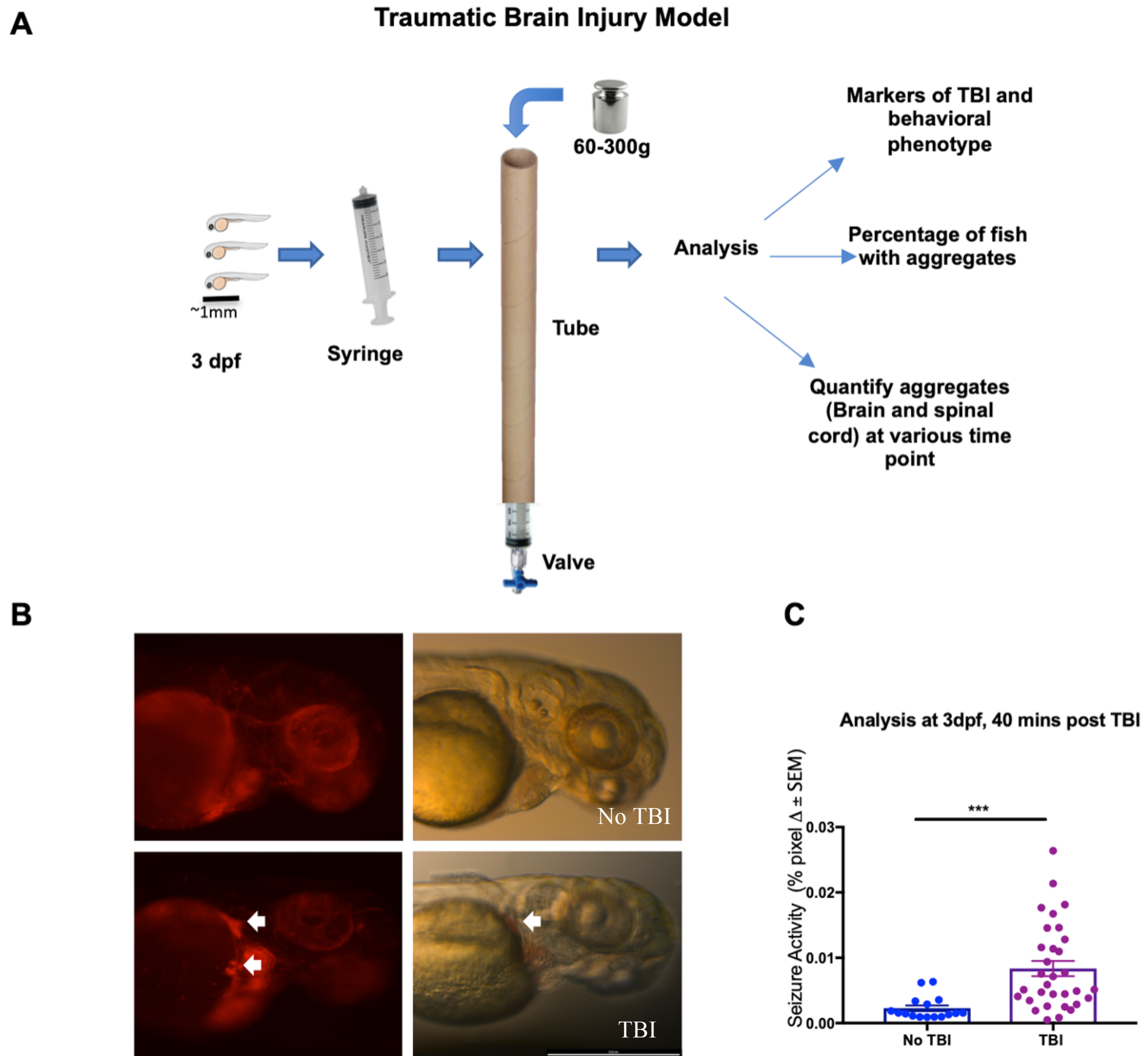
As disruption of neurofilament levels have been used as a marker for axonal damage in multiple neurodegenerative disease, including TBI (Khalil et al., 2018; Siedler et al., 2014), we determined the impact of TBI on neurofilaments distribution in zebrafish larvae via immunostaining using monoclonal NF160 antibody that can detect neurofilaments in zebrafish.

Interestingly, TBI caused a loss in the staining of neurofilaments and disruption in distributions, which are in agreement with immunostaining of neurofilaments in mouse models of TBI (Caner et al., 2004; Huh et al., 2002)(Figure 2.8).

We then evaluated hemorrhage via the use of *Tg[gata1a:DsRed]* larvae that express red fluorescence in blood cells (Traver et al., 2003). Hemorrhage was observed in some larvae when a heavy weight (300g) was used to induce the traumatic brain injury (Figure 2.7B). Additionally, approximately half of the TBI larvae showed abnormalities in blood flow including a temporary reduction or complete absence of blood flow and circulations (video of control 1, https://drive.google.com/file/d/1hANQ6XEjQn9Nami6ttc7UtryHTbJRKO4/view?usp=sharing)(video of TBI 2, https://drive.google.com/file/d/1x0m12ZX_nzwacLn_0UXs4_by2gt5_PSN/view?usp=sharing) in line with abnormalities detected in other TBI models (Bir et al., 2012).

Subsequently, we assessed apoptosis in the TBI larvae, observing that our TBI method induced cell death in larvae as detected by staining for active-Caspase-3 (known also as Cleaved-Caspase-3) (Figure 2.7D). The number of activated-Caspase-3-positive cells was negligible in the control groups compared to a mean of 61.6 cells in TBI larvae (SEM ± 9.17, n=3) and 75 (SEM ± 4, n=2) in positive-control-larvae treated with CPT. These data all align well with existing animal models of TBI with respect to mimicking patient symptoms and support the effectiveness of our method in inducing traumatic injury in larval zebrafish.

Figure 2.7



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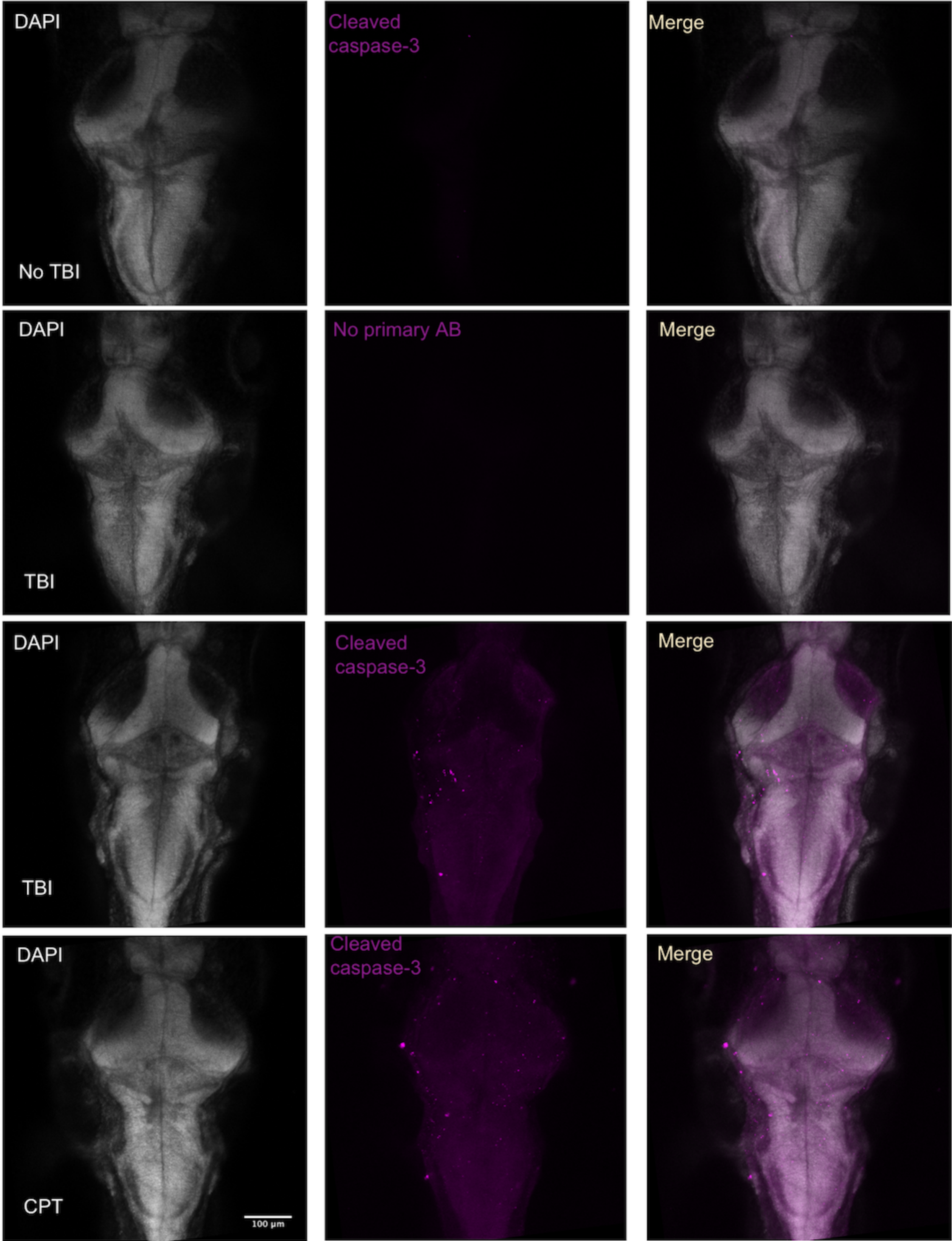


Figure 2. 7: Larvae subjected to traumatic brain injury (TBI) exhibited various markers associated with traumatic blast injury.

(A) Our TBI model for larval zebrafish. To induce blast injury, zebrafish larvae are loaded into a syringe with a stopper. A defined weight is dropped on the syringe plunger from a defined height, producing a pressure wave through the fish body akin to pressure waves experienced during human blast injury. (B) Hemorrhage after TBI was observed in some of the larvae fish using *Tg[gata1a:DsRed]* transgenic zebrafish that express DsRed in erythrocytes as indicated by white arrows. (C) Larvae subjected to the traumatic brain injury display a seizure-like movement that is detected using a tracking software system and significantly higher than the control group (***$p<0.001$). (D) Increased cell death in the brain of larvae subjected to TBI as indicated by immunostaining of activated Caspase-3 (magenta). 4dpf larvae exposed to topoisomerase inhibitor camptothecin (CPT, 3uM) which induce apoptosis, serves as a positive control. Nuclei were stained with DAPI in gray for reference.

Figure 2.8

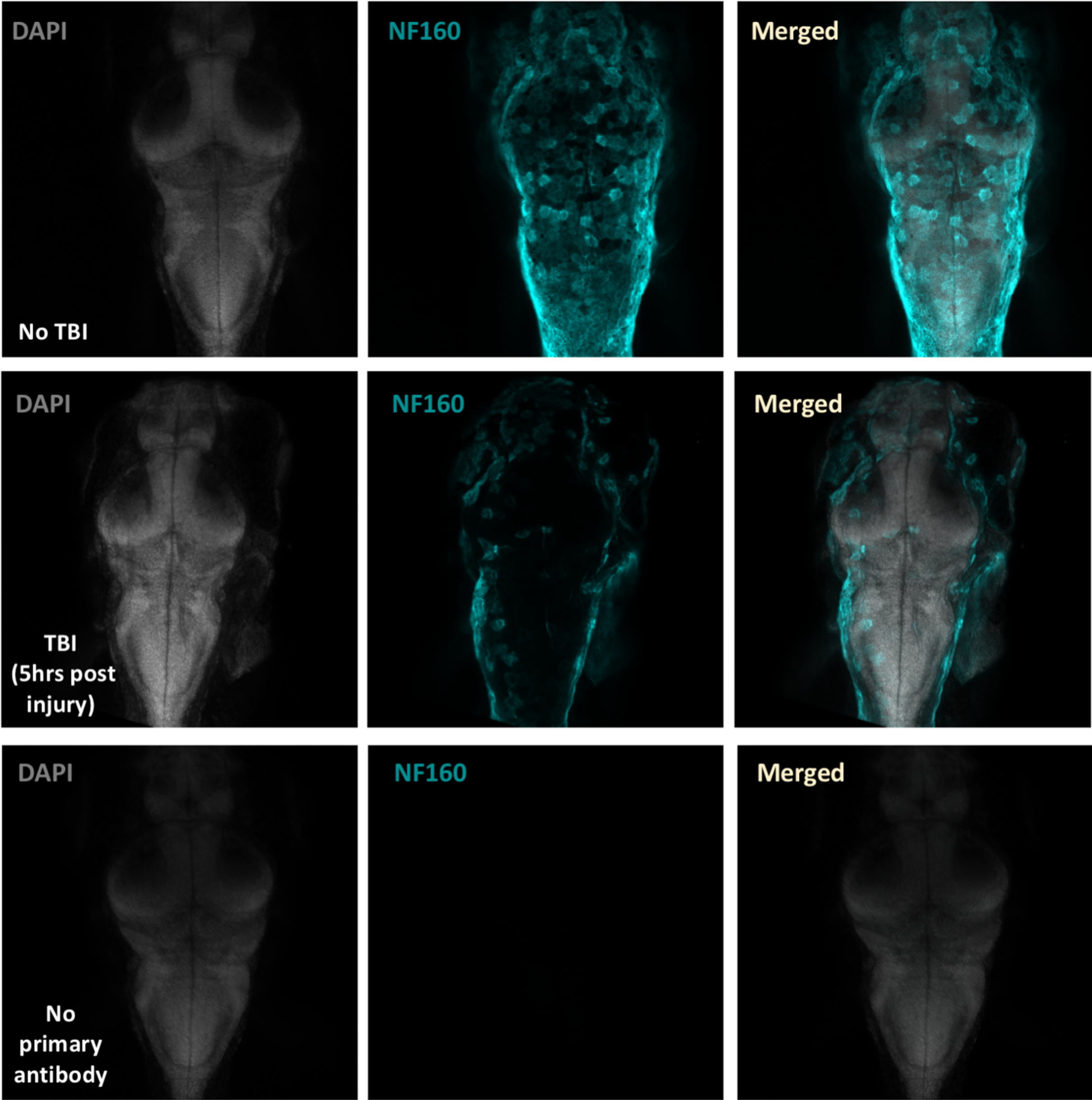


Figure 2. 8 : Disruption of neurofilaments distribution after traumatic brain injury.

Traumatic brain injury caused a loss of neurofilaments labeled neurons and disruption in their distribution after 5 hours of TBI compared to control larvae that was not subjected to TBI. TBI larvae with no primary antibody added was used as a negative control. Nuclei were stained with DAPI in gray for reference.

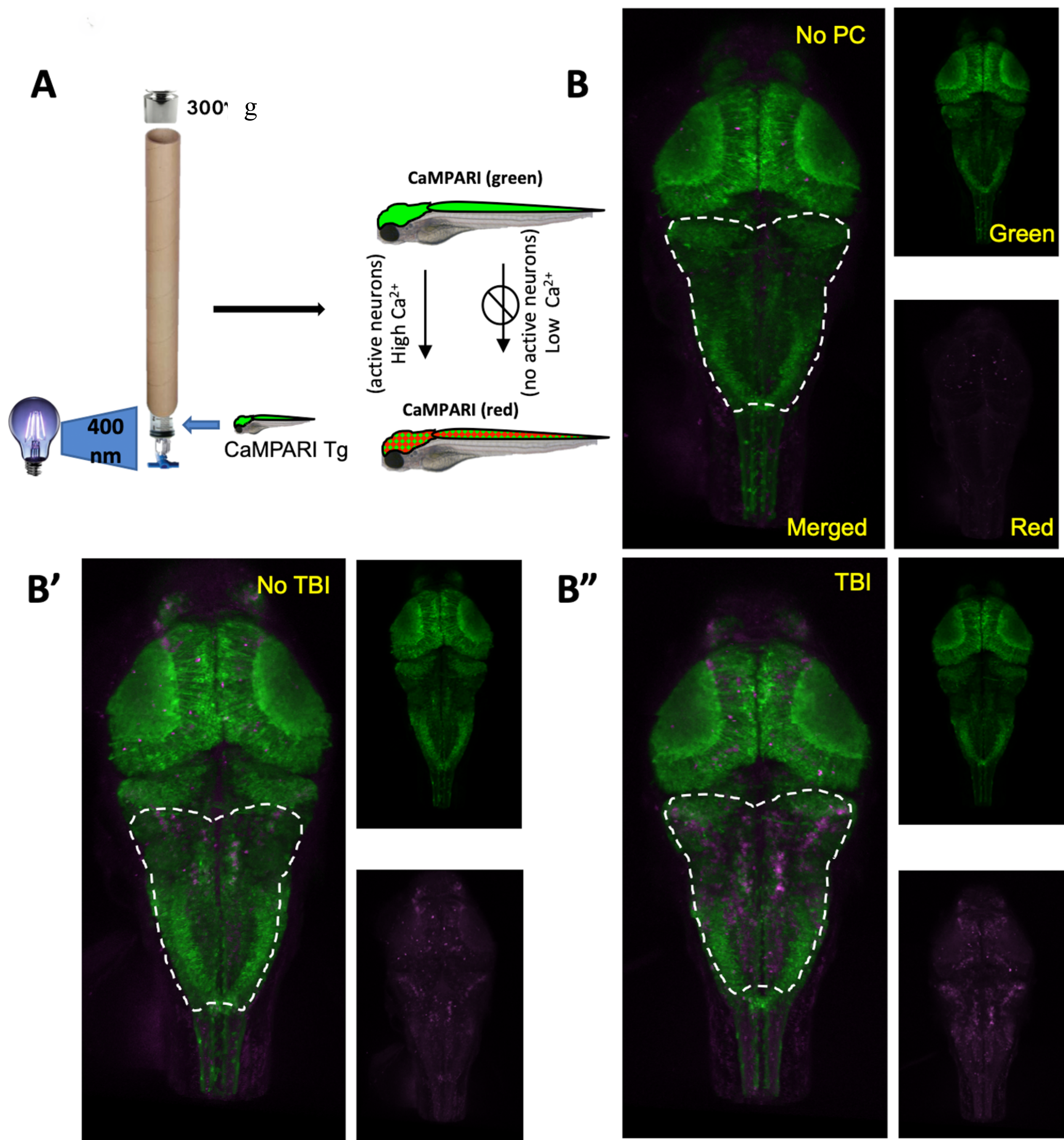
2.4.4 TBI treated larvae exhibited post-traumatic seizure-like behavior and increased neuronal activity during Trauma

Post-traumatic seizures are one of the most frequent conditions associated with traumatic brain injuries and, despite being prevalent, remain poorly understood in TBI patients (Kovacs et al., 2014). Post-traumatic seizures were apparent in a subset (approximately 40%) of zebrafish larvae after they were subjected to TBI. We quantified seizure activity via behavioral tracking software (which we had previously optimized for quantifying seizures in larvae zebrafish (Leighton et al., 2018)) and determined that larvae subjected to the traumatic brain injuries exhibited seizure-like activity that was significantly higher than the control group ($p<0.0007$) (Figure 2.7 C) (TBI Larva with reduced blood flow and seizure, https://drive.google.com/file/d/1hZMuZ-4DrWL_V0GgSTgSzyH_rtT6dejJ/view?usp=sharing).

Seizures are caused by abnormal and excessive neuronal excitability (Stafstrom and Carmant, 2015). To evaluate whether the occurrence of the seizure-like symptoms in our TBI model was caused by a sudden increase in the neuronal activity during the brain trauma, we utilized CaMPARI optogenetic measures. CaMPARI fluoresces green in baseline conditions, and permanently converts to red fluorescent emission if exposed to high calcium levels (i.e. neural activity) coincident with user-defined application of bright 405nm light. We subjected our *Tg[elavl3:CaMPARI]^{ua3144}* larvae to TBI, coincident with brief application of photoconverting light as described in (Figure 2.9 A). An increase in neuronal activity during TBI was evident, especially in the hindbrain region as indicated by enhanced red emission (Figure 2.9 B). CaMPARI allows robust quantification of neural activity expressed as a ratio of red:green fluorescent emission, and confirmed that neuronal excitability increases significantly in response to brain trauma (Figure 2.9 C and D). Notably, this combination of newly introduced methods of

traumatic brain injury being integrated with CaMPARI optogenetic methods offers the rare ability to assess neural activity on un-restrained (free-swimming) subjects during TBI. In sum, our data reveal a substantial burst of neural activity occurs during TBI, and that zebrafish larvae exposed to TBI exhibit a subsequent propensity for spontaneous seizures.

Figure 2.9



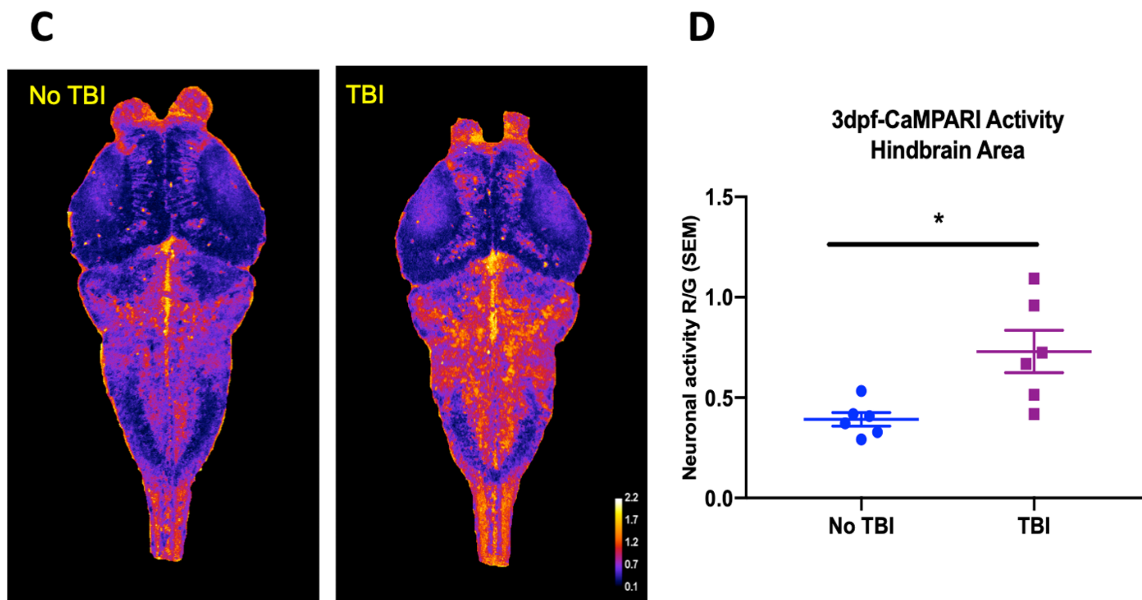


Figure 2. 9: Traumatic brain injury induces neural activity in CaMPARI zebrafish larvae.

(A) Schematic of TBI using CaMPARI (Calcium Modulated Photoactivatable Ratiometric Integrator) to detect neuronal excitability. 3dpf CaMPARI larvae were freely swimming while subjected to TBI, coincident with exposure to 405nm photoconversion light. CaMPARI fluorescence permanently photoconverts from green to red only during neuronal activity (high [Ca2+]), and the ratio of red:green emission is stable and quantifiable via subsequent microscopy. (B) Increased neural activity during TBI is represented by increased red:green emission (red pseudocoloured to magenta) in the hindbrain of larvae (B"), compared to fish not receiving TBI (B') or fish not exposed to photoconverting light ("no PC" in panel B). These representative maximum intensity projection images show dorsal view of zebrafish (anterior at top), including merged, or red or green channels alone. (C) Heatmaps encode the CaMPARI signal (higher neural activity = higher red:green = brighter colours), highlighting location of

increased neural activity during TBI relative to control larvae not receiving TBI. (D) CaMPARI output quantification in the hindbrain area reveals a significant increase in the neuronal excitability during TBI compared to control group not receiving TBI (* $p<0.05$).

2.4.5 Traumatic brain injury on Tau biosensor Tg zebrafish larvae inducing GFP+ puncta

After validating that our TBI method was able to induce traumatic brain injury upon zebrafish larvae, we asked whether TBI can induce tau aggregates in our tau biosensor model. Initially, we evaluated if our TBI method would induce aggregation of fluorescent proteins in models expressing GFP alone or other biosensor proteins such as SOD1:GFP, as a negative control. In traumatic brain injury induced with both light and heavy weights, no GFP+ aggregates were detected in these controls (Figure 2.10 A-C). Similar results were obtained with pt-40 transgenic zebrafish that express GFP around the larva's body (Figure 2.10 D) and other transgenic zebrafish express GFP in motor neurons (data not shown). Further, our Tau4R-GFP fish robustly express a typical GFP variant in the active heart muscle, and this GFP showed no sign of aggregation following TBI. Remarkably, in these same individuals we detected Tau4R-GFP biosensor GFP+ puncta in both brains and spinal cords following TBI (Figure 2.11 A-B). To determine if the severity of tauopathy varies coordinately with severity of the traumatic brain injury, we assessed the impact of varying masses. Although some variability is apparent, a dose-response relationship is evident, such that the 65g, 100g and 300g weights induced more tau aggregates in our tau biosensor compared to the control and 30g weight (Figure 2.12 E). The heaviest weight (300g) induced significantly more tau aggregates versus the control group or the group with the 30g weight ($p<0.01$ and $p<0.001$, respectively). Therefore, we decided to use both the 300 and 65 g weight for the next experiments. We evaluated whether dropping the light weight once or multiple times would affect the number of tau aggregates on the spinal cord as well as dropping the weight once on three consecutive days, perhaps reminiscent of repeated sports injury. We observed a rise in the number tau aggregates when the weight was dropped

multiple times for one day or over three consecutive days, but this increase was not statistically significant (Figure 2.12 B-D).

The GFP+ tau aggregates formed in the brain region following TBI tend to form fused shape similar to spontaneous aggregates (Figure 2.12 A). The aggregates on the spinal cord, however, had similar shape to aggregates detected post brain-injections, but with less brightness in some instances. Multi-day monitoring of individual larvae (Figure 2.13 and 2.14) revealed variation in forming tau aggregates among TBI larvae. We observed tau aggregate abundance over time following traumatic brain injury and found that the average tauopathy significantly increased compared to the control group ($p<0.05$ at 3 dpti and $p<0.01$ at 4 dpti (days post traumatic) (Figure 2.11 C- D). Analysis of distribution of larvae binned into the number of Tau4R-GFP+ puncta at 3 dpti showed that more larvae developed Tau4R-GFP+ puncta compared to the control group (Figure 2.11 E). Considering that many of the larvae subjected to TBI formed Tau4R-GFP+ puncta in the brain that had a fused pattern (Figure 2.12 A), we focused on tau aggregates that formed on the spinal cord as their abundance could be most efficiently quantified compared to aggregates that formed in the brain.

As some of the TBI larvae exhibited seizure-like movements, while some did not seem to move abnormally following TBI, we sought to establish if the post-traumatic seizure activity impacted taupathy abundance in our model. Thus, we sorted the larvae subjected to TBI into groups containing larvae exhibiting the seizure-like behavior and those that displayed no movements. Larvae exhibiting seizure-like behavior developed abundant numbers of spinal cord aggregates in comparison to the other groups (Figure 2.11 F).

Figure 2.10

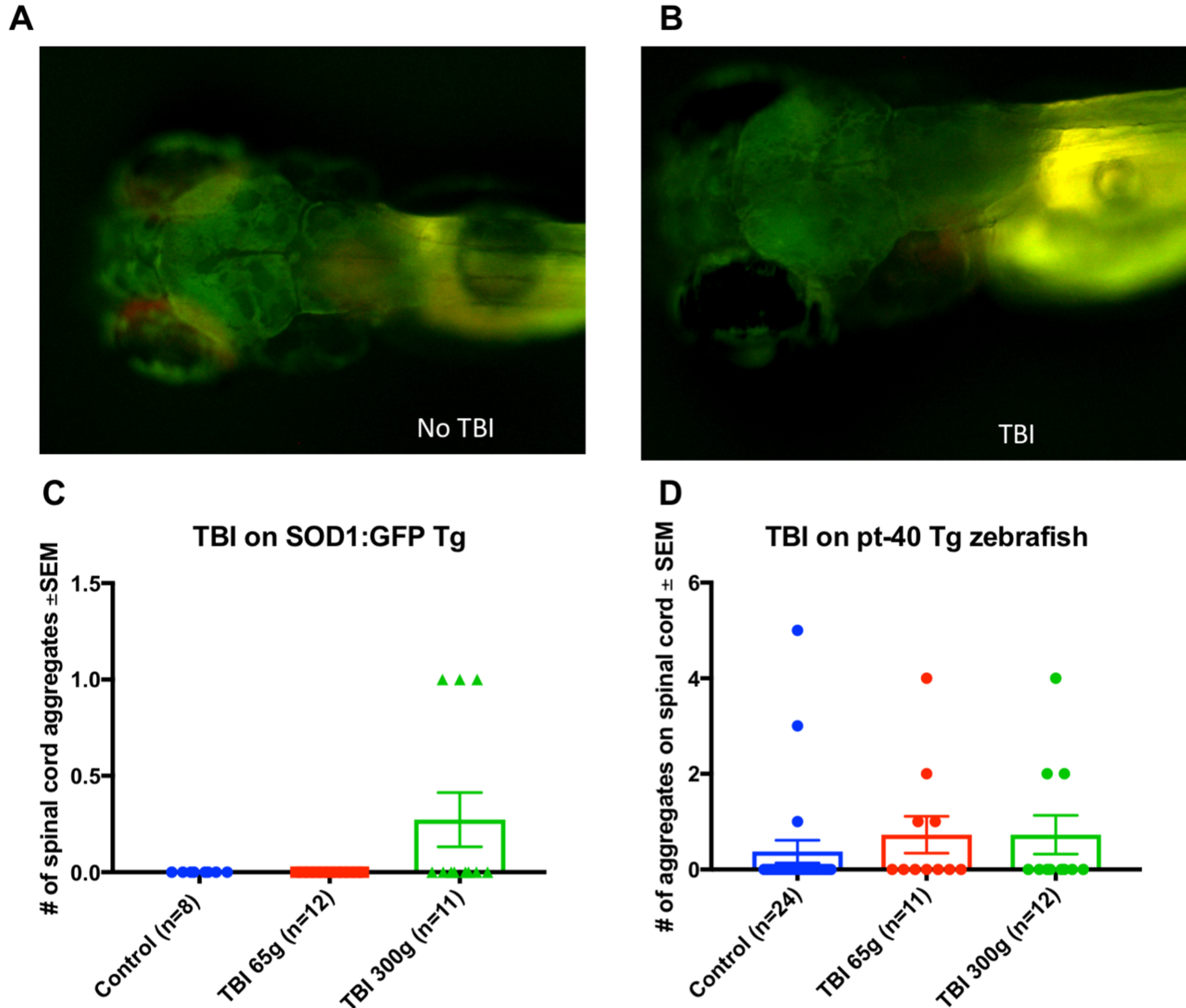
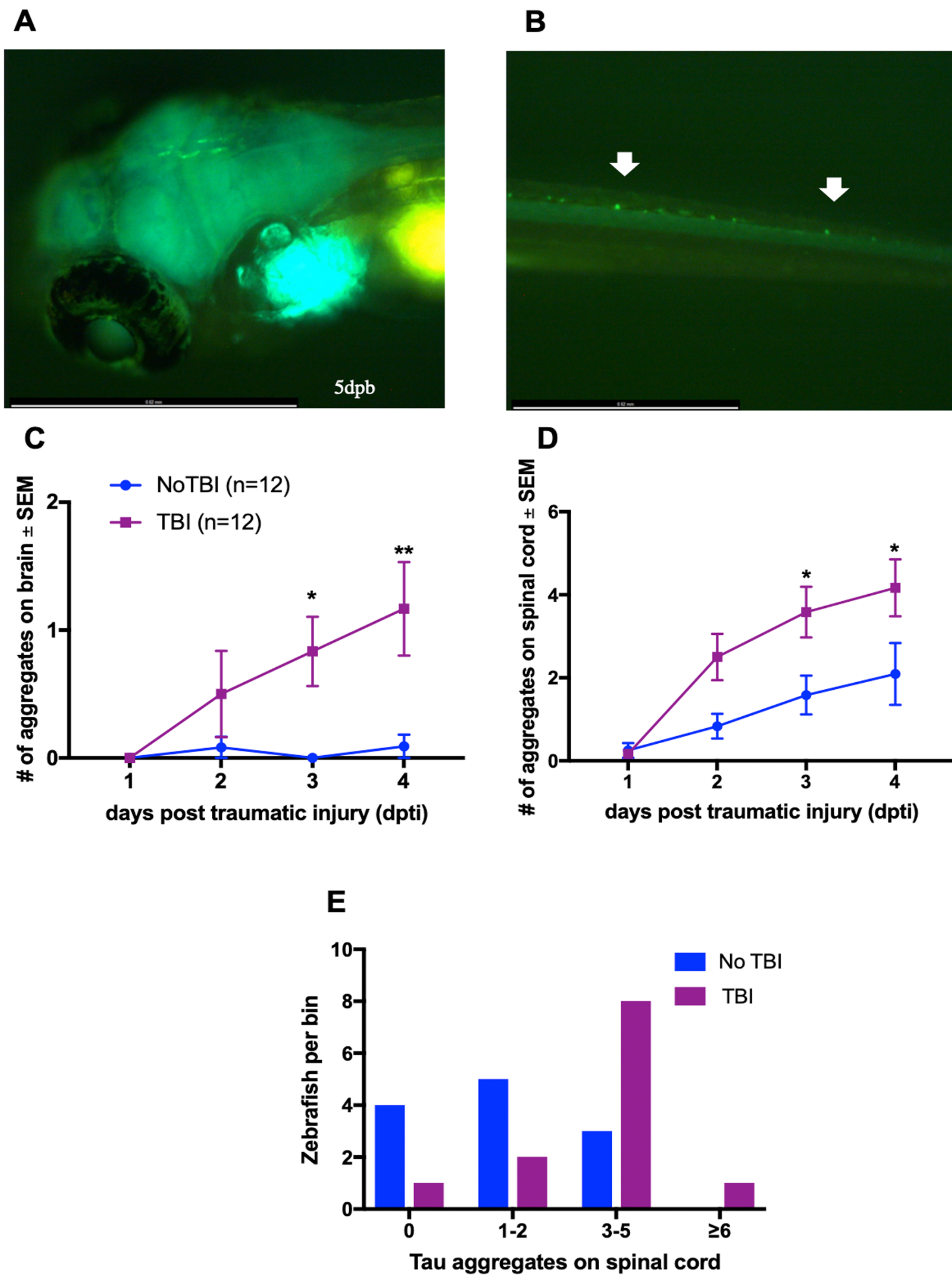


Figure 2. 10: Traumatic brain injury did not induce GFP+ puncta in transgenic larvae zebrafish expressing SOD1:GFP or wildtype GFP. (A and B) SOD1:GFP larvae did not develop any GFP+ aggregates after TBI similar to control larvae (no TBI). Quantification of GFP+ puncta in the spinal cord of SOD1:GFP and Pt-40 (express GFP) larvae shows majority of samples did not develop aggregates post traumatic brain injury.

Figure 2.11



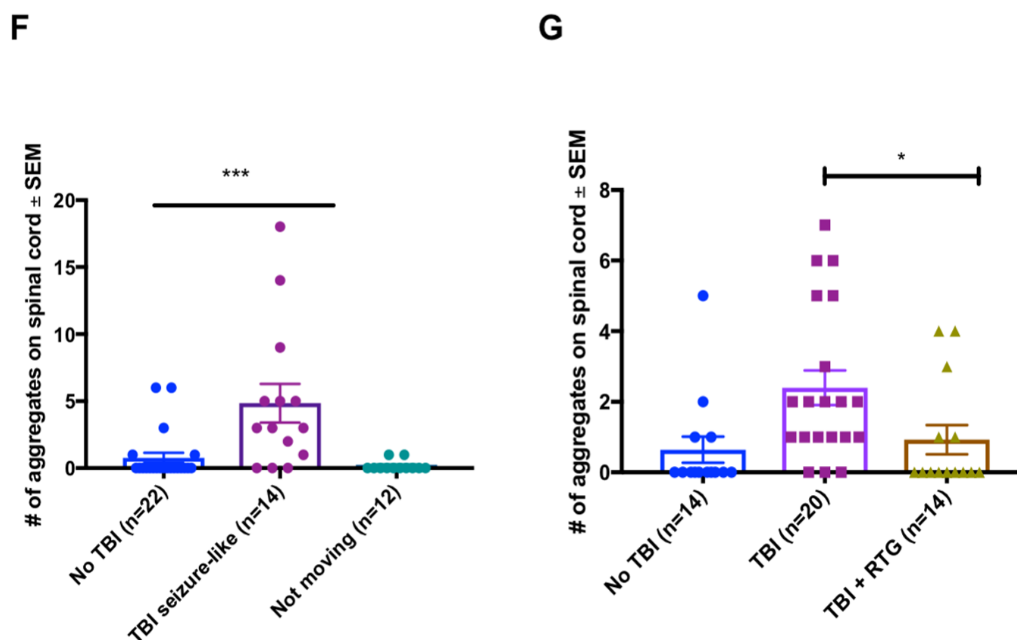


Figure 2. 11: Traumatic brain injury induces tauopathy in larval zebrafish.

(A) Tau4R-GFP biosensor GFP+ puncta are detected in the brain of tau biosensor zebrafish at 5 days post traumatic brain injury (dpti). (B) Tau aggregates formed on the spinal cord as a result of TBI as shown by arrows. (C) Tauopathy significantly increases over time following brain traumatic injury compared to control group (No TBI) (*p<0.05 at 3dpti and **p<0.01 at 4dpti). (D) Quantification of the number of tau aggregates in spinal cord area shows a significant increase over time post brain blast injury compared to control group (*p<0.05 at 3dpti and 4dpti). (E) Analysis of the number of larvae shows that more larvae would develop GFP+ puncta compared to control group. (F) Following TBI larvae displaying seizures tended to develop many more tau aggregates (***p<0.001). (G) Inhibiting post-traumatic seizure with anti-convulsant RTG (10μM) significantly decreased the abundance of GFP+ puncta in the spinal cord (p<0.05).

Figure 2.12

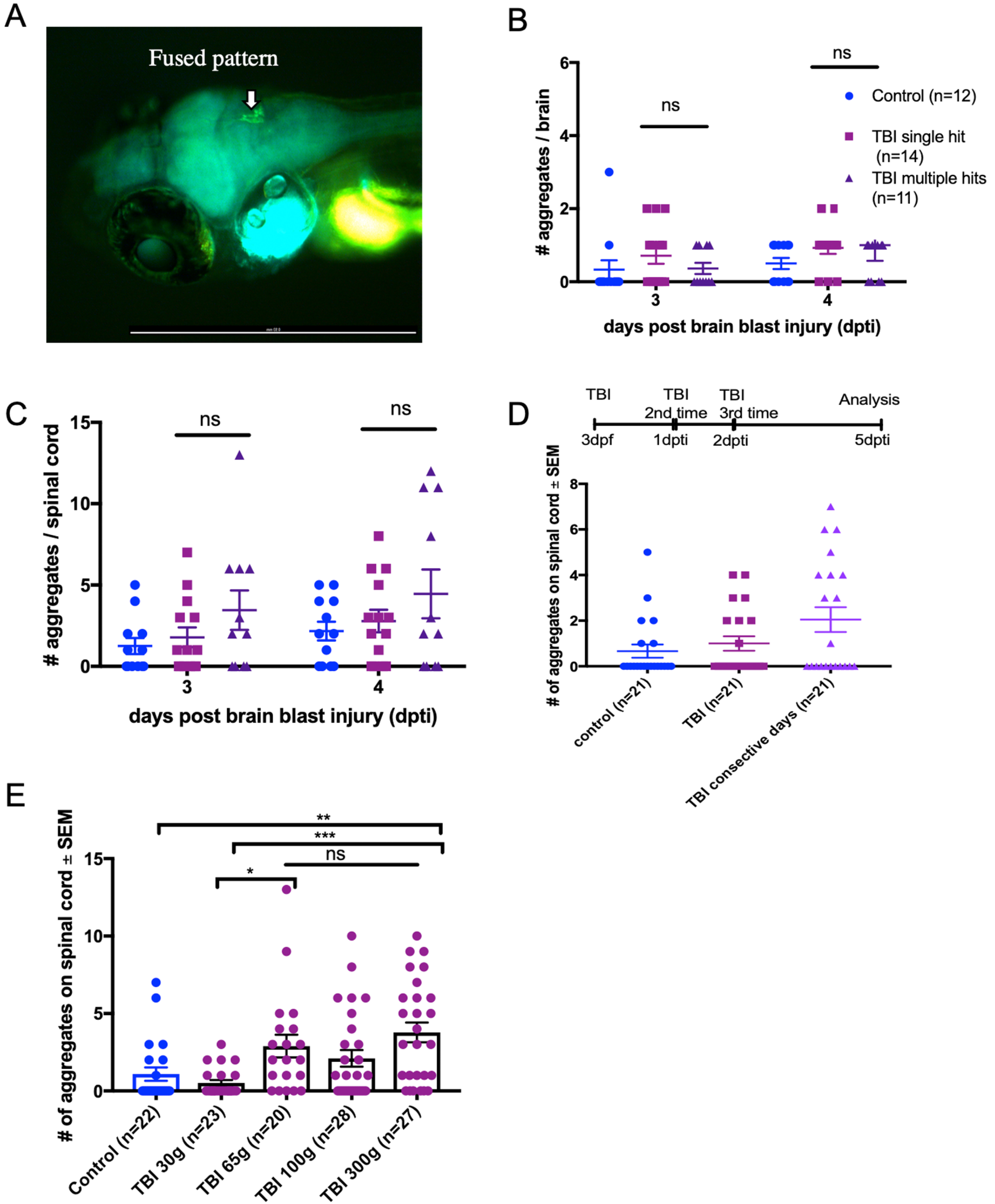


Figure 2. 12: Consistent but modest insignificant increase in tau puncta is observed with an increasing number of successive brain injuries.

Larvae were subjected for either single hit or 5 hits. The number of aggregates both in the brain and the spinal cord were quantified at 3 and 4 days post traumatic brain injury. (A) represent a mean of the number of aggregates in the brain for both conditions while (B) shows the number of the aggregates on the spinal cord as mean. (C) Analysis of the number of fish developing certain number of aggregates indicate that there is an increase in the number of fish developing more than 5 puncta after multiple hits compared to single hit group or control. Statistical analysis shows no significance difference in both conditions. (D) performing TBI over three consecutive days slightly increased the number of tau puncta but the increase was not statistically significant. (E) Analysis of the impact of using varying masses in the TBI method on the formation of tau aggregate shows that 65g, 100g and 300g weights induced more tau aggregates in our tau biosensor compared to the control and 30g weight. Statistical analysis shows significance when the heaviest weight (300g) used compared the control group was not subjected to TBI or the group with the 30g weight ($p<0.01$ and $p<0.001$, respectively).

Figure 2.13

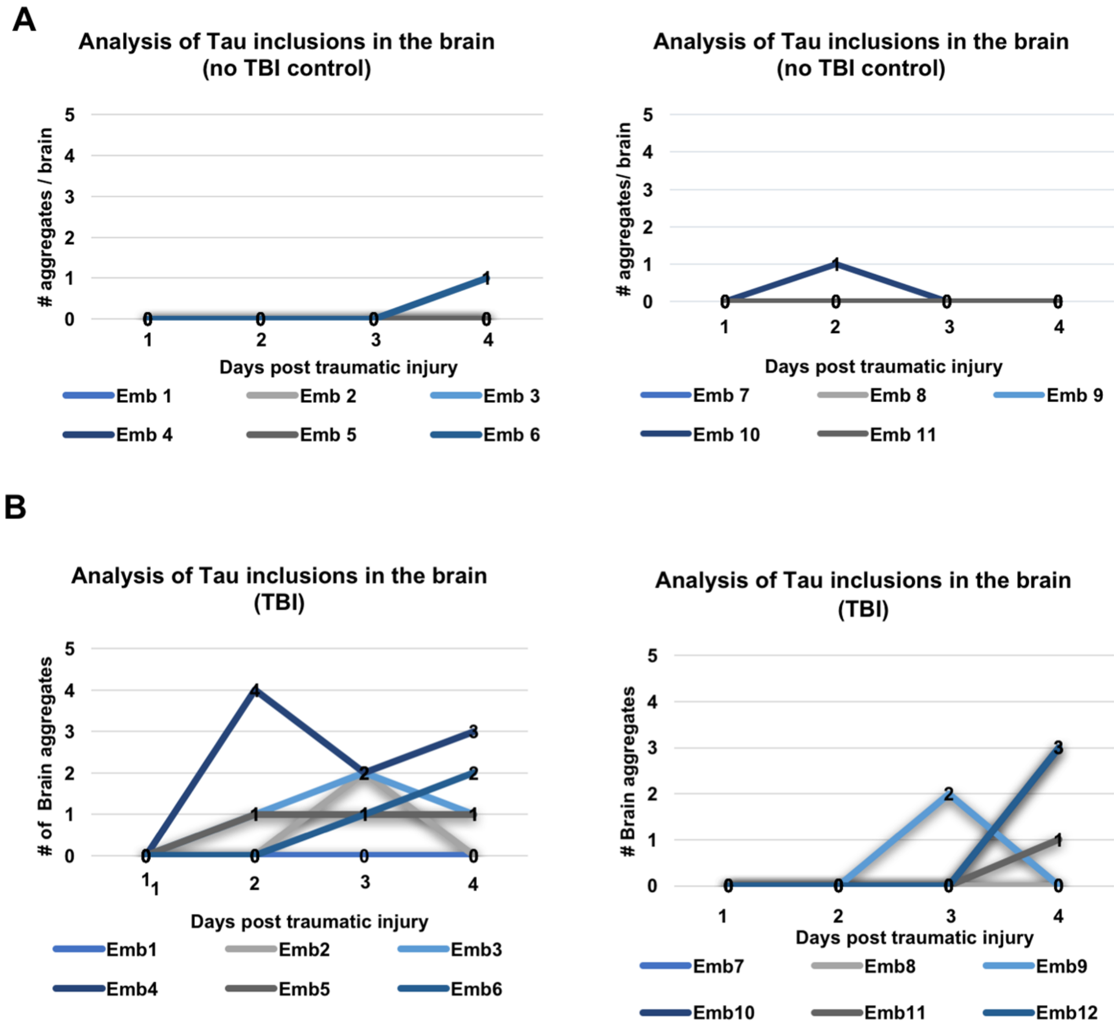
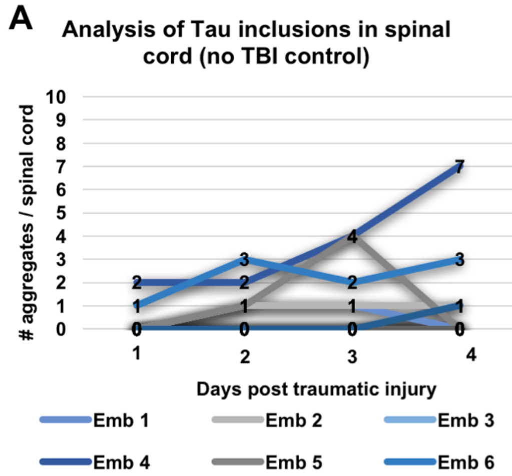


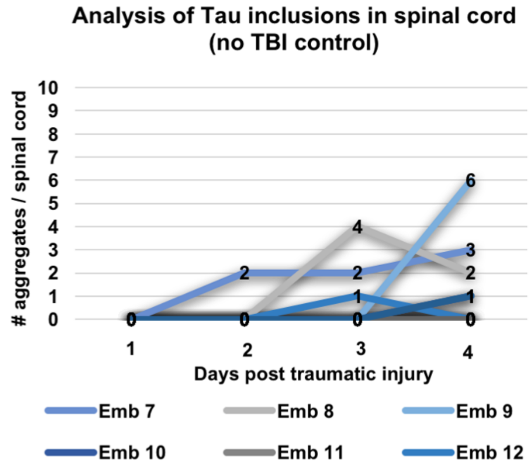
Figure 2. 13: Longitudinal analysis of individual fish after TBI shows various patterns of tau inclusion formation and clearance in their brains.

(A) In the control group (no TBI) most larvae did not develop aggregates, while few either developed aggregates at later time point (3dpi), or developed aggregates that disappear later. (B) larvae subjected to TBI blast injury either developed aggregates at earlier time points and the number of this aggregates would increase gradually, remain the same or display a pattern similar to the control in which the aggregates number decrease at the next time point.

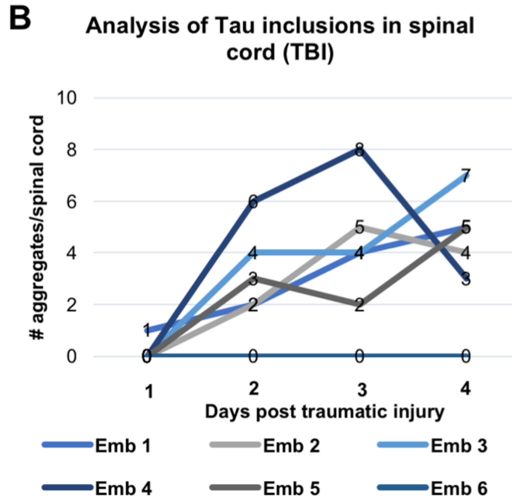
Figure 2.14

A





B



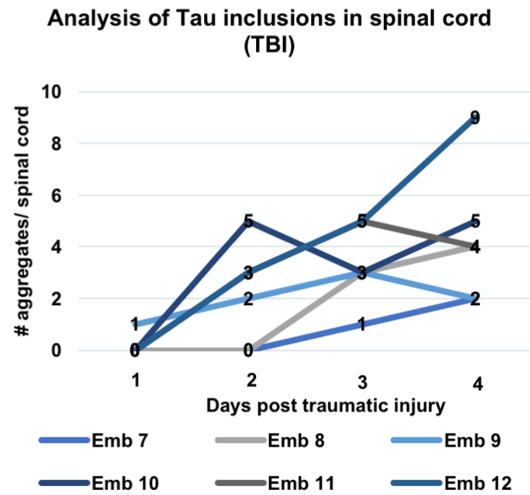


Figure 2. 14: Longitudinal analysis of individual fish after TBI shows various patterns of tau inclusion formation and clearance in their spinal cords.

(A) Representative of the control group (no TBI) wherein some of the larvae did not develop aggregates, while few either developed aggregates at later time point (3dpi), or developed aggregates that disappear later. (B) shows the analysis of larvae subjected to blast injury in which the majority in contrast to the control groups developed aggregates at earlier time points and the number of this aggregates would increase gradually, remain the same or display a pattern similar to the control in which the aggregates number decrease at the next time point but then increase again.

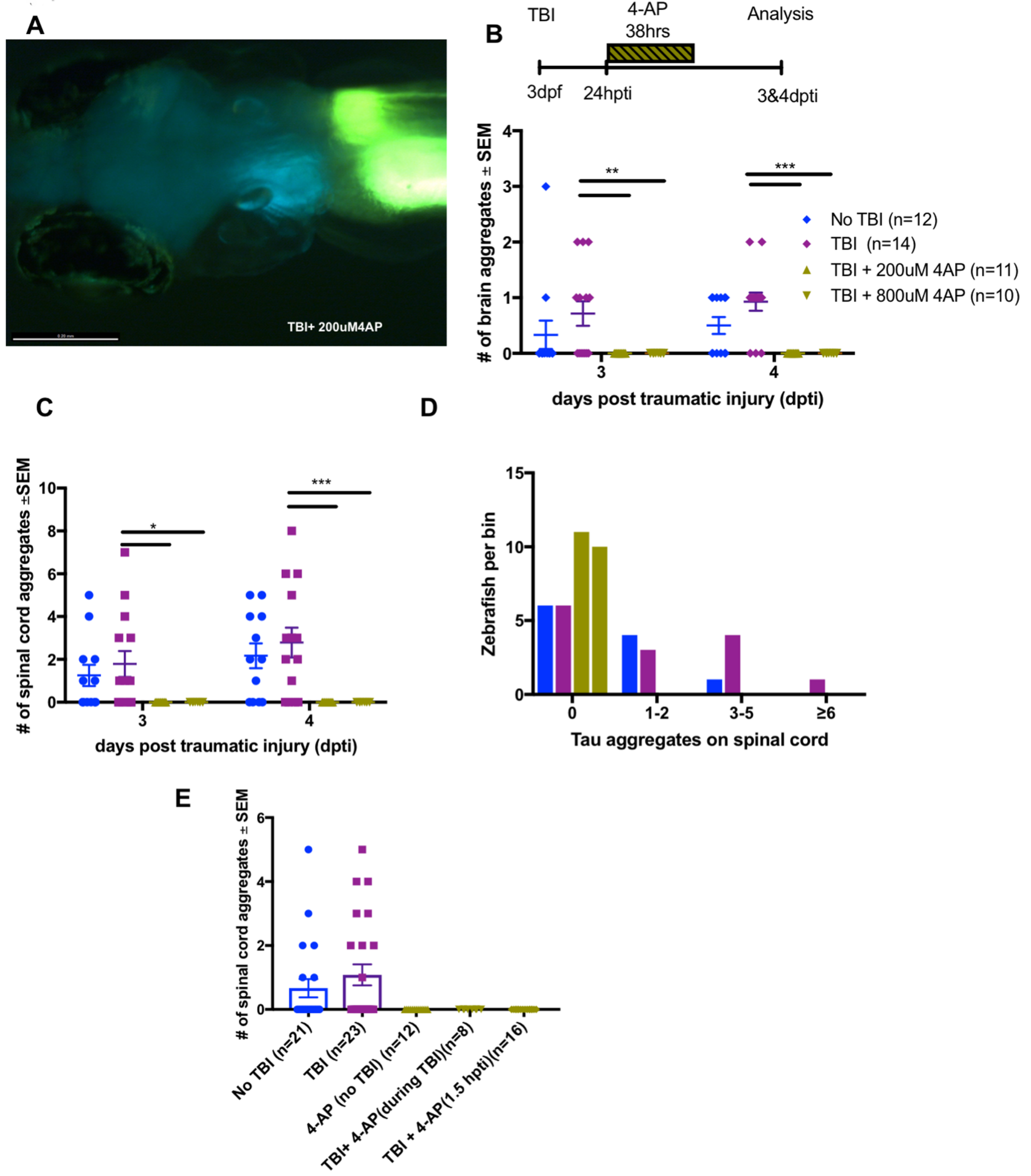
2.4.6 Retigabine and 4-aminopyridine inhibited Tauopathy in a TBI model

We were intrigued that both post-traumatic seizures and tau pathology are prevalent in some TBI patients, and that a causal link between disrupted neural activity and protein misfolding can be demonstrated in at least some neurodegenerative diseases (Kovacs et al., 2014; Sanchez et al., 2018). To disentangle the role of seizure activity in terms of the formation of tau aggregates in our TBI models, we employed convulsant and anti-convulsant drugs to modulate *in vivo* neural activity. Each of these drugs is well-established to behave similarly in zebrafish as in mammals, though it is perhaps notable that our multi-day application here is longer than the acute applications typically considered (Ellis et al., 2012). Based on previous findings regarding the observed increase in tauopathy abundance in TBI larvae that exhibited seizure-like behavior, we hypothesized that decreasing this seizure-like activity will reduce tauopathy. Thus, we applied the anti-convulsant drug Retigabine (RTG), a neuronal potassium channel (KCNQ) opener, to test the impact of decreasing seizures on tau pathology. As expected, we observed a significant decrease in the abundance of tau ($p<0.05$) with many TBI larvae not developing any Tau4R-GFP aggregates (Figure 2.11 G). To increase seizure activity after TBI, we applied 4-aminopyridine (4-AP), a K_v channel blocker and convulsant drug. We predicted that raising the level of seizure activity would elevate tauopathy abundance in our TBI model. We found low doses of 4-AP sometimes met our prediction of increasing the number of aggregates, and though this result was variable between trials it encouraged us to explore further. Surprisingly, higher doses of 4-AP consistently inhibited formation of tau aggregates. Treating TBI larvae with 200 or 800µM of 4-AP for prolonged period (38 hours, beginning 24 hours post TBI) significantly inhibited the abundance of Tau4R-GFP+ puncta in the TBI group (Figure 2.15 A-C). Analysis of the distribution of larvae linked to the number of tau aggregates supported this finding with no

zebrafish larvae developing aggregates in groups treated with 4-AP (Figure 2.15 D). It is worth noting that 4-AP is commonly used on zebrafish but rarely used for prolonged treatment. These results were different from our initial prediction as we were expecting to see more aggregates owing to increased seizure-activity evoked by 4-AP. To evaluate if the time at which treatments are administered plays a role in this unexpected result, we treated larvae with 200 µM 4-AP at earlier time points, specifically during traumatic brain injury and 1.5 hours later. We kept the duration of 4-AP treatment the same as previous experiments (38 hours). We found that administering 4-AP during different time windows relative to the traumatic brain injury did not measurably alter the inhibitory action of 4-AP on the abundance of tau aggregates (Figure 2.15 E). A similar observation was made when the duration of the 4-AP treatment was reduced to 24 hours (Figure 2.15 F).

Next, we considered if this unexpected inhibition of tauopathy by high-dose 4-AP convulsant is a direct consequence of increased neural activity (e.g. perhaps via neural exhaustion). We found that larvae receiving TBI and 4-AP continued to exhibit a lack of tau aggregates when co-treated with anti-convulsant retigabine ($p<0.0001$) (Figure 2.15 G). This suggested that high doses of 4-AP block the formation of tau aggregates via a mechanism independent of its convulsant activity.

Figure 2.15



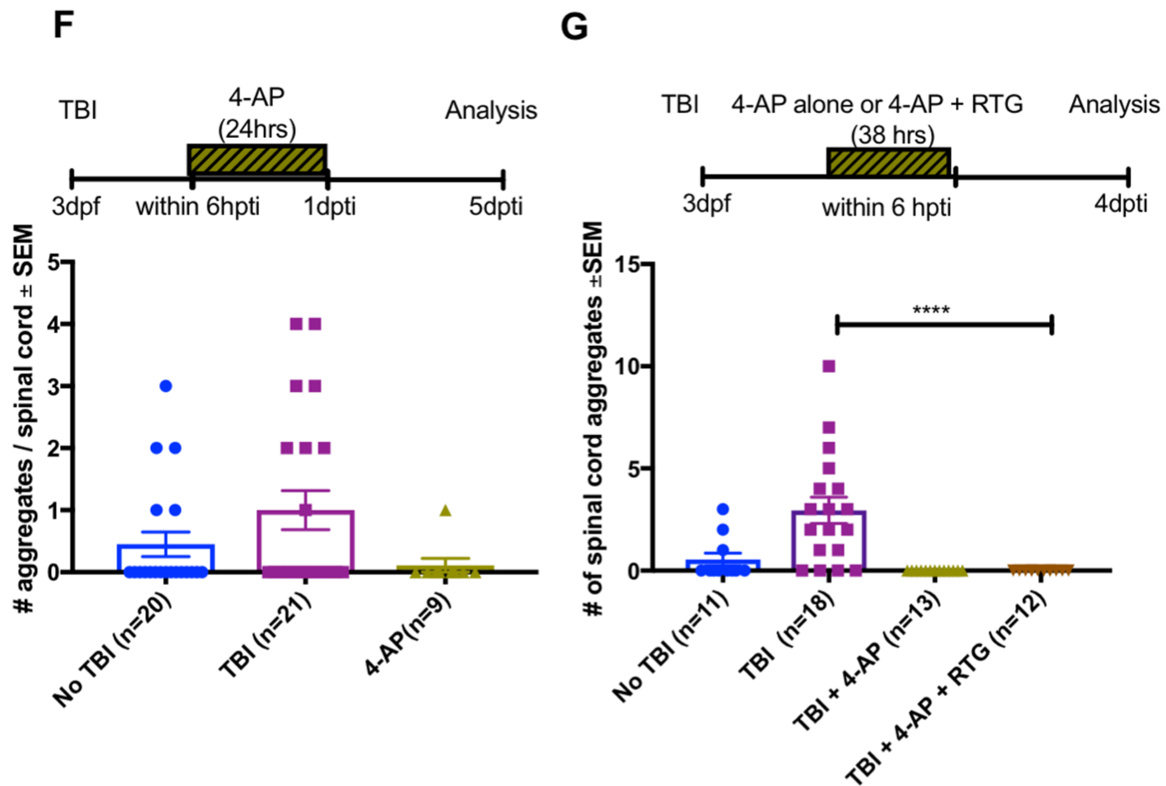


Figure 2. 15: Increase seizure activity using potent convulsant drugs (4-AP) significantly decreased the formation of tau aggregates.

A) Larvae subjected to TBI and treated with 4-aminopyridine (4-AP) show no brain puncta. 4-AP was added to the TBI larvae one day post traumatic brain injury (dpti) and left for 38 hours as displayed in timeline in (B). (B,C) 4-AP significantly reduced the abundance of GFP+ puncta in the brain and spinal cord compared to untreated TBI control (**$p<0.009$ at 3dpti and ***$p<0.0003$ at 4dpti). (D) All larvae treated with 4-AP did not develop any GFP+ puncta (# of aggregate is 0), displayed as distribution of larvae binned into the number of GFP+ puncta they exhibited. (E) Adding drugs at different times post the traumatic brain injury had no effect on the inhibitory action of Tau aggregates evoked by 4-AP. (F) Reducing 4-AP treatments to 24 hours have no impact on the inhibitory effect of 4AP on tauopathy.

2.4.7 Inhibition of dynamin-dependent endocytosis minimized Tau seeding following traumatic brain injury

To resolve a mechanism whereby high doses of 4-AP reduced tau pathology, contrary to our predictions above regarding neural hyperactivity, we considered previous *in vitro* work that demonstrated high concentrations of 4-AP cause reduced endocytosis of synaptic vesicles (Cousin and Robinson, 2000). To examine if the inhibitory actions of 4-AP on the abundance of tau aggregates in our TBI model is caused by inhibition of endocytosis specifically, we treated our tau biosensor larvae post TBI with Pyrimidyn-7 (P7), a dynamin inhibitor that is known to block endocytosis (McGeachie et al., 2013), and analyzed the propagation of tau pathology by quantifying the number of tau inclusions. Owing to the potency of P7 and its impact on the survival of larvae, we treated the larvae with it for 24 hours at just 3 μM. Similar to the findings with 4-AP, P7 treatments significantly inhibited the formation of Tau4R-GFP+ puncta in TBI larvae ($p<0.001$) (Figure 2.16 A). We assessed further the role of endocytosis by employing another dynamin- inhibitor drug, Dango4a, that is less potent than p7 (McCluskey et al., 2013). We obtained similar results in which Dango-4a treatments significantly reduced tauopathy in our TBI model (Figure 2.16 B). To determine if these results are applicable to human tau, we induced traumatic brain injury on larvae expressing both human tau (2N4R) and our tau biosensor reporter and then treated both with either 4-AP or P7. With the exception of the untreated control, both groups treated with 4-AP or P7 exhibited a noticeable reduction in tau aggregate abundance. While the decrease in the case of p7 was not statistically significant, statistical analysis showed significance after 4-AP treatments ($p<0.001$) (Figure 2.16 C and D). These findings confirmed the ability of 4-AP and dynamin inhibitors of reducing human tau aggregates in our TBI larvae.

Figure 2.16

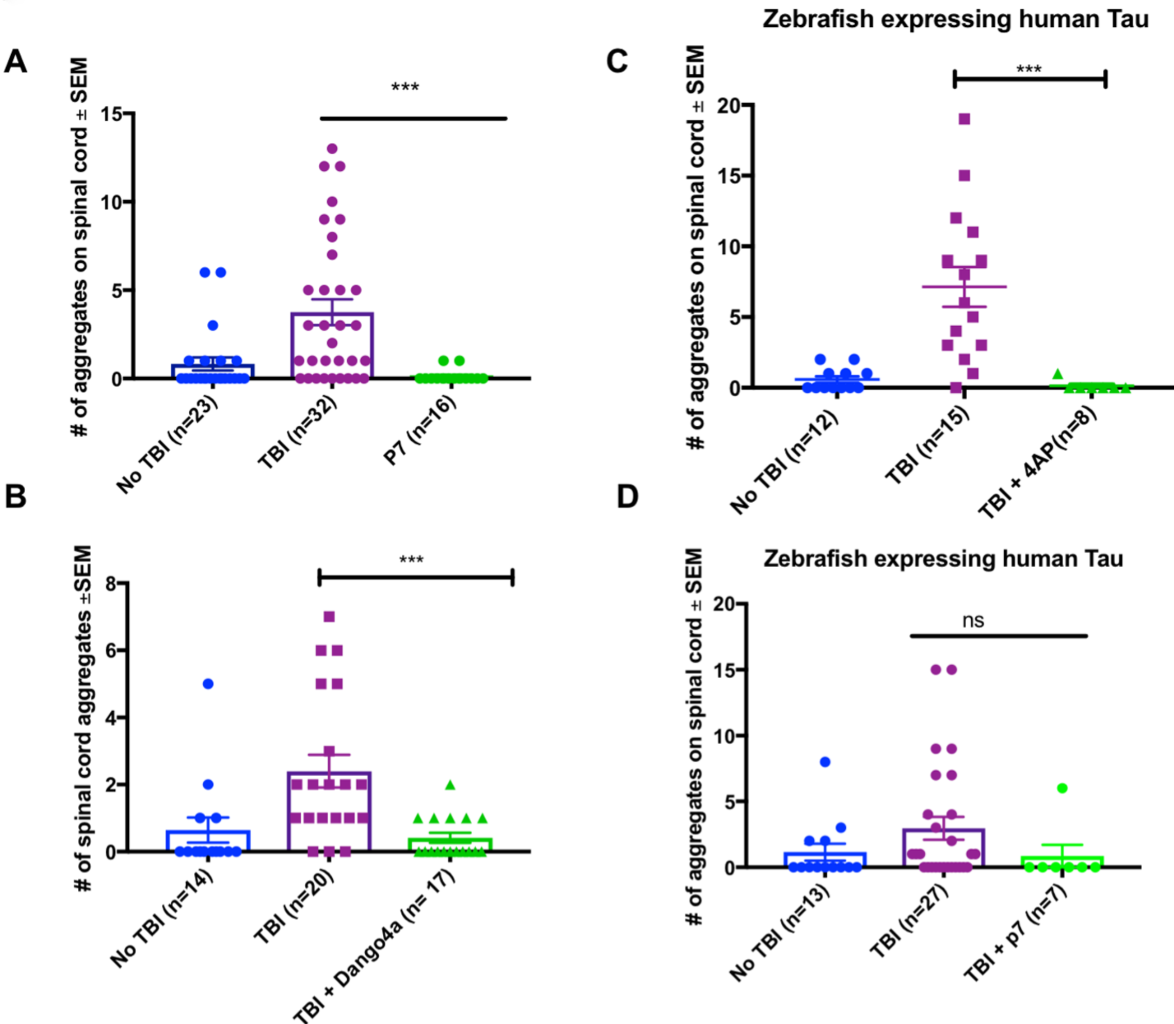


Figure 2. 16: Pharmacological targeted inhibition of endocytosis reduced tauopathy following TBI.

(A) Blocking endocytosis with Pyrimidyn-7 (P7) treatments significantly inhibited the formation of Tau4R-GFP+ puncta following TBI (***$p<0.001$). (B) Dango4a treatment significantly reduced Tau aggregates in the spinal cord (***$p<0.001$) in a manner similar to P7. (C) 4AP treatment significantly inhibited the formation of Tau4R-GFP+ puncta in the spinal cord (***$p<0.001$) of Tau biosensor line that also express human Tau (2N4R) after traumatic brain injury compared to untreated TBI control group. (D) A notable reduction in Tau aggregates was observed in the same line after treatment with p7 drug. Statistical analysis shows no significance difference between groups

2.5 Discussion:

Over the past decade, numerous studies *in vitro* and *in vivo* studies has supported the notion that tau proteins possess prion-like proprieties that permit them to seed conversion in those diseases and spread to synaptically connected regions in tauopathies such as AD and FTD (Ayers et al., 2018; de Calignon et al., 2012; Goedert et al., 2017a; Goedert et al., 2017b; Iba et al., 2015; Woerman et al., 2016). Various possible mechanisms for the transcellular transfer of tau seeds have been proposed including mechanisms of release such as exosomes, or mechanisms of cellular uptake of tau seeds such as endocytosis (Demaegd et al., 2018; Evans et al., 2018; Wang et al., 2017; Wu et al., 2013). Nonetheless, most of the suggested mechanisms were postulated based on *in vitro* evidence as there is a lack of access to appropriate models that could assist in visualizing the prion-like spreading of tau aggregates. Many details regarding the mechanisms and factors involved in the propagation of tau seeds, such as whether one or more mechanisms take place in each tauopathy during the progression of the disease or if there are factors and mechanisms that are unique to each disease, have not been fully explored.

Regarding TBI, a huge knowledge gap exists regarding how tau seeds are released and/or internalized between cells - indeed most of the prion-like properties of tau species in TBI were not assessed until very recently (Woerman et al., 2016; Zanier et al., 2018). Moreover, although there are various forms of TBI and all are considered risk factors for neurodegeneration, the focus in the literature has mostly been directed towards repetitive mild trauma as it is associated with the tauopathy, CTE. The recent revelation that TBI patients, whether they suffered from single or repetitive brain trauma, exhibited similar tau pathology to CTE (Washington et al., 2016; Zanier et al., 2018) makes it intriguing. In our opinion, it would be wise to consider all

forms of TBI as tauopathies. Hence, studying prion-like transmission of tau pathology in the context of TBI is of no lesser importance than studying it in CTE or other tauopathies.

2.5.1 Overview of major findings and significance

In our study, efforts were directed to unraveling mechanistic information about factors involved in prion-like spreading. Specifically, we inspected the role of seizure activity and/or neuronal excitability as well as the role of dynamin-dependent endocytosis during the progression of tauopathy in TBI. We focused our attention on seizure activity in part because seizures frequently occur in TBI patients especially following blast traumatic injury (Englander et al., 2014; Kovacs et al., 2014). We hypothesized that neuronal excitability and seizure activity in TBI can play a role in accelerating the wide dissemination of tau pathology. As such, we undertook two new approaches to test this hypothesis. The first approach was to engineer a novel *in vivo* tau biosensor model in zebrafish that can visualize pathological tau spreading and accumulation within the intact CNS. The tau biosensor zebrafish express human tau4R-GFP reporter protein, and we confirmed its ability to detect tau seeds various sources both *in vivo* and *in vitro*, similar to previously engineered *in vitro* models (Kaufman et al., 2016; Sanders et al., 2014). Our second approach was to introduce and optimize an elegantly simple technique to cause Pressure wave-induced TBI (PW-TBI), similar to human blast TBI, in zebrafish larvae. While the TBI paradigm we developed induces brain injury mimics the one caused by blast-wave and likely would not be identical to other types of TBI, the development of tauopathy is observed in majority of TBI types (Wooten et al., 2019). Our interest in developing methods of inducing injury to larvae zebrafish vs. adults originated from the advantages that larvae zebrafish can provide, which include access to larger numbers of samples and the tractability of those

larvae for high-throughput *in vivo* screening for therapeutic agents (Saleem and Kannan, 2018). Larval zebrafish provide a large economic advantage compared to adults, with respect to cost per individual and space consumed in animal housing. Moreover, treating larval fish (that are accessible early in development due to external fertilization) can be viewed as an ethically favourable replacement (Russell and Burch, 1959) for performing TBI on rodents that typically are not accessible until postnatal stages development. Our data argue that our TBI methods are germane to clinical etiology, because (akin to existing animal models of TBI) we were able to confirm the presence of various markers associated with blast brain injury, such as cell death, axonal damage, blood flow abnormalities, hemorrhage and the occurrence of post-traumatic seizures. The simplicity of the TBI method we introduce makes its adoption very feasible for further work considering genetic and pharmacological study in these diverse and clinically important aspects of TBI.

The post-traumatic seizures apparent in our TBI model led us to consider the neural events occurring during the TBI, and their potential bearing on the correlation between neural activity and tauopathy. Studies that investigate the impact of TBI on neuronal circuits, especially *in vivo*, have been inadequate, with most of the work examining the changes from a few hours to just several days after brain trauma (Bugay et al., 2019). This may be of importance when considering evaluating the reasons behind the developments of post-traumatic seizures and epilepsy. In a controlled cortical impact model of TBI, an initial decrease or loss in neuronal activity is recorded after injury before a rise in neuronal activity is noted (Ping and Jin, 2016) Whether the same takes place establishing the exact moment of impact and in different types of injuries, like blast TBI, remains unexamined. To address this, we performed TBI on zebrafish larvae expressing CaMPARI, an optogenetic reporter of neural activity. CaMPARI is

particularly ideal for this type of question, as its reportage of neural activity (a shift from green to red fluorescence) occurs only during user-defined times and that reportage is relatively permanent. This allowed us to quantify the CNS activity that had occurred during TBI, by characterizing the red: green ration of fluorescent emission after TBI was completed. This approach therefor allows relatively easy access to quantifying neural activity *during injury* in an unencumbered freely-swimming animal. Herein, we garnered major insights using our TBI method on CaMPARI, and we revealed for the first time a snapshot of neurons becoming active at the exact moment of impact of TBI. Unlike previous studies, our results showed an increase in neuronal excitability upon TBI, which may contribute to the frequency of post-traumatic seizures observed in our model, other blast TBI models and TBI patients (Bugay et al., 2019). We believe the capability of CaMPARI is unprecedented, and the platform established here has good potential to reveal what events occur *during* TBI that switch the neural circuitry into a hyperactive state. Regarding tauopathy, the CaMPARI quantification provided us important validation that neural activity was substantively impacted by TBI, supporting our rationale that convulsant and anti-convulsant drug treatments might modulate neural activity and thereby accelerate or decelerate tauopathy accumulation.

As previously mentioned, we noted the occurrence of post-traumatic seizures in most of our TBI samples, which is in agreement with the prevalence of seizures in blast TBI patients and TBI models (Bugay et al., 2019; Kovacs et al., 2014). However, whether post-traumatic seizures contribute to prion-like spreading of tau pathology observed in TBI or not is unknown. Yet, many investigations have supported an association between tau pathology and seizures (Sanchez et al., 2018; Tai et al., 2016). Studies on epileptic human temporal structure revealed accumulation of tau aggregates (Sanchez et al., 2018). In 3XTg AD mice, induced chronic

epilepsy was associated with changes of inter-neuronal p-tau expression (Yan et al., 2012). Additionally, data obtained from post-mortem analysis of patient tissues with AD and drug resistance epilepsy uncovered a link between symptomatic seizures and increased Braak stages as well as accelerated tau accumulation (Thom et al., 2011). Interestingly, the presence of tau deposits in epileptic patients and the similarity of its pathology to CTE suggest a conceivable role for seizures influencing the progression of tau pathology in a similar manner to TBI (Puvenna et al., 2016). Indeed, our data here support the ability of seizure activity to enhance tau abundance in our TBI model. This finding is in line with observations in a patient with epilepsy and a history of head injury, in which progressive tau pathology was noted (Geddes et al., 1999; Thom et al., 2011). On the other hand, the aforementioned results also raise the possibility of benefits of reducing post-traumatic seizures on the progression of tauopathy. Certainly our findings do provide tentative support for the previous statement - reducing seizure activity after TBI in our model using anti-convulsant drugs significantly reduced tau abundance, providing further evidence of the relationship between seizures and tauopathy in TBI (Figure 2.11 G).

Unexpectedly, our data demonstrated inducing seizures for a long period of time using 4-AP convulsant drugs inhibited tauopathy in our model. 4-AP is a voltage-gated potassium channel blocker that enhances neuronal firing activity and has been used often on zebrafish for seizure studies (Kasatkina, 2016; Liu and Baraban, 2019; Lundh, 1978; Winter et al., 2017). Yet, 4-AP is rarely administered for prolonged treatments such as those we deployed here, e.g. past studies rarely exceed 1hour of 4-AP (Winter et al., 2017). Thus, we considered that the prolonged stimulation with 4-AP in addition to the high dosages applied may have affected the dynamin-dependent endocytosis, in turn affecting the progression of tauopathy in our TBI model. Our proposition is reinforced by the fact that *in vitro* work has disclosed that higher

concentrations of 4-AP and prolonged stimulation is shown to associate with inhibition of dynamin, which is important for the endocytosis of synaptic vesicles at the nerve terminals (Cousin and Robinson, 2000). The inhibition of endocytosis observed in that study was independent of 4-AP-dependent seizure activity. We also confirmed this in our TBI model as blocking seizures induced by 4-AP did not affect the results and we still detected a significant decrease in tauopathy. As our data were obtained exclusively from prolonged 4-AP treatments, further work concentrating on comparing the effects of short durations of 4-AP treatments on tauopathy progression and the impact of administering 4-AP after formation of tau aggregates in TBI is valuable.

We further emphasized the role of dynamin-dependent endocytosis in the prion-like progression of tau pathology in TBI with potent endocytosis inhibitors that target dynamin. Dynamin is a GTPase involved in two mechanisms of endocytosis that are important for synaptic vesicle transport (Singh et al., 2017). Empirical work on human stem cell-derived neurons has indicated that tau aggregates are internalized via dynamin-dependent endocytosis and that blocking other endocytosis pathways independent of dynamin, such as bulk endocytosis and macropincytosis, did not disrupt tau uptake (Evans et al., 2018). On the contrary, inhibiting dynamin significantly decreased the internalization of tau aggregates. Our results are in line with the previously mentioned findings that show tau progression in TBI models depends on dynamin-dependent endocytic pathways - blocking them with two different inhibitors dramatically lessened the abundance of tau seeds. Hence, our findings not only provide validation of *in vitro* works *in vivo*, but also provide valuable knowledge for understanding more

about mechanisms underlying prion-like spreading of tau seeds in TBI and CTE that could aid in developing therapeutic strategies.

2.5.2 Limitations of the experimental approach

Despite the sensitivity of our *in vivo* biosensor zebrafish in the detection of exogenous tau seeds, we observed the formation of spontaneous tau aggregates occasionally in certain fish part of our control groups. Fortunately, that number of fish and the abundance of tau seeds in those larvae was always below the threshold compared to treatments. These spontaneous aggregates could be based on genetic variations that have been documented in zebrafish (Balik-Meisner et al., 2018; Guryev et al., 2006). Although methods for selection and isolation of larvae with the least incidence of developing spontaneous aggregates were implemented in this study and considered for future work, analyzing those larvae could be of interest, especially for comprehending the impact of genetic variation on the development of tauopathy in our zebrafish model. It could also provide an avenue for studying spontaneous non-familial tauopathies. With regards to our PW-TBI method, the simplicity of our methods renders them easier to adopt for other studies. However, further optimizations that aim towards increasing consistency and reducing variability among TBI samples are recommended and probably necessary for studies that seek to investigate small effect sizes.

2.5.3 Conclusion

We propose a role of seizures and dynamin-dependent endocytosis in the prion-like progression of tauopathy following TBI using novel *in vivo* models. Currently, no treatments are available applicable to all tauopathies. As such, developing new tools and models are required

for the mechanistic understanding of the pathogenesis of tauopathies, such as CTE and TBI. Specifically, creating *in vivo* models that can help elucidate the mechanisms underlying the prion-like spreading of tau pathologies are required as this information may establish important targets for therapeutic intervention.

3. Chapter 3: Conclusions and Future directions

Conclusions, implications and future directions

3.1 Main conclusion

In this thesis, the role of seizure and neural activity in the aggregation and spreading of tau pathology in TBI models was interrogated using two novel approaches: the engineering of a novel tauopathy biosensor model in zebrafish and the development of the first TBI method suitable for zebrafish larvae. In Chapter 2, we validated both models and established an essential role of both seizure and dynamin-dependent endocytosis in the prion-like propagation of tau in TBI. This was supported by the finding that blocking either one significantly minimized tau abundance in our TBI model. The findings not only provide new mechanistic insights into tau propagation in TBI, but also present more interesting questions that can act as a framework for future studies. The work presented in Chapter 2 indicates the advantages of the development of both zebrafish models as complements to the available *in vitro* models, as it enables further in-depth study of pathology in a vibrant brain with proper cell–cell interactions in which the role of neural activity and seizure can be properly assessed.

In this final chapter, I will address some technical limitations as well as some of the future applications of the tau biosensor and TBI models and some of the interesting hypotheses, questions, and aims that could be tested in future.

3.2 Implications of tau biosensor model

3.2.1 Analysis of tau strains using tau biosensor zebrafish models

Chapter 1 reviews evidence supporting the existence of various tau strains (Narasimhan et al., 2017; Sanders et al., 2014). There is also the potential that multiple tau strains can exist in one patient (Morales et al., 2007; Schoch et al., 2006), but the factors that influence the

dominance of one strain vs. another remain unclear. The competition between strains is an interesting area that was recently investigated as one of the new therapeutic strategies for prion diseases (Asante et al., 2015). One study showed that the introduction of non-toxic or less toxic prion strain protected against the propagation of the toxic strain (Asante et al., 2015). This therapeutic approach may be a really promising application to the prion-like propagation of tau in tauopathies; however, it requires further examination in appropriate models. The tauopathy biosensor model can be implemented in such studies, as it allows the study of strain-specific pathology by investigating the rate of progression and cell type affected, and the morphology of tau seeds *in vivo*. Thus, it may provide further insight as to whether such approaches could be applicable to tauopathies. The model has potential for neurobehavioral measurements, hence providing further information on strain-specific defects, and could be adapted for compound screening that rescues the neurological phenotype induced by specific strains.

Another interesting experimental approach in the prion field is the passaging of transmitted protein aggregates to a new host in order to prove the existence of specific strains. This experimental approach has been employed widely in the prion field ,but few studies have utilized this approach with tau (Sanders et al., 2014). It would be interesting to examine whether passaging the aggregated tau we observed in our tauopathy biosensor model after TBI and injections of brain homogenate to either naïve tauopathy biosensor zebrafish larvae and our tauopathy model will reveal more information regarding tau strains.

3.2.2 The potential therapeutic of Anti-convulsant drugs for TBI

Post-traumatic seizure (PTS), as discussed in Chapter 1 and 2, is one of the serious complications observed after TBI which can occur within the first week of the injury (Annegers

et al., 1998; Kovacs et al., 2014). Post-traumatic seizures that occur after brain injury could contribute to secondary damage by affecting the intracranial pressure (mostly increasing it) and causing excessive neurotransmitter release, which may further worsen existing damage (Vespa et al., 2007). Thus, early administration of an anti-convulsant after head injury is considered an important step for minimizing brain damage by preventing early seizures (Schierhout and Roberts, 1998). Aside from seizure, anti-convulsants have neuroprotective effects in some conditions, such as hypoxia, as seen by a reduction in neuronal damage in neonatal rats following hypoxia (Vartanian et al., 1996). Additionally, anti-convulsants seem to offer a promising potential therapeutic approach for some neurodegenerative diseases such as ALS as they can reduce abnormal neuronal excitability (Wainger et al., 2014; Welty et al., 1995). However, whether the use of anti-convulsants can affect tau spreading in TBI has not been fully addressed. Interestingly, our data suggest a promising potential for the use of the anti-convulsant RTG, used in some ALS studies (Wainger et al., 2014), in reducing tauopathy in TBI. This effect is likely due to inhibition of seizure, which is associated with more tau aggregation in our TBI model. However, further examination is needed to validate these findings and confirm the potential for the use of an anti-convulsant. This could include examining the time of drug administration (whether before or after Tau aggregation), assessing the duration of treatments (short-term vs long term) and lastly, examining the effects of other anti-convulsants to compare their efficiency in reducing tau aggregates.

3.2.3 The role of pathological tau on vision

An interesting observation when analyzing tau aggregation in the tau biosensor model is the presence of aggregates in the eye of some larvae (Figure 3.1). The accumulation of tau in the

retina and optic nerve have been indicated by some studies (Chiasseu et al., 2017; Chiasseu et al., 2016; Hart et al., 2016). Recent work has shown the accumulation of hyper-phosphorylated tau in the retina of AD mouse model (Chiasseu et al., 2017). This accumulation of tau in the retina precedes the behavioural defects and tau pathology in the brain. Additionally, tau alterations were detected in the retinal ganglion cells (RGC) and promoted early neuronal dysfunctions, suggesting a potential role of pathological tau in visual deficits in some AD patients(Chiasseu et al., 2017). Taking these findings into consideration, the larvae with tau aggregates in their eye may serve as a model to disentangle the impact of the accumulation of tau in the eye on the visual functions. Characterization of the tau pathological species in the eye may provide important information for the development of diagnostic tools for neurodegenerative disease such as AD (Hart et al., 2016; Wright et al., 2019).

3.3 Possible limitations of using zebrafish larvae:

As mentioned in Chapter 2, the use of larval zebrafish to model TBI and tauopathy spreading is favourable because larval zebrafish allow for large sample sizes and are an ethical replacement, as any procedures performed on zebrafish larvae before they are fully developed (5-6 days post-fertilization [dpf]) do not require ethical approval (Geisler et al., 2017). However, there are several limitations associated with using zebrafish larvae compared to adult zebrafish when studying tauopathies. The first limitation is the short duration of analysis at the larval stage. For the experiments described in this thesis, most larvae were analyzed from 2 to 7 dpf, as they must be transferred to the nursery at 7 to 9 dpf and no further analysis is possible until they reach maturity and are released from the nursery at approximately 2 months of age. Another limitation

is that some phenotypes associated with later stages of the disease may not be apparent at the larval stage. This is an especially important point to raise because most tauopathies, including CTE and AD, are usually detected during adulthood, not during embryonic development. There are two ways to address this limitation. One approach is to subject larvae to TBI or to inject larvae with pathological tau fibrils, then examine phenotypes such as the spreading of tauopathy and behavioural deficits during adulthood. This approach may be valuable when studying the long-term effects of TBI or tauopathy on cognition. The second is to induce TBI or perform tau injections on adult zebrafish instead of larvae, then analyze the aforementioned phenotypes. Notably, despite the limitations of performing TBI and analyzing its effects on zebrafish larvae, this approach may be valuable in investigating the impact of TBI on fetuses during pregnancy or on children and determining how this type of injury may affect development and cognitive functions.

3.4 Future directions

3.4.1 Link between changes in neural activity after TBI and after tau aggregation

Changes in neuronal excitability are often noted in neurodegenerative disease such as Alzheimer's disease (AD), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis (ALS) (Davis et al., 2014; Eisen et al., 1993; Garcia-Cabrero et al., 2013). These changes in neuronal excitability are also observed in a very recently developed animal model of TBI (Bugay et al., 2019). Such changes are often associated in some cases with neuronal dysfunction and degeneration (Siskova et al., 2014). However, how the changes contribute to the pathogenesis of TBI is not well understood. In Chapter 2, we were able to obtain a snapshot of the changes in neuronal activity that occur during brain trauma, which to our knowledge, have not been

documented before. The increased neuronal excitability we observed was in the hind brain area. Interestingly, tau 4R-GFP aggregates that formed in the brain were also observed by the ventricle wall in the same area. This observation sparks the question of whether the changes in neuronal excitability are behind the neuronal vulnerability of this region to tau aggregation. Additionally, are there various changes affecting neural excitability over the course of brain trauma (does neuronal excitability change after the trauma or after tau aggregation)? One way of answering the latter question is by out-crossing our CaMPARI fish with the tauopathy biosensor transgenic fish. Then, TBI is induced in the larvae fish with both reporters, and neuronal activity at various time points after the trauma, including before and after tau aggregation is detected, is analyzed.

3.4.2 *In vivo* live imaging of tau spreading

To understand more about the kinetics of tau pathology spread and to assess the validity of therapeutic strategies that may reduce or minimize tauopathy propagation, there is a need for models in which the rate and location of spreading in the living brain can be tracked over time. While various mouse models support prion-like transmission and tau seeding, assessing the kinetics in mice via live imaging techniques was quite a challenge, as mouse neural tissues are not visible, so the animals are often sacrificed at certain time points for analysis (Narasimhan et al., 2017; Wegmann et al., 2019). One of the advantages of engineering the tauopathy biosensor in zebrafish is the ability to perform live imaging without sacrificing animals. In Chapter 2, our data suggest the movement of tau aggregates over time. However, the kinetics of spreading was not assessed. Thus, performing live imaging after injecting tau seeds or inducing TBI would be

our next step, as this type of imaging could reveal more information regarding the kinetics of tau spreading in various conditions.

3.4.3 In-depth characterization of tau pathology observed in tau biosensor model

Identifying tau species observed after injections and in TBI models

In our work, tau aggregation was shown in the form of green fluorescent protein (GFP)+ve puncta. However, it is unclear whether these puncta captured all tau aggregation events occurring in our models. Thus, there is a need for future work focusing on analyzing tau aggregates via various antibodies against tau [e.g., AT8 for phospo tau, or anti-pS396-tau antibody C10.2 (Rosenqvist et al., 2018), which targets tau competent seeds] to identify the types of tau seeds detected by our reporter *in vivo* post-injection. Moreover, observing phospho-tau co-localized with the Tau4R-GFP aggregates would further validate that the GFP aggregates faithfully represent the pathobiological mechanisms occurring in human tauopathy.

For TBI, a recent finding indicates that *cis* p-Tau, a precursor of tau pathology that appears before the development of tauopathy, is the early driver of neurodegeneration in TBI (Albayram et al., 2018; Kondo et al., 2015). Thus, it may be better to perform immunohistochemistry (IHC) using an anti-*cis* antibody instead of AT8 on TBI models to verify if *cis*-Tau is involved in tau aggregation in our model.

Characterization of cells containing the aggregates

One important area of this in-depth investigation will include identification the type of cells in which tau aggregates are observed. Although we think that the aggregates observed along the spinal cord are in the sensory motor neurons, conformation of cell type is essential.

This could be done via immunohistochemistry (IHC) using an anti-zebrafish neuronal marker antibody or anti-NeuN antibody to label neurons and the anti-4C4 antibody that labels microglia and macrophages in zebrafish as referred to here (Steen et al., 1989).

Investigating another possible *in vivo* route of Tau spreading

As mentioned in Chapter 1, *in vivo* studies suggest the spreading of tau pathology to an adjacent area can occur trans-synaptically (Liu et al., 2012). Another alternative route could be spreading through microglia. Microglia are macrophages that play an important role in maintaining the homeostasis of the CNS by constantly surveying the microenvironment and responding to any injury or microbial infections by proliferating and exhibiting phagocytic activity that allow the phagocytosis of dying cell debris and protein aggregates (Kim and de Vellis, 2005). The role of microglia in Alzheimer's disease (Ghoshal et al., 2001; Serrano-Pozo et al., 2011) and spreading of tau pathology has recently started to unravel (Asai et al., 2015). This recent work has shown that inhibiting microglia reduces the progression of tau pathology in Tg mice expressing human Tau P301L and tau was suggested to get released from microglia via exosomes (Asai et al., 2015). Whether microglia can facilitate tau propagation in TBI or not has not been investigated. One way to address this question is to ablate the microglia in our TBI model and monitor tau aggregation and spreading. There are two approaches to study the role of microglia in our TBI zebrafish model. The first approach is by out crossing our Tau biosensor to *irf8*$^{-/-}$ zebrafish mutant which lack microglia (Hamilton et al., 2016) and then subjecting the tau biosensor larva with the mutations to TBI. The second approach is treating larva subjected to TBI with the pharmacological inhibitor BLZ945 which inhibits the CSF-1 receptor (CSF-1R) on microglia resulting in microglia reduction (Hamilton et al., 2016). Subsequently, analysis of tauopathy progression and spreading will be performed.

3.4.4 Role of zebrafish Mapt (microtubule-associated protein tau) proteins in the efficiency of the tau biosensor

It is worth considering that zebrafish have two paralogs of the *MAPT* gene (*mapta* and *maptb*) (Chen et al., 2009). Both genes share a sequence identity of 62% and 58%, respectively, with the human *MAPT*, particularly at the tubulin binding regions and C-terminal (Chen et al., 2009). To date, little is known about zebrafish Mapt proteins. Particularly, the role of zebrafish mapt proteins on the efficiency of the tauopathy biosensor model we engineered is unknown, as is whether they are involved in (required for) tauopathy spread and propagation, or if they can interfere with some strains of human or mouse tau. To address these hypotheses, CRISPR/Cas9 engineering of *mapta* and *maptb* knockout fish could be done. Once the *mapta* and/or *maptb* knockout fish is generated, it will be crossed to the tau biosensor reporter and used to assess the aggregation and spreading of tau introduced via brain injections.

Figure 3.1

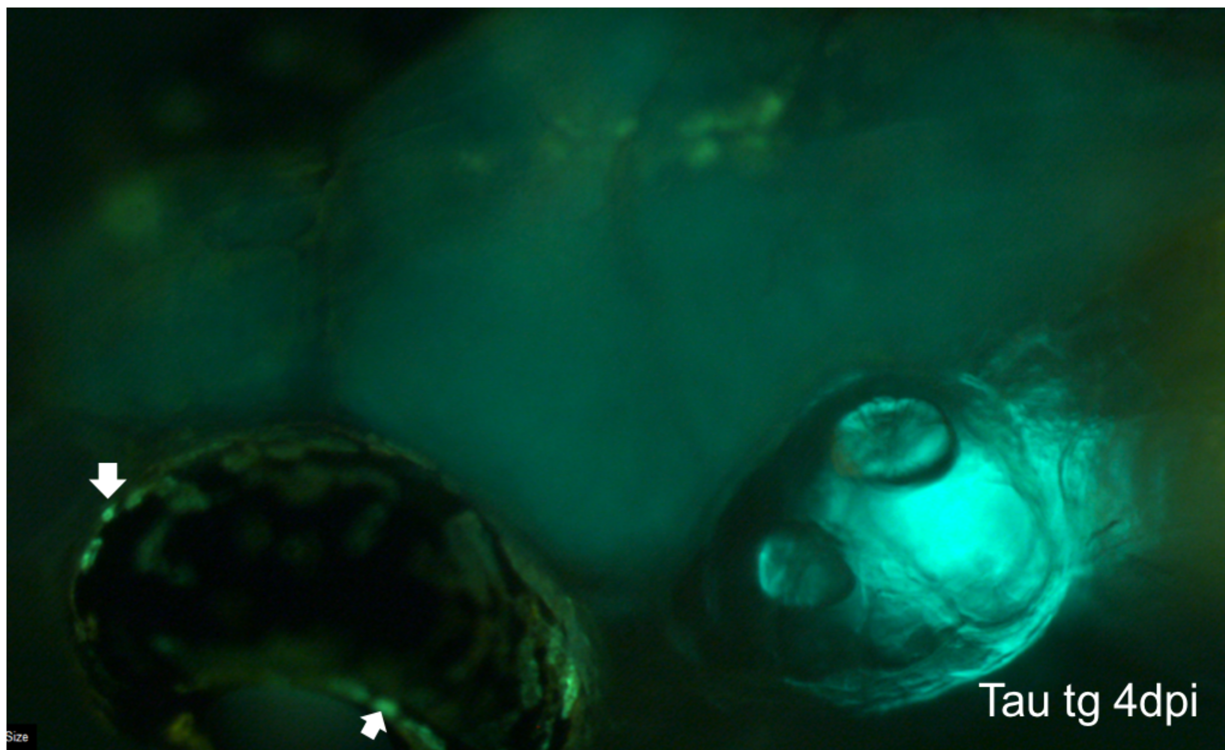


Figure 3. 1: The presence of aggregates in the eye of some larvae injected with mouse brain homogenate loaded with pathological human tau aggregates.

Arrow points to Tau4R-GFP+ve puncta around pigmented eye.

Bibliography:

Adlard, P.A., and Cummings, B.J. (2004). Alzheimer's disease--a sum greater than its parts? Neurobiol Aging 25, 725-733; discussion 743-726.

Aguzzi, A. (2009). Cell biology: Beyond the prion principle. Nature 459, 924-925.

Ahmed, Z., Cooper, J., Murray, T.K., Garn, K., McNaughton, E., Clarke, H., Parhizkar, S., Ward, M.A., Cavallini, A., Jackson, S.*, et al*. (2014). A novel in vivo model of tau propagation with rapid and progressive neurofibrillary tangle pathology: the pattern of spread is determined by connectivity, not proximity. Acta Neuropathol 127, 667-683.

Alavi Naini, S.M., and Soussi-Yanicostas, N. (2015). Tau Hyperphosphorylation and Oxidative Stress, a Critical Vicious Circle in Neurodegenerative Tauopathies? Oxid Med Cell Longev 2015, 151979.

Albayram, O., Angeli, P., Bernstein, E., Baxley, S., Gao, Z., Lu, K.P., and Zhou, X.Z. (2018). Targeting Prion-like Cis Phosphorylated Tau Pathology in Neurodegenerative Diseases. J Alzheimers Dis Parkinsonism 8.

Allen, B., Ingram, E., Takao, M., Smith, M.J., Jakes, R., Virdee, K., Yoshida, H., Holzer, M., Craxton, M., Emson, P.C.*, et al*. (2002). Abundant tau filaments and nonapoptotic neurodegeneration in transgenic mice expressing human P301S tau protein. J Neurosci 22, 9340-9351.

Amyere, M., Mettlen, M., Van Der Smissen, P., Platek, A., Payrastre, B., Veithen, A., and Courtoy, P.J. (2002). Origin, originality, functions, subversions and molecular signalling of macropinocytosis. Int J Med Microbiol 291, 487-494.

Annegers, J.F., Hauser, W.A., Coan, S.P., and Rocca, W.A. (1998). A population-based study of seizures after traumatic brain injuries. N Engl J Med 338, 20-24.

Arendt, T., Stieler, J., Strijkstra, A.M., Hut, R.A., Rudiger, J., Van der Zee, E.A., Harkany, T., Holzer, M., and Hartig, W. (2003). Reversible paired helical filament-like phosphorylation of tau is an adaptive process associated with neuronal plasticity in hibernating animals. J Neurosci 23, 6972-6981.

Arnold, C.S., Johnson, G.V., Cole, R.N., Dong, D.L., Lee, M., and Hart, G.W. (1996). The microtubule-associated protein tau is extensively modified with O-linked N-acetylglucosamine. J Biol Chem 271, 28741-28744.

Asai, H., Ikezu, S., Tsunoda, S., Medalla, M., Luebke, J., Haydar, T., Wolozin, B., Butovsky, O., Kugler, S., and Ikezu, T. (2015). Depletion of microglia and inhibition of exosome synthesis halt tau propagation. Nat Neurosci 18, 1584-1593.

Asante, E.A., Smidak, M., Grimshaw, A., Houghton, R., Tomlinson, A., Jeelani, A., Jakubcova, T., Hamdan, S., Richard-Londt, A., Linehan, J.M*., et al*. (2015). A naturally occurring variant of the human prion protein completely prevents prion disease. Nature 522, 478-481.

Asikainen, I., Kaste, M., and Sarna, S. (1999). Early and late posttraumatic seizures in traumatic brain injury rehabilitation patients: brain injury factors causing late seizures and influence of seizures on long-term outcome. Epilepsia 40, 584-589.

Audouard, E., Houben, S., Masaracchia, C., Yilmaz, Z., Suain, V., Authelet, M., De Decker, R., Buee, L., Boom, A., Leroy, K*., et al*. (2016). High-Molecular-Weight Paired Helical Filaments from Alzheimer Brain Induces Seeding of Wild-Type Mouse Tau into an Argyrophilic 4R Tau Pathology in Vivo. Am J Pathol 186, 2709-2722.

Avila, J., Jimenez, J.S., Sayas, C.L., Bolos, M., Zabala, J.C., Rivas, G., and Hernandez, F. (2016). Tau Structures. Front Aging Neurosci 8, 262.

Ayers, J.I., Giasson, B.I., and Borchelt, D.R. (2018). Prion-like Spreading in Tauopathies. Biol Psychiatry 83, 337-346.

Baas, P.W., and Qiang, L. (2019). Tau: It's Not What You Think. Trends Cell Biol 29, 452-461.

Baker, M., Litvan, I., Houlden, H., Adamson, J., Dickson, D., Perez-Tur, J., Hardy, J., Lynch, T., Bigio, E., and Hutton, M. (1999). Association of an extended haplotype in the tau gene with progressive supranuclear palsy. Hum Mol Genet 8, 711-715.

Baker, M., Mackenzie, I.R., Pickering-Brown, S.M., Gass, J., Rademakers, R., Lindholm, C., Snowden, J., Adamson, J., Sadovnick, A.D., Rollinson, S.*, et al*. (2006). Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17. Nature 442, 916-919.

Balik-Meisner, M., Truong, L., Scholl, E.H., Tanguay, R.L., and Reif, D.M. (2018). Population genetic diversity in zebrafish lines. Mamm Genome 29, 90-100.

Bancher, C., Brunner, C., Lassmann, H., Budka, H., Jellinger, K., Wiche, G., Seitelberger, F., Grundke-Iqbal, I., Iqbal, K., and Wisniewski, H.M. (1989). Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary tangles in Alzheimer's disease. Brain Res 477, 90-99.

Barbier, P., Zejneli, O., Martinho, M., Lasorsa, A., Belle, V., Smet-Nocca, C., Tsvetkov, P.O., Devred, F., and Landrieu, I. (2019). Role of Tau as a Microtubule-Associated Protein: Structural and Functional Aspects. Front Aging Neurosci 11, 204.

Bekris, L.M., Yu, C.E., Bird, T.D., and Tsuang, D.W. (2010). Genetics of Alzheimer disease. J Geriatr Psychiatry Neurol 23, 213-227.

Berger, Z., Roder, H., Hanna, A., Carlson, A., Rangachari, V., Yue, M., Wszolek, Z., Ashe, K., Knight, J., Dickson, D.*, et al*. (2007). Accumulation of pathological tau species and memory loss in a conditional model of tauopathy. J Neurosci 27, 3650-3662.

Bieniek, K.F., Blessing, M.M., Heckman, M.G., Diehl, N.N., Serie, A.M., Paolini, M.A., 2nd, Boeve, B.F., Savica, R., Reichard, R.R., and Dickson, D.W. (2020). Association between contact sports participation and chronic traumatic encephalopathy: a retrospective cohort study. Brain Pathol 30, 63-74.

Bir, C., Vandevord, P., Shen, Y., Raza, W., and Haacke, E.M. (2012). Effects of variable blast pressures on blood flow and oxygen saturation in rat brain as evidenced using MRI. Magn Reson Imaging 30, 527-534.

Blennow, K., Hardy, J., and Zetterberg, H. (2012). The neuropathology and neurobiology of traumatic brain injury. Neuron 76, 886-899.

Borg, J., Holm, L., Cassidy, J.D., Peloso, P.M., Carroll, L.J., von Holst, H., Ericson, K., and Injury, W.H.O.C.C.T.F.o.M.T.B. (2004). Diagnostic procedures in mild traumatic brain injury: results of the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. J Rehabil Med, 61-75.

Boutajangout, A., Boom, A., Leroy, K., and Brion, J.P. (2004). Expression of tau mRNA and soluble tau isoforms in affected and non-affected brain areas in Alzheimer's disease. FEBS Lett 576, 183-189.

Braak, E., Braak, H., and Mandelkow, E.M. (1994). A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. Acta Neuropathol 87, 554-567.

Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82, 239-259.

Braak, H., and Del Tredici, K. (2011). Alzheimer's pathogenesis: is there neuron-to-neuron propagation? Acta Neuropathol 121, 589-595.

Buch, K., Riemenschneider, M., Bartenstein, P., Willoch, F., Muller, U., Schmolke, M., Nolde, T., Steinmann, C., Guder, W.G., and Kurz, A. (1998). [Tau protein. A potential biological indicator for early detection of Alzheimer disease]. Nervenarzt 69, 379-385.

Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A., and Hof, P.R. (2000). Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res Brain Res Rev 33, 95-130.

Bugay, V., Bozdemir, E., Vigil, F.A., Holstein, D.M., Chun, S.H., Elliot, W., Sprague, C., Cavazos, J.E., Zamora, D.O., Rule, G.*, et al*. (2019). A mouse model of repetitive blast traumatic brain injury reveals post-trauma seizures and increased neuronal excitability. J Neurotrauma.

Bugiani, O., Murrell, J.R., Giaccone, G., Hasegawa, M., Ghigo, G., Tabaton, M., Morbin, M., Primavera, A., Carella, F., Solaro, C.*, et al*. (1999). Frontotemporal dementia and corticobasal degeneration in a family with a P301S mutation in tau. J Neuropathol Exp Neurol 58, 667-677.

Bukar Maina, M., Al-Hilaly, Y.K., and Serpell, L.C. (2016). Nuclear Tau and Its Potential Role in Alzheimer's Disease. Biomolecules 6, 9.

Caffrey, T.M., Joachim, C., and Wade-Martins, R. (2008). Haplotype-specific expression of the N-terminal exons 2 and 3 at the human MAPT locus. Neurobiol Aging 29, 1923-1929.

Calafate, S., Buist, A., Miskiewicz, K., Vijayan, V., Daneels, G., de Strooper, B., de Wit, J., Verstreken, P., and Moechars, D. (2015). Synaptic Contacts Enhance Cell-to-Cell Tau Pathology Propagation. Cell Rep 11, 1176-1183.

Calafate, S., Flavin, W., Verstreken, P., and Moechars, D. (2016). Loss of Bin1 Promotes the Propagation of Tau Pathology. Cell Rep 17, 931-940.

Caner, H., Can, A., Atalay, B., Erdogan, B., Albayrak, A.H., Kilinc, K., Bavbek, M., and Altinors, N. (2004). Early effects of mild brain trauma on the cytoskeletal proteins neurofilament160 and MAP2, and the preventive effects of mexilitine. Acta Neurochir (Wien) 146, 611-621; discussion 621.

Castellani, R.J., and Perry, G. (2019). Tau Biology, Tauopathy, Traumatic Brain Injury, and Diagnostic Challenges. J Alzheimers Dis 67, 447-467.

Castillo-Carranza, D.L., Gerson, J.E., Sengupta, U., Guerrero-Munoz, M.J., Lasagna-Reeves, C.A., and Kayed, R. (2014). Specific targeting of tau oligomers in Htau mice prevents cognitive impairment and tau toxicity following injection with brain-derived tau oligomeric seeds. J Alzheimers Dis 40 Suppl 1, S97-S111.

Cernak, I., and Noble-Haeusslein, L.J. (2010). Traumatic brain injury: an overview of pathobiology with emphasis on military populations. J Cereb Blood Flow Metab 30, 255-266.

Chai, X., Dage, J.L., and Citron, M. (2012). Constitutive secretion of tau protein by an unconventional mechanism. Neurobiol Dis 48, 356-366.

Chauhan, N.B. (2014). Chronic neurodegenerative consequences of traumatic brain injury. Restor Neurol Neurosci 32, 337-365.

Chen, J., Yu, J.T., Wojta, K., Wang, H.F., Zetterberg, H., Blennow, K., Yokoyama, J.S., Weiner, M.W., Kramer, J.H., Rosen, H., *et al.* (2017). Genome-wide association study identifies MAPT locus influencing human plasma tau levels. Neurology 88, 669-676.

Chen, M., Martins, R.N., and Lardelli, M. (2009). Complex splicing and neural expression of duplicated tau genes in zebrafish embryos. J Alzheimers Dis 18, 305-317.

Chen, S., Li, B., Grundke-Iqbal, I., and Iqbal, K. (2008). I1PP2A affects tau phosphorylation via association with the catalytic subunit of protein phosphatase 2A. J Biol Chem 283, 10513-10521.

Cheng, J.S., Craft, R., Yu, G.Q., Ho, K., Wang, X., Mohan, G., Mangnitsky, S., Ponnusamy, R., and Mucke, L. (2014). Tau reduction diminishes spatial learning and memory deficits after mild repetitive traumatic brain injury in mice. PLoS One 9, e115765.

Chiasseu, M., Alarcon-Martinez, L., Belforte, N., Quintero, H., Dotigny, F., Destroismaisons, L., Vande Velde, C., Panayi, F., Louis, C., and Di Polo, A. (2017). Tau accumulation in the retina promotes early neuronal dysfunction and precedes brain pathology in a mouse model of Alzheimer's disease. Mol Neurodegener 12, 58.

Chiasseu, M., Cueva Vargas, J.L., Destroismaisons, L., Vande Velde, C., Leclerc, N., and Di Polo, A. (2016). Tau Accumulation, Altered Phosphorylation, and Missorting Promote Neurodegeneration in Glaucoma. J Neurosci 36, 5785-5798.

Chiti, F., and Dobson, C.M. (2006). Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem 75, 333-366.

Choi, D.S., Kim, D.K., Kim, Y.K., and Gho, Y.S. (2013). Proteomics, transcriptomics and lipidomics of exosomes and ectosomes. Proteomics 13, 1554-1571.

Cicchetti, F., Lacroix, S., Cisbani, G., Vallieres, N., Saint-Pierre, M., St-Amour, I., Tolouei, R., Skepper, J.N., Hauser, R.A., Mantovani, D.*, et al*. (2014). Mutant huntingtin is present in neuronal grafts in Huntington disease patients. Ann Neurol 76, 31-42.

Clavaguera, F., Akatsu, H., Fraser, G., Crowther, R.A., Frank, S., Hench, J., Probst, A., Winkler, D.T., Reichwald, J., Staufenbiel, M.*, et al*. (2013). Brain homogenates from human tauopathies induce tau inclusions in mouse brain. Proc Natl Acad Sci U S A 110, 9535-9540.

Clavaguera, F., Bolmont, T., Crowther, R.A., Abramowski, D., Frank, S., Probst, A., Fraser, G., Stalder, A.K., Beibel, M., Staufenbiel, M.*, et al*. (2009). Transmission and spreading of tauopathy in transgenic mouse brain. Nat Cell Biol 11, 909-913.

Clavaguera, F., Hench, J., Goedert, M., and Tolnay, M. (2015). Invited review: Prion-like transmission and spreading of tau pathology. Neuropathol Appl Neurobiol 41, 47-58.

Clavaguera, F., Hench, J., Lavenir, I., Schweighauser, G., Frank, S., Goedert, M., and Tolnay, M. (2014). Peripheral administration of tau aggregates triggers intracerebral tauopathy in transgenic mice. Acta Neuropathol 127, 299-301.

Cleveland, D.W., Hwo, S.Y., and Kirschner, M.W. (1977). Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. J Mol Biol 116, 207-225.

Cohen, T.J., Guo, J.L., Hurtado, D.E., Kwong, L.K., Mills, I.P., Trojanowski, J.Q., and Lee, V.M. (2011). The acetylation of tau inhibits its function and promotes pathological tau aggregation. Nat Commun 2, 252.

Collinge, J., and Clarke, A.R. (2007). A general model of prion strains and their pathogenicity. Science 318, 930-936.

Corrigan, J.D., Wolfe, M., Mysiw, W.J., Jackson, R.D., and Bogner, J.A. (2003). Early identification of mild traumatic brain injury in female victims of domestic violence. Am J Obstet Gynecol 188, S71-76.

Corsellis, J.A., Bruton, C.J., and Freeman-Browne, D. (1973). The aftermath of boxing. Psychol Med 3, 270-303.

Cousin, M.A., and Robinson, P.J. (2000). Ca(2+) influx inhibits dynamin and arrests synaptic vesicle endocytosis at the active zone. J Neurosci 20, 949-957.

Cowan, C.M., Bossing, T., Page, A., Shepherd, D., and Mudher, A. (2010). Soluble hyper-phosphorylated tau causes microtubule breakdown and functionally compromises normal tau in vivo. Acta Neuropathol 120, 593-604.

Cowan, C.M., and Mudher, A. (2013). Are tau aggregates toxic or protective in tauopathies? Front Neurol 4, 114.

Crespo-Biel, N., Canudas, A.M., Camins, A., and Pallas, M. (2007). Kainate induces AKT, ERK and cdk5/GSK3beta pathway deregulation, phosphorylates tau protein in mouse hippocampus. Neurochem Int 50, 435-442.

Cruz-Haces, M., Tang, J., Acosta, G., Fernandez, J., and Shi, R. (2017). Pathological correlations between traumatic brain injury and chronic neurodegenerative diseases. Transl Neurodegener 6, 20.

Cuello, A.C. (2005). Intracellular and extracellular Abeta, a tale of two neuropathologies. Brain Pathol 15, 66-71.

Davis, K.E., Fox, S., and Gigg, J. (2014). Increased hippocampal excitability in the 3xTgAD mouse model for Alzheimer's disease in vivo. PLoS One 9, e91203.

Dawson, H.N., Ferreira, A., Eyster, M.V., Ghoshal, N., Binder, L.I., and Vitek, M.P. (2001). Inhibition of neuronal maturation in primary hippocampal neurons from tau deficient mice. J Cell Sci 114, 1179-1187.

de Calignon, A., Polydoro, M., Suarez-Calvet, M., William, C., Adamowicz, D.H., Kopeikina, K.J., Pitstick, R., Sahara, N., Ashe, K.H., Carlson, G.A., *et al*. (2012). Propagation of tau pathology in a model of early Alzheimer's disease. Neuron 73, 685-697.

DeKosky, S.T., Blennow, K., Ikonomovic, M.D., and Gandy, S. (2013). Acute and chronic traumatic encephalopathies: pathogenesis and biomarkers. Nat Rev Neurol 9, 192-200.

Demaegd, K., Schymkowitz, J., and Rousseau, F. (2018). Transcellular Spreading of Tau in Tauopathies. Chembiochem 19, 2424-2432.

Devkota, S. (2018). The road less traveled: strategies to enhance the frequency of homology-directed repair (HDR) for increased efficiency of CRISPR/Cas-mediated transgenesis. BMB Rep 51, 437-443.

Dewan, M.C., Rattani, A., Gupta, S., Baticulon, R.E., Hung, Y.C., Punchak, M., Agrawal, A., Adeleye, A.O., Shrime, M.G., Rubiano, A.M., *et al*. (2018). Estimating the global incidence of traumatic brain injury. J Neurosurg, 1-18.

Dhandapani, S., Manju, D., Sharma, B., and Mahapatra, A. (2012). Prognostic significance of age in traumatic brain injury. J Neurosci Rural Pract 3, 131-135.

Dorsett, C.R., McGuire, J.L., DePasquale, E.A., Gardner, A.E., Floyd, C.L., and McCullumsmith, R.E. (2017). Glutamate Neurotransmission in Rodent Models of Traumatic Brain Injury. J Neurotrauma 34, 263-272.

Dorval, V., and Fraser, P.E. (2006). Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein. J Biol Chem 281, 9919-9924.

Dotti, C.G., Banker, G.A., and Binder, L.I. (1987). The expression and distribution of the microtubule-associated proteins tau and microtubule-associated protein 2 in hippocampal neurons in the rat in situ and in cell culture. Neuroscience 23, 121-130.

Dubourg, C., Sanlaville, D., Doco-Fenzy, M., Le Caignec, C., Missirian, C., Jaillard, S., Schluth-Bolard, C., Landais, E., Boute, O., Philip, N., *et al*. (2011). Clinical and molecular

characterization of 17q21.31 microdeletion syndrome in 14 French patients with mental retardation. Eur J Med Genet 54, 144-151.

Dugger, B.N., Whiteside, C.M., Maarouf, C.L., Walker, D.G., Beach, T.G., Sue, L.I., Garcia, A., Dunckley, T., Meechoovet, B., Reiman, E.M., and Roher, A.E. (2016). The Presence of Select Tau Species in Human Peripheral Tissues and Their Relation to Alzheimer's Disease. J Alzheimers Dis 51, 345-356.

Dujardin, S., Begard, S., Caillierez, R., Lachaud, C., Delattre, L., Carrier, S., Loyens, A., Galas, M.C., Bousset, L., Melki, R., *et al*. (2014a). Ectosomes: a new mechanism for non-exosomal secretion of tau protein. PLoS One 9, e100760.

Dujardin, S., Lecolle, K., Caillierez, R., Begard, S., Zommer, N., Lachaud, C., Carrier, S., Dufour, N., Auregan, G., Winderickx, J., *et al*. (2014b). Neuron-to-neuron wild-type Tau protein transfer through a trans-synaptic mechanism: relevance to sporadic tauopathies. Acta Neuropathol Commun 2, 14.

DuVal, M.G., Oel, A.P., and Allison, W.T. (2014). gdf6a Is Required for Cone Photoreceptor Subtype Differentiation and for the Actions of tbx2b in Determining Rod Versus Cone Photoreceptor Fate. Plos One 9.

Ebneth, A., Godemann, R., Stamer, K., Illenberger, S., Trinczek, B., and Mandelkow, E. (1998). Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. J Cell Biol 143, 777-794.

Edwards, G., 3rd, Zhao, J., Dash, P.K., Soto, C., and Moreno-Gonzalez, I. (2019). Traumatic Brain Injury Induces Tau Aggregation and Spreading. J Neurotrauma.

Edwards, G., 3rd, Zhao, J., Dash, P.K., Soto, C., and Moreno-Gonzalez, I. (2020). Traumatic Brain Injury Induces Tau Aggregation and Spreading. J Neurotrauma 37, 80-92.

Eisen, A., Pant, B., and Stewart, H. (1993). Cortical excitability in amyotrophic lateral sclerosis: a clue to pathogenesis. Can J Neurol Sci 20, 11-16.

Ellis, L.D., Seibert, J., and Soanes, K.H. (2012). Distinct models of induced hyperactivity in zebrafish larvae. Brain Res 1449, 46-59.

Englander, J., Cifu, D.X., and Diaz-Arrastia, R. (2014). Information/education page. Seizures and traumatic brain injury. Arch Phys Med Rehabil 95, 1223-1224.

Eskandari-Sedighi, G., Daude, N., Gapeshina, H., Sanders, D.W., Kamali-Jamil, R., Yang, J., Shi, B., Wille, H., Ghetti, B., Diamond, M.I., *et al*. (2017). The CNS in inbred transgenic models of 4-repeat Tauopathy develops consistent tau seeding capacity yet focal and diverse patterns of protein deposition. Mol Neurodegener 12, 72.

Esquerda-Canals, G., Montoliu-Gaya, L., Guell-Bosch, J., and Villegas, S. (2017). Mouse Models of Alzheimer's Disease. J Alzheimers Dis 57, 1171-1183.

Evans, L.D., Wassmer, T., Fraser, G., Smith, J., Perkinton, M., Billinton, A., and Livesey, F.J. (2018). Extracellular Monomeric and Aggregated Tau Efficiently Enter Human Neurons through Overlapping but Distinct Pathways. Cell Rep 22, 3612-3624.

Falcon, B., Cavallini, A., Angers, R., Glover, S., Murray, T.K., Barnham, L., Jackson, S., O'Neill, M.J., Isaacs, A.M., Hutton, M.L., *et al*. (2015). Conformation determines the seeding potencies of native and recombinant Tau aggregates. J Biol Chem 290, 1049-1065.

Falcon, B., Zhang, W., Murzin, A.G., Murshudov, G., Garringer, H.J., Vidal, R., Crowther, R.A., Ghetti, B., Scheres, S.H.W., and Goedert, M. (2018). Structures of filaments from Pick's disease reveal a novel tau protein fold. Nature 561, 137-140.

Feany, M.B., Mattiace, L.A., and Dickson, D.W. (1996). Neuropathologic overlap of progressive supranuclear palsy, Pick's disease and corticobasal degeneration. J Neuropathol Exp Neurol 55, 53-67.

Fein, J.A., Sokolow, S., Miller, C.A., Vinters, H.V., Yang, F., Cole, G.M., and Gylys, K.H. (2008). Co-localization of amyloid beta and tau pathology in Alzheimer's disease synaptosomes. Am J Pathol 172, 1683-1692.

Ferrer, I., Lopez-Gonzalez, I., Carmona, M., Arregui, L., Dalfo, E., Torrejon-Escribano, B., Diehl, R., and Kovacs, G.G. (2014). Glial and neuronal tau pathology in tauopathies: characterization of disease-specific phenotypes and tau pathology progression. J Neuropathol Exp Neurol 73, 81-97.

Fevrier, B., and Raposo, G. (2004). Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr Opin Cell Biol 16, 415-421.

Fischer, D., Mukrasch, M.D., Biernat, J., Bibow, S., Blackledge, M., Griesinger, C., Mandelkow, E., and Zweckstetter, M. (2009). Conformational changes specific for pseudophosphorylation at serine 262 selectively impair binding of tau to microtubules. Biochemistry 48, 10047-10055.

Fisher, S., Grice, E.A., Vinton, R.M., Bessling, S.L., Urasaki, A., Kawakami, K., and McCallion, A.S. (2006). Evaluating the biological relevance of putative enhancers using Tol2 transposon-mediated transgenesis in zebrafish. Nat Protoc 1, 1297-1305.

Fitzpatrick, A.W.P., Falcon, B., He, S., Murzin, A.G., Murshudov, G., Garringer, H.J., Crowther, R.A., Ghetti, B., Goedert, M., and Scheres, S.H.W. (2017). Cryo-EM structures of tau filaments from Alzheimer's disease. Nature 547, 185-190.

Flach, K., Hilbrich, I., Schiffmann, A., Gartner, U., Kruger, M., Leonhardt, M., Waschipky, H., Wick, L., Arendt, T., and Holzer, M. (2012). Tau oligomers impair artificial membrane integrity and cellular viability. J Biol Chem 287, 43223-43233.

Fleisch, V.C., Leighton, P.L., Wang, H., Pillay, L.M., Ritzel, R.G., Bhinder, G., Roy, B., Tierney, K.B., Ali, D.W., Waskiewicz, A.J., and Allison, W.T. (2013). Targeted mutation of the gene encoding prion protein in zebrafish reveals a conserved role in neuron excitability. Neurobiol Dis 55, 11-25.

Fontaine, S.N., Zheng, D., Sabbagh, J.J., Martin, M.D., Chaput, D., Darling, A., Trotter, J.H., Stothert, A.R., Nordhues, B.A., Lussier, A., *et al*. (2016). DnaJ/Hsc70 chaperone complexes control the extracellular release of neurodegenerative-associated proteins. EMBO J 35, 1537-1549.

Forde, C.T., Karri, S.K., Young, A.M., and Ogilvy, C.S. (2014). Predictive markers in traumatic brain injury: opportunities for a serum biosignature. Br J Neurosurg 28, 8-15.

Forrest, S.L., Kril, J.J., Stevens, C.H., Kwok, J.B., Hallupp, M., Kim, W.S., Huang, Y., McGinley, C.V., Werka, H., Kiernan, M.C., *et al*. (2018). Retiring the term FTDP-17 as MAPT mutations are genetic forms of sporadic frontotemporal tauopathies. Brain 141, 521-534.

Fosque, B.F., Sun, Y., Dana, H., Yang, C.T., Ohyama, T., Tadross, M.R., Patel, R., Zlatic, M., Kim, D.S., Ahrens, M.B., *et al*. (2015). Neural circuits. Labeling of active neural circuits in vivo with designed calcium integrators. Science 347, 755-760.

Foster, N.L., Wilhelmsen, K., Sima, A.A., Jones, M.Z., D'Amato, C.J., and Gilman, S. (1997). Frontotemporal dementia and parkinsonism linked to chromosome 17: a consensus conference. Conference Participants. Ann Neurol 41, 706-715.

Frost, B., Jacks, R.L., and Diamond, M.I. (2009). Propagation of tau misfolding from the outside to the inside of a cell. J Biol Chem 284, 12845-12852.

Fu, T.S., Jing, R., McFaull, S.R., and Cusimano, M.D. (2016). Health & Economic Burden of Traumatic Brain Injury in the Emergency Department. Can J Neurol Sci 43, 238-247.

Gagnon, J.A., Valen, E., Thyme, S.B., Huang, P., Akhmetova, L., Pauli, A., Montague, T.G., Zimmerman, S., Richter, C., and Schier, A.F. (2014). Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. PLoS One 9, e98186.

Garcia-Cabrero, A.M., Guerrero-Lopez, R., Giraldez, B.G., Llorens-Martin, M., Avila, J., Serratosa, J.M., and Sanchez, M.P. (2013). Hyperexcitability and epileptic seizures in a model of frontotemporal dementia. Neurobiol Dis 58, 200-208.

Gardner, R.C., and Yaffe, K. (2015). Epidemiology of mild traumatic brain injury and neurodegenerative disease. Mol Cell Neurosci 66, 75-80.

Gartner, U., Janke, C., Holzer, M., Vanmechelen, E., and Arendt, T. (1998). Postmortem changes in the phosphorylation state of tau-protein in the rat brain. Neurobiol Aging 19, 535-543.

Geddes, J.F., Vowles, G.H., Nicoll, J.A., and Revesz, T. (1999). Neuronal cytoskeletal changes are an early consequence of repetitive head injury. Acta Neuropathol 98, 171-178.

Geisler, R., Kohler, A., Dickmeis, T., and Strahle, U. (2017). Archiving of zebrafish lines can reduce animal experiments in biomedical research. EMBO Rep 18, 1-2.

Gendron, T.F., and Petrucelli, L. (2009). The role of tau in neurodegeneration. Mol Neurodegener 4, 13.

Gerson, J., Castillo-Carranza, D.L., Sengupta, U., Bodani, R., Prough, D.S., DeWitt, D.S., Hawkins, B.E., and Kayed, R. (2016). Tau Oligomers Derived from Traumatic Brain Injury Cause Cognitive Impairment and Accelerate Onset of Pathology in Htau Mice. J Neurotrauma 33, 2034-2043.

Gerson, J.E., Sengupta, U., Lasagna-Reeves, C.A., Guerrero-Munoz, M.J., Troncoso, J., and Kayed, R. (2014). Characterization of tau oligomeric seeds in progressive supranuclear palsy. Acta Neuropathol Commun 2, 73.

Ghetti, B., Oblak, A.L., Boeve, B.F., Johnson, K.A., Dickerson, B.C., and Goedert, M. (2015). Invited review: Frontotemporal dementia caused by microtubule-associated protein tau gene (MAPT) mutations: a chameleon for neuropathology and neuroimaging. Neuropathol Appl Neurobiol 41, 24-46.

Ghoshal, N., Garcia-Sierra, F., Fu, Y., Beckett, L.A., Mufson, E.J., Kuret, J., Berry, R.W., and Binder, L.I. (2001). Tau-66: evidence for a novel tau conformation in Alzheimer's disease. J Neurochem 77, 1372-1385.

Giza, C.C., and Hovda, D.A. (2014). The new neurometabolic cascade of concussion. Neurosurgery 75 Suppl 4, S24-33.

Goedert, M., Eisenberg, D.S., and Crowther, R.A. (2017a). Propagation of Tau Aggregates and Neurodegeneration. Annu Rev Neurosci 40, 189-210.

Goedert, M., Ghetti, B., and Spillantini, M.G. (2012). Frontotemporal dementia: implications for understanding Alzheimer disease. Cold Spring Harb Perspect Med 2, a006254.

Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A., and Cohen, P. (1997). Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases. FEBS Lett 409, 57-62.

Goedert, M., and Jakes, R. (1990). Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. EMBO J 9, 4225-4230.

Goedert, M., Jakes, R., Spillantini, M.G., Hasegawa, M., Smith, M.J., and Crowther, R.A. (1996). Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. Nature 383, 550-553.

Goedert, M., Masuda-Suzukake, M., and Falcon, B. (2017b). Like prions: the propagation of aggregated tau and alpha-synuclein in neurodegeneration. Brain 140, 266-278.

Goedert, M., and Spillantini, M.G. (2017). Propagation of Tau aggregates. Mol Brain 10, 18.

Goedert, M., Spillantini, M.G., Jakes, R., Rutherford, D., and Crowther, R.A. (1989). Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3, 519-526.

Gomez-Ramos, A., Diaz-Hernandez, M., Rubio, A., Diaz-Hernandez, J.I., Miras-Portugal, M.T., and Avila, J. (2009). Characteristics and consequences of muscarinic receptor activation by tau protein. Eur Neuropsychopharmacol 19, 708-717.

Gong, C.X., Singh, T.J., Grundke-Iqbal, I., and Iqbal, K. (1993). Phosphoprotein phosphatase activities in Alzheimer disease brain. J Neurochem 61, 921-927.

Goodson, H.V., and Jonasson, E.M. (2018). Microtubules and Microtubule-Associated Proteins. Cold Spring Harb Perspect Biol 10.

Gorgoraptis, N., Li, L.M., Whittington, A., Zimmerman, K.A., Maclean, L.M., McLeod, C., Ross, E., Heslegrave, A., Zetterberg, H., Passchier, J., *et al*. (2019). In vivo detection of cerebral tau pathology in long-term survivors of traumatic brain injury. Sci Transl Med 11.

Gotz, J., Halliday, G., and Nisbet, R.M. (2019). Molecular Pathogenesis of the Tauopathies. Annu Rev Pathol 14, 239-261.

Gousset, K., Schiff, E., Langevin, C., Marijanovic, Z., Caputo, A., Browman, D.T., Chenouard, N., de Chaumont, F., Martino, A., Enninga, J., *et al*. (2009). Prions hijack tunnelling nanotubes for intercellular spread. Nat Cell Biol 11, 328-336.

Graves, A.B., White, E., Koepsell, T.D., Reifler, B.V., van Belle, G., Larson, E.B., and Raskind, M. (1990). The association between head trauma and Alzheimer's disease. Am J Epidemiol 131, 491-501.

Grundke-Iqbal, I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M., and Binder, L.I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U S A 83, 4913-4917.

Guo, J.L., and Lee, V.M. (2011). Seeding of normal Tau by pathological Tau conformers drives pathogenesis of Alzheimer-like tangles. J Biol Chem 286, 15317-15331.

Guo, J.L., Narasimhan, S., Changolkar, L., He, Z., Stieber, A., Zhang, B., Gathagan, R.J., Iba, M., McBride, J.D., Trojanowski, J.Q., and Lee, V.M. (2016). Unique pathological tau conformers from Alzheimer's brains transmit tau pathology in nontransgenic mice. J Exp Med 213, 2635-2654.

Guo, Z., Cupples, L.A., Kurz, A., Auerbach, S.H., Volicer, L., Chui, H., Green, R.C., Sadovnick, A.D., Duara, R., DeCarli, C., *et al*. (2000). Head injury and the risk of AD in the MIRAGE study. Neurology 54, 1316-1323.

Guryev, V., Koudijs, M.J., Berezikov, E., Johnson, S.L., Plasterk, R.H., van Eeden, F.J., and Cuppen, E. (2006). Genetic variation in the zebrafish. Genome Res 16, 491-497.

Gutzman, J.H., and Sive, H. (2009). Zebrafish brain ventricle injection. J Vis Exp.

Hamilton, L., Astell, K.R., Velikova, G., and Sieger, D. (2016). A Zebrafish Live Imaging Model Reveals Differential Responses of Microglia Toward Glioblastoma Cells In Vivo. Zebrafish 13, 523-534.

Hanger, D.P., Anderton, B.H., and Noble, W. (2009). Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. Trends Mol Med 15, 112-119.

Hanger, D.P., Byers, H.L., Wray, S., Leung, K.Y., Saxton, M.J., Seereeram, A., Reynolds, C.H., Ward, M.A., and Anderton, B.H. (2007). Novel phosphorylation sites in tau from Alzheimer brain support a role for casein kinase 1 in disease pathogenesis. J Biol Chem 282, 23645-23654.

Hanwell, D., Hutchinson, S.A., Collymore, C., Bruce, A.E., Louis, R., Ghalami, A., Allison, W.T., Ekker, M., Eames, B.F., Childs, S., *et al*. (2016). Restrictions on the Importation of Zebrafish into Canada Associated with Spring Viremia of Carp Virus. Zebrafish 13 Suppl 1, S153-163.

Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato-Yoshitake, R., Takei, Y., Noda, T., and Hirokawa, N. (1994). Altered microtubule organization in small-calibre axons of mice lacking tau protein. Nature 369, 488-491.

Harris, J.A., Koyama, A., Maeda, S., Ho, K., Devidze, N., Dubal, D.B., Yu, G.Q., Masliah, E., and Mucke, L. (2012). Human P301L-mutant tau expression in mouse entorhinal-hippocampal network causes tau aggregation and presynaptic pathology but no cognitive deficits. PLoS One 7, e45881.

Hart, N.J., Koronyo, Y., Black, K.L., and Koronyo-Hamaoui, M. (2016). Ocular indicators of Alzheimer's: exploring disease in the retina. Acta Neuropathol 132, 767-787.

Hawkins, B.E., Krishnamurthy, S., Castillo-Carranza, D.L., Sengupta, U., Prough, D.S., Jackson, G.R., DeWitt, D.S., and Kayed, R. (2013). Rapid accumulation of endogenous tau oligomers in a rat model of traumatic brain injury: possible link between traumatic brain injury and sporadic tauopathies. J Biol Chem 288, 17042-17050.

Hay, J., Johnson, V.E., Smith, D.H., and Stewart, W. (2016). Chronic Traumatic Encephalopathy: The Neuropathological Legacy of Traumatic Brain Injury. Annu Rev Pathol 11, 21-45.

Hemphill, M.A., Dauth, S., Yu, C.J., Dabiri, B.E., and Parker, K.K. (2015). Traumatic brain injury and the neuronal microenvironment: a potential role for neuropathological mechanotransduction. Neuron 85, 1177-1192.

Heusermann, W., Hean, J., Trojer, D., Steib, E., von Bueren, S., Graff-Meyer, A., Genoud, C., Martin, K., Pizzato, N., Voshol, J., *et al*. (2016). Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER. J Cell Biol 213, 173-184.

Himmler, A., Drechsel, D., Kirschner, M.W., and Martin, D.W., Jr. (1989). Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. Mol Cell Biol 9, 1381-1388.

Hirokawa, N., Shiomura, Y., and Okabe, S. (1988). Tau proteins: the molecular structure and mode of binding on microtubules. J Cell Biol 107, 1449-1459.

Hisano, Y., Sakuma, T., Nakade, S., Ohga, R., Ota, S., Okamoto, H., Yamamoto, T., and Kawahara, A. (2015). Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. Sci Rep 5, 8841.

Holmes, B.B., DeVos, S.L., Kfoury, N., Li, M., Jacks, R., Yanamandra, K., Ouidja, M.O., Brodsky, F.M., Marasa, J., Bagchi, D.P., *et al*. (2013). Heparan sulfate proteoglycans mediate internalization and propagation of specific proteopathic seeds. Proc Natl Acad Sci U S A 110, E3138-3147.

Holmes, B.B., and Diamond, M.I. (2014). Prion-like properties of Tau protein: the importance of extracellular Tau as a therapeutic target. J Biol Chem 289, 19855-19861.

Horiguchi, T., Uryu, K., Giasson, B.I., Ischiropoulos, H., LightFoot, R., Bellmann, C., Richter-Landsberg, C., Lee, V.M., and Trojanowski, J.Q. (2003). Nitration of tau protein is linked to neurodegeneration in tauopathies. Am J Pathol 163, 1021-1031.

Houlden, H., Baker, M., Morris, H.R., MacDonald, N., Pickering-Brown, S., Adamson, J., Lees, A.J., Rossor, M.N., Quinn, N.P., Kertesz, A., *et al*. (2001). Corticobasal degeneration and progressive supranuclear palsy share a common tau haplotype. Neurology 56, 1702-1706.

Huber, B.R., Meabon, J.S., Martin, T.J., Mourad, P.D., Bennett, R., Kraemer, B.C., Cernak, I., Petrie, E.C., Emery, M.J., Swenson, E.R., *et al*. (2013). Blast exposure causes early and persistent aberrant phospho- and cleaved-tau expression in a murine model of mild blast-induced traumatic brain injury. J Alzheimers Dis 37, 309-323.

Huh, J.W., Laurer, H.L., Raghupathi, R., Helfaer, M.A., and Saatman, K.E. (2002). Rapid loss and partial recovery of neurofilament immunostaining following focal brain injury in mice. Exp Neurol 175, 198-208.

Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., *et al*. (1998). Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393, 702-705.

Iba, M., Guo, J.L., McBride, J.D., Zhang, B., Trojanowski, J.Q., and Lee, V.M. (2013). Synthetic tau fibrils mediate transmission of neurofibrillary tangles in a transgenic mouse model of Alzheimer's-like tauopathy. J Neurosci 33, 1024-1037.

Iba, M., McBride, J.D., Guo, J.L., Zhang, B., Trojanowski, J.Q., and Lee, V.M. (2015). Tau pathology spread in PS19 tau transgenic mice following locus coeruleus (LC) injections of synthetic tau fibrils is determined by the LC's afferent and efferent connections. Acta Neuropathol 130, 349-362.

Ilieva, H., Polymenidou, M., and Cleveland, D.W. (2009). Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol 187, 761-772.

Iliff, J.J., Chen, M.J., Plog, B.A., Zeppenfeld, D.M., Soltero, M., Yang, L., Singh, I., Deane, R., and Nedergaard, M. (2014). Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. J Neurosci 34, 16180-16193.

Inglese, M., Makani, S., Johnson, G., Cohen, B.A., Silver, J.A., Gonen, O., and Grossman, R.I. (2005). Diffuse axonal injury in mild traumatic brain injury: a diffusion tensor imaging study. J Neurosurg 103, 298-303.

Irwin, D.J. (2016). Tauopathies as clinicopathological entities. Parkinsonism Relat Disord 22 Suppl 1, S29-33.

Ittner, L.M., Ke, Y.D., Delerue, F., Bi, M., Gladbach, A., van Eersel, J., Wolfing, H., Chieng, B.C., Christie, M.J., Napier, I.A., *et al*. (2010). Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models. Cell 142, 387-397.

Iwata, M., Watanabe, S., Yamane, A., Miyasaka, T., and Misonou, H. (2019). Regulatory mechanisms for the axonal localization of tau protein in neurons. Mol Biol Cell 30, 2441-2457.

Iyer, A., Lapointe, N.E., Zielke, K., Berdynski, M., Guzman, E., Barczak, A., Chodakowska-Zebrowska, M., Barcikowska, M., Feinstein, S., and Zekanowski, C. (2013). A novel MAPT mutation, G55R, in a frontotemporal dementia patient leads to altered Tau function. PLoS One 8, e76409.

Jafari, S., Etminan, M., Aminzadeh, F., and Samii, A. (2013). Head injury and risk of Parkinson disease: a systematic review and meta-analysis. Mov Disord 28, 1222-1229.

Jarrett, J.T., and Lansbury, P.T., Jr. (1993). Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell 73, 1055-1058.

Jeganathan, S., von Bergen, M., Brutlach, H., Steinhoff, H.J., and Mandelkow, E. (2006). Global hairpin folding of tau in solution. Biochemistry 45, 2283-2293.

Jeganathan, S., von Bergen, M., Mandelkow, E.M., and Mandelkow, E. (2008). The natively unfolded character of tau and its aggregation to Alzheimer-like paired helical filaments. Biochemistry 47, 10526-10539.

Jiang, Z., Cote, J., Kwon, J.M., Goate, A.M., and Wu, J.Y. (2000). Aberrant splicing of tau pre-mRNA caused by intronic mutations associated with the inherited dementia frontotemporal dementia with parkinsonism linked to chromosome 17. Mol Cell Biol 20, 4036-4048.

Johnson, N.R., Condello, C., Guan, S., Oehler, A., Becker, J., Gavidia, M., Carlson, G.A., Giles, K., and Prusiner, S.B. (2017). Evidence for sortilin modulating regional accumulation of human tau prions in transgenic mice. Proc Natl Acad Sci U S A 114, E11029-E11036.

Johnson, V.E., Stewart, J.E., Begbie, F.D., Trojanowski, J.Q., Smith, D.H., and Stewart, W. (2013). Inflammation and white matter degeneration persist for years after a single traumatic brain injury. Brain 136, 28-42.

Johnson, V.E., Stewart, W., and Smith, D.H. (2012). Widespread tau and amyloid-beta pathology many years after a single traumatic brain injury in humans. Brain Pathol 22, 142-149.

Jordan, B.D. (2013). The clinical spectrum of sport-related traumatic brain injury. Nat Rev Neurol 9, 222-230.

Jordan, B.D., Relkin, N.R., Ravdin, L.D., Jacobs, A.R., Bennett, A., and Gandy, S. (1997). Apolipoprotein E epsilon4 associated with chronic traumatic brain injury in boxing. JAMA 278, 136-140.

Kadavath, H., Hofele, R.V., Biernat, J., Kumar, S., Tepper, K., Urlaub, H., Mandelkow, E., and Zweckstetter, M. (2015). Tau stabilizes microtubules by binding at the interface between tubulin heterodimers. Proc Natl Acad Sci U S A 112, 7501-7506.

Kampers, T., Friedhoff, P., Biernat, J., Mandelkow, E.M., and Mandelkow, E. (1996). RNA stimulates aggregation of microtubule-associated protein tau into Alzheimer-like paired helical filaments. FEBS Lett 399, 344-349.

Kanemaru, K., Takio, K., Miura, R., Titani, K., and Ihara, Y. (1992). Fetal-type phosphorylation of the tau in paired helical filaments. J Neurochem 58, 1667-1675.

Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325, 733-736.

Karch, C.M., Jeng, A.T., and Goate, A.M. (2012). Extracellular Tau levels are influenced by variability in Tau that is associated with tauopathies. J Biol Chem 287, 42751-42762.

Kasatkina, L.A. (2016). 4-capital A, Cyrillicminopyridine sequesters intracellular Ca(2+) which triggers exocytosis in excitable and non-excitable cells. Sci Rep 6, 34749.

Katsinelos, T., Zeitler, M., Dimou, E., Karakatsani, A., Muller, H.M., Nachman, E., Steringer, J.P., Ruiz de Almodovar, C., Nickel, W., and Jahn, T.R. (2018). Unconventional Secretion Mediates the Trans-cellular Spreading of Tau. Cell Rep 23, 2039-2055.

Kaufman, S.K., Sanders, D.W., Thomas, T.L., Ruchinskas, A.J., Vaquer-Alicea, J., Sharma, A.M., Miller, T.M., and Diamond, M.I. (2016). Tau Prion Strains Dictate Patterns of Cell Pathology, Progression Rate, and Regional Vulnerability In Vivo. Neuron 92, 796-812.

Ke, Y.D., Suchowerska, A.K., van der Hoven, J., De Silva, D.M., Wu, C.W., van Eersel, J., Ittner, A., and Ittner, L.M. (2012). Lessons from tau-deficient mice. Int J Alzheimers Dis 2012, 873270.

Kempf, M., Clement, A., Faissner, A., Lee, G., and Brandt, R. (1996). Tau binds to the distal axon early in development of polarity in a microtubule- and microfilament-dependent manner. J Neurosci 16, 5583-5592.

Khalil, M., Teunissen, C.E., Otto, M., Piehl, F., Sormani, M.P., Gattringer, T., Barro, C., Kappos, L., Comabella, M., Fazekas, F., *et al*. (2018). Neurofilaments as biomarkers in neurological disorders. Nat Rev Neurol 14, 577-589.

Khlistunova, I., Biernat, J., Wang, Y., Pickhardt, M., von Bergen, M., Gazova, Z., Mandelkow, E., and Mandelkow, E.M. (2006). Inducible expression of Tau repeat domain in cell models of tauopathy: aggregation is toxic to cells but can be reversed by inhibitor drugs. J Biol Chem 281, 1205-1214.

Kim, S.U., and de Vellis, J. (2005). Microglia in health and disease. J Neurosci Res 81, 302-313.

Kimura, T., Whitcomb, D.J., Jo, J., Regan, P., Piers, T., Heo, S., Brown, C., Hashikawa, T., Murayama, M., Seok, H., *et al*. (2014). Microtubule-associated protein tau is essential for long-term depression in the hippocampus. Philos Trans R Soc Lond B Biol Sci 369, 20130144.

Kondo, A., Shahpasand, K., Mannix, R., Qiu, J., Moncaster, J., Chen, C.H., Yao, Y., Lin, Y.M., Driver, J.A., Sun, Y., *et al*. (2015). Antibody against early driver of neurodegeneration cis P-tau blocks brain injury and tauopathy. Nature 523, 431-436.

Kopeikina, K.J., Hyman, B.T., and Spires-Jones, T.L. (2012). Soluble forms of tau are toxic in Alzheimer's disease. Transl Neurosci 3, 223-233.

Kopke, E., Tung, Y.C., Shaikh, S., Alonso, A.C., Iqbal, K., and Grundke-Iqbal, I. (1993). Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. J Biol Chem 268, 24374-24384.

Korkut, C., Li, Y., Koles, K., Brewer, C., Ashley, J., Yoshihara, M., and Budnik, V. (2013). Regulation of postsynaptic retrograde signaling by presynaptic exosome release. Neuron 77, 1039-1046.

Kosik, K.S., Joachim, C.L., and Selkoe, D.J. (1986). Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease. Proc Natl Acad Sci U S A 83, 4044-4048.

Kouri, N., Carlomagno, Y., Baker, M., Liesinger, A.M., Caselli, R.J., Wszolek, Z.K., Petrucelli, L., Boeve, B.F., Parisi, J.E., Josephs, K.A., *et al*. (2014). Novel mutation in MAPT exon 13 (p.N410H) causes corticobasal degeneration. Acta Neuropathol 127, 271-282.

Kovacs, G.G. (2017). Tauopathies. Handb Clin Neurol 145, 355-368.

Kovacs, S.K., Leonessa, F., and Ling, G.S. (2014). Blast TBI Models, Neuropathology, and Implications for Seizure Risk. Front Neurol 5, 47.

Kuchibhotla, K.V., Wegmann, S., Kopeikina, K.J., Hawkes, J., Rudinskiy, N., Andermann, M.L., Spires-Jones, T.L., Bacskai, B.J., and Hyman, B.T. (2014). Neurofibrillary tangle-bearing neurons are functionally integrated in cortical circuits in vivo. Proc Natl Acad Sci U S A 111, 510-514.

Kuret, J., Chirita, C.N., Congdon, E.E., Kannanayakal, T., Li, G., Necula, M., Yin, H., and Zhong, Q. (2005). Pathways of tau fibrillization. Biochim Biophys Acta 1739, 167-178.

Kwan, K.M., Fujimoto, E., Grabher, C., Mangum, B.D., Hardy, M.E., Campbell, D.S., Parant, J.M., Yost, H.J., Kanki, J.P., and Chien, C.B. (2007). The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Dev Dyn 236, 3088-3099.

LaPointe, N.E., Morfini, G., Pigino, G., Gaisina, I.N., Kozikowski, A.P., Binder, L.I., and Brady, S.T. (2009). The amino terminus of tau inhibits kinesin-dependent axonal transport: implications for filament toxicity. J Neurosci Res 87, 440-451.

Lasagna-Reeves, C.A., Castillo-Carranza, D.L., Sengupta, U., Clos, A.L., Jackson, G.R., and Kayed, R. (2011). Tau oligomers impair memory and induce synaptic and mitochondrial dysfunction in wild-type mice. Mol Neurodegener 6, 39.

Lasagna-Reeves, C.A., Castillo-Carranza, D.L., Sengupta, U., Guerrero-Munoz, M.J., Kiritoshi, T., Neugebauer, V., Jackson, G.R., and Kayed, R. (2012a). Alzheimer brain-derived tau oligomers propagate pathology from endogenous tau. Sci Rep 2, 700.

Lasagna-Reeves, C.A., Castillo-Carranza, D.L., Sengupta, U., Sarmiento, J., Troncoso, J., Jackson, G.R., and Kayed, R. (2012b). Identification of oligomers at early stages of tau aggregation in Alzheimer's disease. FASEB J 26, 1946-1959.

Lee, G. (2005). Tau and src family tyrosine kinases. Biochim Biophys Acta 1739, 323-330.

Lee, G., Cowan, N., and Kirschner, M. (1988). The primary structure and heterogeneity of tau protein from mouse brain. Science 239, 285-288.

Lee, G., and Leugers, C.J. (2012). Tau and tauopathies. Prog Mol Biol Transl Sci 107, 263-293.

Lee, G., Newman, S.T., Gard, D.L., Band, H., and Panchamoorthy, G. (1998). Tau interacts with src-family non-receptor tyrosine kinases. J Cell Sci 111 (Pt 21), 3167-3177.

Lee, G., Thangavel, R., Sharma, V.M., Litersky, J.M., Bhaskar, K., Fang, S.M., Do, L.H., Andreadis, A., Van Hoesen, G., and Ksiezak-Reding, H. (2004). Phosphorylation of tau by fyn: implications for Alzheimer's disease. J Neurosci 24, 2304-2312.

Lee, H., Wintermark, M., Gean, A.D., Ghajar, J., Manley, G.T., and Mukherjee, P. (2008). Focal lesions in acute mild traumatic brain injury and neurocognitive outcome: CT versus 3T MRI. J Neurotrauma 25, 1049-1056.

Leighton, P.L., and Allison, W.T. (2016). Protein Misfolding in Prion and Prion-Like Diseases: Reconsidering a Required Role for Protein Loss-of-Function. J Alzheimers Dis 54, 3-29.

Leighton, P.L.A., Kanyo, R., Neil, G.J., Pollock, N.M., and Allison, W.T. (2018). Prion gene paralogs are dispensable for early zebrafish development and have nonadditive roles in seizure susceptibility. J Biol Chem 293, 12576-12592.

Lewis, J., and Dickson, D.W. (2016). Propagation of tau pathology: hypotheses, discoveries, and yet unresolved questions from experimental and human brain studies. Acta Neuropathol 131, 27-48.

Liang, Z., Liu, F., Iqbal, K., Grundke-Iqbal, I., and Gong, C.X. (2009). Dysregulation of tau phosphorylation in mouse brain during excitotoxic damage. J Alzheimers Dis 17, 531-539.

Liliang, P.C., Liang, C.L., Lu, K., Wang, K.W., Weng, H.C., Hsieh, C.H., Tsai, Y.D., and Chen, H.J. (2010). Relationship between injury severity and serum tau protein levels in traumatic brain injured rats. Resuscitation 81, 1205-1208.

Lim, J., and Yue, Z. (2015). Neuronal aggregates: formation, clearance, and spreading. Dev Cell 32, 491-501.

Lindwall, G., and Cole, R.D. (1984). Phosphorylation affects the ability of tau protein to promote microtubule assembly. J Biol Chem 259, 5301-5305.

Liu, J., and Baraban, S.C. (2019). Network Properties Revealed during Multi-Scale Calcium Imaging of Seizure Activity in Zebrafish. eNeuro 6.

Liu, L., Drouet, V., Wu, J.W., Witter, M.P., Small, S.A., Clelland, C., and Duff, K. (2012). Trans-synaptic spread of tau pathology in vivo. PLoS One 7, e31302.

LoPresti, P., Szuchet, S., Papasozomenos, S.C., Zinkowski, R.P., and Binder, L.I. (1995). Functional implications for the microtubule-associated protein tau: localization in oligodendrocytes. Proc Natl Acad Sci U S A 92, 10369-10373.

Lucke-Wold, B.P., Nguyen, L., Turner, R.C., Logsdon, A.F., Chen, Y.W., Smith, K.E., Huber, J.D., Matsumoto, R., Rosen, C.L., Tucker, E.S., and Richter, E. (2015). Traumatic brain injury and epilepsy: Underlying mechanisms leading to seizure. Seizure 33, 13-23.

Lundh, H. (1978). Effects of 4-aminopyridine on neuromuscular transmission. Brain Res 153, 307-318.

Maeda, S., Sahara, N., Saito, Y., Murayama, S., Ikai, A., and Takashima, A. (2006). Increased levels of granular tau oligomers: an early sign of brain aging and Alzheimer's disease. Neurosci Res 54, 197-201.

Magnoni, S., Esparza, T.J., Conte, V., Carbonara, M., Carrabba, G., Holtzman, D.M., Zipfel, G.J., Stocchetti, N., and Brody, D.L. (2012). Tau elevations in the brain extracellular space correlate with reduced amyloid-beta levels and predict adverse clinical outcomes after severe traumatic brain injury. Brain 135, 1268-1280.

Maheras, A.L., Dix, B., Carmo, O.M.S., Young, A.E., Gill, V.N., Sun, J.L., Booker, A.R., Thomason, H.A., Ibrahim, A.E., Stanislaw, L., *et al*. (2018). Genetic Pathways of Neuroregeneration in a Novel Mild Traumatic Brain Injury Model in Adult Zebrafish. eNeuro 5.

Maina, M.B., Bailey, L.J., Doherty, A.J., and Serpell, L.C. (2018a). The Involvement of Abeta42 and Tau in Nucleolar and Protein Synthesis Machinery Dysfunction. Front Cell Neurosci 12, 220.

Maina, M.B., Bailey, L.J., Wagih, S., Biasetti, L., Pollack, S.J., Quinn, J.P., Thorpe, J.R., Doherty, A.J., and Serpell, L.C. (2018b). The involvement of tau in nucleolar transcription and the stress response. Acta Neuropathol Commun 6, 70.

Mandelkow, E.M., Biernat, J., Drewes, G., Gustke, N., Trinczek, B., and Mandelkow, E. (1995). Tau domains, phosphorylation, and interactions with microtubules. Neurobiol Aging 16, 355-362; discussion 362-353.

Mandelkow, E.M., and Mandelkow, E. (2012). Biochemistry and cell biology of tau protein in neurofibrillary degeneration. Cold Spring Harb Perspect Med 2, a006247.

Mannix, R., and Meehan, W.P., III (2015). Evaluating the Effects of APOE4 after Mild Traumatic Brain Injury in Experimental Models. In Brain Neurotrauma: Molecular, Neuropsychological, and Rehabilitation Aspects, F.H. Kobeissy, ed. (Boca Raton (FL)).

Mansuroglu, Z., Benhelli-Mokrani, H., Marcato, V., Sultan, A., Violet, M., Chauderlier, A., Delattre, L., Loyens, A., Talahari, S., Begard, S., *et al*. (2016). Loss of Tau protein affects the structure, transcription and repair of neuronal pericentromeric heterochromatin. Sci Rep 6, 33047.

Masel, B.E., and DeWitt, D.S. (2010). Traumatic brain injury: a disease process, not an event. J Neurotrauma 27, 1529-1540.

Matsuo, E.S., Shin, R.W., Billingsley, M.L., Van deVoorde, A., O'Connor, M., Trojanowski, J.Q., and Lee, V.M. (1994). Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. Neuron 13, 989-1002.

Matsuo, Y., Tomita, S., Tsuneoka, Y., Furukawa, A., and Ichikawa, Y. (1992). Molecular cloning and nucleotide sequences of bovine hepato-ferredoxin cDNA; identical primary structures of hepato- and adreno-ferredoxins. Int J Biochem 24, 289-295.

Mattson, M.P., and Magnus, T. (2006). Ageing and neuronal vulnerability. Nat Rev Neurosci 7, 278-294.

McCluskey, A., Daniel, J.A., Hadzic, G., Chau, N., Clayton, E.L., Mariana, A., Whiting, A., Gorgani, N.N., Lloyd, J., Quan, A.*, et al*. (2013). Building a better dynasore: the dyngo compounds potently inhibit dynamin and endocytosis. Traffic 14, 1272-1289.

McCutcheon, V., Park, E., Liu, E., Sobhebidari, P., Tavakkoli, J., Wen, X.Y., and Baker, A.J. (2017). A Novel Model of Traumatic Brain Injury in Adult Zebrafish Demonstrates Response to Injury and Treatment Comparable with Mammalian Models. J Neurotrauma 34, 1382-1393.

McGeachie, A.B., Odell, L.R., Quan, A., Daniel, J.A., Chau, N., Hill, T.A., Gorgani, N.N., Keating, D.J., Cousin, M.A., van Dam, E.M.*, et al*. (2013). Pyrimidyn compounds: dual-action small molecule pyrimidine-based dynamin inhibitors. ACS Chem Biol 8, 1507-1518.

McKee, A.C., Abdolmohammadi, B., and Stein, T.D. (2018). The neuropathology of chronic traumatic encephalopathy. Handb Clin Neurol 158, 297-307.

McKee, A.C., Cairns, N.J., Dickson, D.W., Folkerth, R.D., Keene, C.D., Litvan, I., Perl, D.P., Stein, T.D., Vonsattel, J.P., Stewart, W.*, et al*. (2016). The first NINDS/NIBIB consensus meeting to define neuropathological criteria for the diagnosis of chronic traumatic encephalopathy. Acta Neuropathol 131, 75-86.

McKee, A.C., and Daneshvar, D.H. (2015). The neuropathology of traumatic brain injury. Handb Clin Neurol 127, 45-66.

McKee, A.C., Stein, T.D., Kiernan, P.T., and Alvarez, V.E. (2015). The neuropathology of chronic traumatic encephalopathy. Brain Pathol 25, 350-364.

McKee, A.C., Stern, R.A., Nowinski, C.J., Stein, T.D., Alvarez, V.E., Daneshvar, D.H., Lee, H.S., Wojtowicz, S.M., Hall, G., Baugh, C.M., *et al*. (2013). The spectrum of disease in chronic traumatic encephalopathy. Brain 136, 43-64.

Meaney, D.F., and Smith, D.H. (2011). Biomechanics of concussion. Clin Sports Med 30, 19-31, vii.

Medina, M., Hernandez, F., and Avila, J. (2016). New Features about Tau Function and Dysfunction. Biomolecules 6.

Melkova, K., Zapletal, V., Narasimhan, S., Jansen, S., Hritz, J., Skrabana, R., Zweckstetter, M., Ringkjobing Jensen, M., Blackledge, M., and Zidek, L. (2019). Structure and Functions of Microtubule Associated Proteins Tau and MAP2c: Similarities and Differences. Biomolecules 9.

Merezhko, M., Brunello, C.A., Yan, X., Vihinen, H., Jokitalo, E., Uronen, R.L., and Huttunen, H.J. (2018). Secretion of Tau via an Unconventional Non-vesicular Mechanism. Cell Rep 25, 2027-2035 e2024.

Michel, C.H., Kumar, S., Pinotsi, D., Tunnacliffe, A., St George-Hyslop, P., Mandelkow, E., Mandelkow, E.M., Kaminski, C.F., and Kaminski Schierle, G.S. (2014). Extracellular monomeric tau protein is sufficient to initiate the spread of tau protein pathology. J Biol Chem 289, 956-967.

Mirbaha, H., Holmes, B.B., Sanders, D.W., Bieschke, J., and Diamond, M.I. (2015). Tau Trimers Are the Minimal Propagation Unit Spontaneously Internalized to Seed Intracellular Aggregation. J Biol Chem 290, 14893-14903.

Moraga, D.M., Nunez, P., Garrido, J., and Maccioni, R.B. (1993). A tau fragment containing a repetitive sequence induces bundling of actin filaments. J Neurochem 61, 979-986.

Morales, R. (2017). Prion strains in mammals: Different conformations leading to disease. PLoS Pathog 13, e1006323.

Morales, R., Abid, K., and Soto, C. (2007). The prion strain phenomenon: molecular basis and unprecedented features. Biochim Biophys Acta 1772, 681-691.

Mori, H., Kondo, J., and Ihara, Y. (1987). Ubiquitin is a component of paired helical filaments in Alzheimer's disease. Science 235, 1641-1644.

Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Watanabe, A., Titani, K., and Ihara, Y. (1995). Hyperphosphorylation of tau in PHF. Neurobiol Aging 16, 365-371; discussion 371-380.

Morozova, O.A., March, Z.M., Robinson, A.S., and Colby, D.W. (2013). Conformational features of tau fibrils from Alzheimer's disease brain are faithfully propagated by unmodified recombinant protein. Biochemistry 52, 6960-6967.

Morris, M., Knudsen, G.M., Maeda, S., Trinidad, J.C., Ioanoviciu, A., Burlingame, A.L., and Mucke, L. (2015). Tau post-translational modifications in wild-type and human amyloid precursor protein transgenic mice. Nat Neurosci 18, 1183-1189.

Mudher, A., Colin, M., Dujardin, S., Medina, M., Dewachter, I., Alavi Naini, S.M., Mandelkow, E.M., Mandelkow, E., Buee, L., Goedert, M., and Brion, J.P. (2017). What is the evidence that tau pathology spreads through prion-like propagation? Acta Neuropathol Commun 5, 99.

Mukrasch, M.D., Bibow, S., Korukottu, J., Jeganathan, S., Biernat, J., Griesinger, C., Mandelkow, E., and Zweckstetter, M. (2009). Structural polymorphism of 441-residue tau at single residue resolution. PLoS Biol 7, e34.

Mukrasch, M.D., von Bergen, M., Biernat, J., Fischer, D., Griesinger, C., Mandelkow, E., and Zweckstetter, M. (2007). The "jaws" of the tau-microtubule interaction. J Biol Chem 282, 12230-12239.

Musa, A., Lehrach, H., and Russo, V.A. (2001). Distinct expression patterns of two zebrafish homologues of the human APP gene during embryonic development. Dev Genes Evol 211, 563-567.

Nakagawa, A., Manley, G.T., Gean, A.D., Ohtani, K., Armonda, R., Tsukamoto, A., Yamamoto, H., Takayama, K., and Tominaga, T. (2011). Mechanisms of primary blast-induced traumatic brain injury: insights from shock-wave research. J Neurotrauma 28, 1101-1119.

Nakamura, K., Greenwood, A., Binder, L., Bigio, E.H., Denial, S., Nicholson, L., Zhou, X.Z., and Lu, K.P. (2012). Proline isomer-specific antibodies reveal the early pathogenic tau conformation in Alzheimer's disease. Cell 149, 232-244.

Narasimhan, S., Guo, J.L., Changolkar, L., Stieber, A., McBride, J.D., Silva, L.V., He, Z., Zhang, B., Gathagan, R.J., Trojanowski, J.Q., and Lee, V.M.Y. (2017). Pathological Tau Strains from Human Brains Recapitulate the Diversity of Tauopathies in Nontransgenic Mouse Brain. J Neurosci 37, 11406-11423.

Nelson, P.T., Stefansson, K., Gulcher, J., and Saper, C.B. (1996). Molecular evolution of tau protein: implications for Alzheimer's disease. J Neurochem 67, 1622-1632.

Neve, R.L., Harris, P., Kosik, K.S., Kurnit, D.M., and Donlon, T.A. (1986). Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. Brain Res 387, 271-280.

Newman, M., Ebrahimie, E., and Lardelli, M. (2014). Using the zebrafish model for Alzheimer's disease research. Front Genet 5, 189.

Nguyen, R., Fiest, K.M., McChesney, J., Kwon, C.S., Jette, N., Frolkis, A.D., Atta, C., Mah, S., Dhaliwal, H., Reid, A., *et al.* (2016). The International Incidence of Traumatic Brain Injury: A Systematic Review and Meta-Analysis. Can J Neurol Sci 43, 774-785.

Nicolas, M., and Hassan, B.A. (2014). Amyloid precursor protein and neural development. Development 141, 2543-2548.

Nilsson, P., Saito, T., and Saido, T.C. (2014). New mouse model of Alzheimer's. ACS Chem Neurosci 5, 499-502.

Nizynski, B., Dzwolak, W., and Nieznanski, K. (2017). Amyloidogenesis of Tau protein. Protein Sci 26, 2126-2150.

Ojo, J.O., Mouzon, B., Algamal, M., Leary, P., Lynch, C., Abdullah, L., Evans, J., Mullan, M., Bachmeier, C., Stewart, W., and Crawford, F. (2016). Chronic Repetitive Mild Traumatic Brain Injury Results in Reduced Cerebral Blood Flow, Axonal Injury, Gliosis, and Increased T-Tau and Tau Oligomers. J Neuropathol Exp Neurol 75, 636-655.

Oka, T., Tagawa, K., Ito, H., and Okazawa, H. (2011). Dynamic changes of the phosphoproteome in postmortem mouse brains. PLoS One 6, e21405.

Olesen, O.F., Kawabata-Fukui, H., Yoshizato, K., and Noro, N. (2002). Molecular cloning of XTP, a tau-like microtubule-associated protein from Xenopus laevis tadpoles. Gene 283, 299-309.

Orr, M.E., Sullivan, A.C., and Frost, B. (2017). A Brief Overview of Tauopathy: Causes, Consequences, and Therapeutic Strategies. Trends Pharmacol Sci 38, 637-648.

Ota, S., Hisano, Y., Ikawa, Y., and Kawahara, A. (2014). Multiple genome modifications by the CRISPR/Cas9 system in zebrafish. Genes Cells 19, 555-564.

Parton, R.G., and Dotti, C.G. (1993). Cell biology of neuronal endocytosis. J Neurosci Res 36, 1-9.

Patterson, K.R., Remmers, C., Fu, Y., Brooker, S., Kanaan, N.M., Vana, L., Ward, S., Reyes, J.F., Philibert, K., Glucksman, M.J., and Binder, L.I. (2011). Characterization of prefibrillar Tau oligomers *in vitro* and in Alzheimer disease. J Biol Chem 286, 23063-23076.

Peeraer, E., Bottelbergs, A., Van Kolen, K., Stancu, I.C., Vasconcelos, B., Mahieu, M., Duytschaever, H., Ver Donck, L., Torremans, A., Sluydts, E., *et al*. (2015). Intracerebral injection of preformed synthetic tau fibrils initiates widespread tauopathy and neuronal loss in the brains of tau transgenic mice. Neurobiol Dis 73, 83-95.

Peineau, S., Taghibiglou, C., Bradley, C., Wong, T.P., Liu, L., Lu, J., Lo, E., Wu, D., Saule, E., Bouschet, T., *et al.* (2007). LTP inhibits LTD in the hippocampus via regulation of GSK3beta. Neuron 53, 703-717.

Perez, M., Avila, J., and Hernandez, F. (2019). Propagation of Tau via Extracellular Vesicles. Front Neurosci 13, 698.

Perl, D.P. (2010). Neuropathology of Alzheimer's disease. Mt Sinai J Med 77, 32-42.

Pickett, E.K., Henstridge, C.M., Allison, E., Pitstick, R., Pooler, A., Wegmann, S., Carlson, G., Hyman, B.T., and Spires-Jones, T.L. (2017). Spread of tau down neural circuits precedes synapse and neuronal loss in the rTgTauEC mouse model of early Alzheimer's disease. Synapse 71.

Ping, X., and Jin, X. (2016). Transition from Initial Hypoactivity to Hyperactivity in Cortical Layer V Pyramidal Neurons after Traumatic Brain Injury In Vivo. J Neurotrauma 33, 354-361.

Pittman, A.M., Fung, H.C., and de Silva, R. (2006). Untangling the tau gene association with neurodegenerative disorders. Hum Mol Genet 15 Spec No 2, R188-195.

Pittman, A.M., Myers, A.J., Abou-Sleiman, P., Fung, H.C., Kaleem, M., Marlowe, L., Duckworth, J., Leung, D., Williams, D., Kilford, L., *et al.* (2005). Linkage disequilibrium fine mapping and haplotype association analysis of the tau gene in progressive supranuclear palsy and corticobasal degeneration. J Med Genet 42, 837-846.

Planel, E., Miyasaka, T., Launey, T., Chui, D.H., Tanemura, K., Sato, S., Murayama, O., Ishiguro, K., Tatebayashi, Y., and Takashima, A. (2004). Alterations in glucose metabolism induce hypothermia leading to tau hyperphosphorylation through differential inhibition of kinase and phosphatase activities: implications for Alzheimer's disease. J Neurosci 24, 2401-2411.

Planel, E., Richter, K.E., Nolan, C.E., Finley, J.E., Liu, L., Wen, Y., Krishnamurthy, P., Herman, M., Wang, L., Schachter, J.B., *et al.* (2007). Anesthesia leads to tau hyperphosphorylation through inhibition of phosphatase activity by hypothermia. J Neurosci 27, 3090-3097.

Plassman, B.L., Havlik, R.J., Steffens, D.C., Helms, M.J., Newman, T.N., Drosdick, D., Phillips, C., Gau, B.A., Welsh-Bohmer, K.A., Burke, J.R., *et al*. (2000). Documented head injury in early adulthood and risk of Alzheimer's disease and other dementias. Neurology 55, 1158-1166.

Pokrishevsky, E., McAlary, L., Farrawell, N.E., Zhao, B., Sher, M., Yerbury, J.J., and Cashman, N.R. (2018). Tryptophan 32-mediated SOD1 aggregation is attenuated by pyrimidine-like compounds in living cells. Sci Rep 8, 15590.

Polanco, J.C., Scicluna, B.J., Hill, A.F., and Gotz, J. (2016). Extracellular Vesicles Isolated from the Brains of rTg4510 Mice Seed Tau Protein Aggregation in a Threshold-dependent Manner. J Biol Chem 291, 12445-12466.

Polymenidou, M., and Cleveland, D.W. (2012). Prion-like spread of protein aggregates in neurodegeneration. J Exp Med 209, 889-893.

Pooler, A.M., Phillips, E.C., Lau, D.H., Noble, W., and Hanger, D.P. (2013). Physiological release of endogenous tau is stimulated by neuronal activity. EMBO Rep 14, 389-394.

Poorkaj, P., Bird, T.D., Wijsman, E., Nemens, E., Garruto, R.M., Anderson, L., Andreadis, A., Wiederholt, W.C., Raskind, M., and Schellenberg, G.D. (1998). Tau is a candidate gene for chromosome 17 frontotemporal dementia. Ann Neurol 43, 815-825.

Prezel, E., Elie, A., Delaroche, J., Stoppin-Mellet, V., Bosc, C., Serre, L., Fourest-Lieuvin, A., Andrieux, A., Vantard, M., and Arnal, I. (2018). Tau can switch microtubule network organizations: from random networks to dynamic and stable bundles. Mol Biol Cell 29, 154-165.

Prusiner, S.B. (1982). Novel proteinaceous infectious particles cause scrapie. Science 216, 136-144.

Prusiner, S.B. (1998). Prions. Proc Natl Acad Sci U S A 95, 13363-13383.

Puvenna, V., Engeler, M., Banjara, M., Brennan, C., Schreiber, P., Dadas, A., Bahrami, A., Solanki, J., Bandyopadhyay, A., Morris, J.K., *et al*. (2016). Is phosphorylated tau unique to

chronic traumatic encephalopathy? Phosphorylated tau in epileptic brain and chronic traumatic encephalopathy. Brain Res 1630, 225-240.

Qiang, L., Sun, X., Austin, T.O., Muralidharan, H., Jean, D.C., Liu, M., Yu, W., and Baas, P.W. (2018). Tau Does Not Stabilize Axonal Microtubules but Rather Enables Them to Have Long Labile Domains. Curr Biol 28, 2181-2189 e2184.

Rademakers, R., Melquist, S., Cruts, M., Theuns, J., Del-Favero, J., Poorkaj, P., Baker, M., Sleegers, K., Crook, R., De Pooter, T., *et al*. (2005). High-density SNP haplotyping suggests altered regulation of tau gene expression in progressive supranuclear palsy. Hum Mol Genet 14, 3281-3292.

Ramkumar, A., Jong, B.Y., and Ori-McKenney, K.M. (2018). ReMAPping the microtubule landscape: How phosphorylation dictates the activities of microtubule-associated proteins. Dev Dyn 247, 138-155.

Rao, V.R., and Parko, K.L. (2015). Clinical approach to posttraumatic epilepsy. Semin Neurol 35, 57-63.

Regan, P., Piers, T., Yi, J.H., Kim, D.H., Huh, S., Park, S.J., Ryu, J.H., Whitcomb, D.J., and Cho, K. (2015). Tau phosphorylation at serine 396 residue is required for hippocampal LTD. J Neurosci 35, 4804-4812.

Regan, P., Whitcomb, D.J., and Cho, K. (2017). Physiological and Pathophysiological Implications of Synaptic Tau. Neuroscientist 23, 137-151.

Ren, Y., and Sahara, N. (2013). Characteristics of tau oligomers. Front Neurol 4, 102.

Rimel, R.W., Giordani, B., Barth, J.T., Boll, T.J., and Jane, J.A. (1981). Disability caused by minor head injury. Neurosurgery 9, 221-228.

Rodriguez, L., Mohamed, N.V., Desjardins, A., Lippe, R., Fon, E.A., and Leclerc, N. (2017). Rab7A regulates tau secretion. J Neurochem 141, 592-605.

Rosenqvist, N., Asuni, A.A., Andersson, C.R., Christensen, S., Daechsel, J.A., Egebjerg, J., Falsig, J., Helboe, L., Jul, P., Kartberg, F., *et al*. (2018). Highly specific and selective anti-pS396-tau antibody C10.2 targets seeding-competent tau. Alzheimers Dement (N Y) 4, 521-534.

Rossi, G., Conconi, D., Panzeri, E., Redaelli, S., Piccoli, E., Paoletta, L., Dalpra, L., and Tagliavini, F. (2013). Mutations in MAPT gene cause chromosome instability and introduce copy number variations widely in the genome. J Alzheimers Dis 33, 969-982.

Rubenstein, R., Chang, B., Davies, P., Wagner, A.K., Robertson, C.S., and Wang, K.K. (2015). A novel, ultrasensitive assay for tau: potential for assessing traumatic brain injury in tissues and biofluids. J Neurotrauma 32, 342-352.

Russell, W.M.S., and Burch, R.L. (1959). The principles of humane experimental technique (London,: Methuen).

Rustom, A., Saffrich, R., Markovic, I., Walther, P., and Gerdes, H.H. (2004). Nanotubular highways for intercellular organelle transport. Science 303, 1007-1010.

Saleem, S., and Kannan, R.R. (2018). Zebrafish: an emerging real-time model system to study Alzheimer's disease and neurospecific drug discovery. Cell Death Discov 4, 45.

Salehi, A., Zhang, J.H., and Obenaus, A. (2017). Response of the cerebral vasculature following traumatic brain injury. J Cereb Blood Flow Metab 37, 2320-2339.

Salinsky, M., Storzbach, D., Goy, E., and Evrard, C. (2015). Traumatic brain injury and psychogenic seizures in veterans. J Head Trauma Rehabil 30, E65-70.

Saman, S., Kim, W., Raya, M., Visnick, Y., Miro, S., Saman, S., Jackson, B., McKee, A.C., Alvarez, V.E., Lee, N.C., and Hall, G.F. (2012). Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. J Biol Chem 287, 3842-3849.

Samsonov, A., Yu, J.Z., Rasenick, M., and Popov, S.V. (2004). Tau interaction with microtubules in vivo. J Cell Sci 117, 6129-6141.

Sanchez, M.P., Garcia-Cabrero, A.M., Sanchez-Elexpuru, G., Burgos, D.F., and Serratosa, J.M. (2018). Tau-Induced Pathology in Epilepsy and Dementia: Notions from Patients and Animal Models. Int J Mol Sci 19.

Sanders, D.W., Kaufman, S.K., DeVos, S.L., Sharma, A.M., Mirbaha, H., Li, A., Barker, S.J., Foley, A.C., Thorpe, J.R., Serpell, L.C., *et al*. (2014). Distinct tau prion strains propagate in cells and mice and define different tauopathies. Neuron 82, 1271-1288.

Santa-Maria, I., Varghese, M., Ksiezak-Reding, H., Dzhun, A., Wang, J., and Pasinetti, G.M. (2012). Paired helical filaments from Alzheimer disease brain induce intracellular accumulation of Tau protein in aggresomes. J Biol Chem 287, 20522-20533.

Santacruz, K., Lewis, J., Spires, T., Paulson, J., Kotilinek, L., Ingelsson, M., Guimaraes, A., DeTure, M., Ramsden, M., McGowan, E., *et al*. (2005). Tau suppression in a neurodegenerative mouse model improves memory function. Science 309, 476-481.

Saxena, S., and Caroni, P. (2011). Selective neuronal vulnerability in neurodegenerative diseases: from stressor thresholds to degeneration. Neuron 71, 35-48.

Scheckel, C., and Aguzzi, A. (2018). Prions, prionoids and protein misfolding disorders. Nat Rev Genet 19, 405-418.

Schierhout, G., and Roberts, I. (1998). Prophylactic antiepileptic agents after head injury: a systematic review. J Neurol Neurosurg Psychiatry 64, 108-112.

Schmitt, S., and Dichter, M.A. (2015). Electrophysiologic recordings in traumatic brain injury. Handb Clin Neurol 127, 319-339.

Schoch, G., Seeger, H., Bogousslavsky, J., Tolnay, M., Janzer, R.C., Aguzzi, A., and Glatzel, M. (2006). Analysis of prion strains by PrPSc profiling in sporadic Creutzfeldt-Jakob disease. PLoS Med 3, e14.

Schofield, P.W., Tang, M., Marder, K., Bell, K., Dooneief, G., Chun, M., Sano, M., Stern, Y., and Mayeux, R. (1997). Alzheimer's disease after remote head injury: an incidence study. J Neurol Neurosurg Psychiatry 62, 119-124.

Serrano-Pozo, A., Mielke, M.L., Gomez-Isla, T., Betensky, R.A., Growdon, J.H., Frosch, M.P., and Hyman, B.T. (2011). Reactive glia not only associates with plaques but also parallels tangles in Alzheimer's disease. Am J Pathol 179, 1373-1384.

Shafiei, S.S., Guerrero-Munoz, M.J., and Castillo-Carranza, D.L. (2017). Tau Oligomers: Cytotoxicity, Propagation, and Mitochondrial Damage. Front Aging Neurosci 9, 83.

Shaw, N.A. (2002). The neurophysiology of concussion. Prog Neurobiol 67, 281-344.

Shaw-Smith, C., Pittman, A.M., Willatt, L., Martin, H., Rickman, L., Gribble, S., Curley, R., Cumming, S., Dunn, C., Kalaitzopoulos, D., *et al.* (2006). Microdeletion encompassing MAPT at chromosome 17q21.3 is associated with developmental delay and learning disability. Nat Genet 38, 1032-1037.

Shively, S., Scher, A.I., Perl, D.P., and Diaz-Arrastia, R. (2012). Dementia resulting from traumatic brain injury: what is the pathology? Arch Neurol 69, 1245-1251.

Siedler, D.G., Chuah, M.I., Kirkcaldie, M.T., Vickers, J.C., and King, A.E. (2014). Diffuse axonal injury in brain trauma: insights from alterations in neurofilaments. Front Cell Neurosci 8, 429.

Simic, G., Babic Leko, M., Wray, S., Harrington, C., Delalle, I., Jovanov-Milosevic, N., Bazadona, D., Buee, L., de Silva, R., Di Giovanni, G., *et al.* (2016). Tau Protein Hyperphosphorylation and Aggregation in Alzheimer's Disease and Other Tauopathies, and Possible Neuroprotective Strategies. Biomolecules 6, 6.

Simon, D., Garcia-Garcia, E., Gomez-Ramos, A., Falcon-Perez, J.M., Diaz-Hernandez, M., Hernandez, F., and Avila, J. (2012). Tau overexpression results in its secretion via membrane vesicles. Neurodegener Dis 10, 73-75.

Singh, M., Jadhav, H.R., and Bhatt, T. (2017). Dynamin Functions and Ligands: Classical Mechanisms Behind. Mol Pharmacol 91, 123-134.

Siskova, Z., Justus, D., Kaneko, H., Friedrichs, D., Henneberg, N., Beutel, T., Pitsch, J., Schoch, S., Becker, A., von der Kammer, H., and Remy, S. (2014). Dendritic structural degeneration is functionally linked to cellular hyperexcitability in a mouse model of Alzheimer's disease. Neuron 84, 1023-1033.

Sokolow, S., Henkins, K.M., Bilousova, T., Gonzalez, B., Vinters, H.V., Miller, C.A., Cornwell, L., Poon, W.W., and Gylys, K.H. (2015). Pre-synaptic C-terminal truncated tau is released from cortical synapses in Alzheimer's disease. J Neurochem 133, 368-379.

Song, J., Combs, C.K., Pilcher, W.H., Song, L.Y., Utal, A.K., and Coleman, P.D. (1997). Low initial tau phosphorylation in human brain biopsy samples. Neurobiol Aging 18, 475-481.

Sotiropoulos, I., Galas, M.C., Silva, J.M., Skoulakis, E., Wegmann, S., Maina, M.B., Blum, D., Sayas, C.L., Mandelkow, E.M., Mandelkow, E., *et al*. (2017). Atypical, non-standard functions of the microtubule associated Tau protein. Acta Neuropathol Commun 5, 91.

Spillantini, M.G., and Goedert, M. (2013). Tau pathology and neurodegeneration. Lancet Neurol 12, 609-622.

Spillantini, M.G., Goedert, M., Crowther, R.A., Murrell, J.R., Farlow, M.R., and Ghetti, B. (1997). Familial multiple system tauopathy with presenile dementia: a disease with abundant neuronal and glial tau filaments. Proc Natl Acad Sci U S A 94, 4113-4118.

Spires, T.L., Orne, J.D., SantaCruz, K., Pitstick, R., Carlson, G.A., Ashe, K.H., and Hyman, B.T. (2006). Region-specific dissociation of neuronal loss and neurofibrillary pathology in a mouse model of tauopathy. Am J Pathol 168, 1598-1607.

Stafstrom, C.E., and Carmant, L. (2015). Seizures and epilepsy: an overview for neuroscientists. Cold Spring Harb Perspect Med 5.

Stamer, K., Vogel, R., Thies, E., Mandelkow, E., and Mandelkow, E.M. (2002). Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress. J Cell Biol 156, 1051-1063.

Stanford, P.M., Halliday, G.M., Brooks, W.S., Kwok, J.B., Storey, C.E., Creasey, H., Morris, J.G., Fulham, M.J., and Schofield, P.R. (2000). Progressive supranuclear palsy pathology caused by a novel silent mutation in exon 10 of the tau gene: expansion of the disease phenotype caused by tau gene mutations. Brain 123 (Pt 5), 880-893.

Steen, P., Kalghatgi, L., and Constantine-Paton, M. (1989). Monoclonal antibody markers for amphibian oligodendrocytes and neurons. J Comp Neurol 289, 467-480.

Strich, S.J. (1956). Diffuse degeneration of the cerebral white matter in severe dementia following head injury. J Neurol Neurosurg Psychiatry 19, 163-185.

Swanson, E., Breckenridge, L., McMahon, L., Som, S., McConnell, I., and Bloom, G.S. (2017). Extracellular Tau Oligomers Induce Invasion of Endogenous Tau into the Somatodendritic Compartment and Axonal Transport Dysfunction. J Alzheimers Dis 58, 803-820.

Tai, H.C., Serrano-Pozo, A., Hashimoto, T., Frosch, M.P., Spires-Jones, T.L., and Hyman, B.T. (2012). The synaptic accumulation of hyperphosphorylated tau oligomers in Alzheimer disease is associated with dysfunction of the ubiquitin-proteasome system. Am J Pathol 181, 1426-1435.

Tai, X.Y., Koepp, M., Duncan, J.S., Fox, N., Thompson, P., Baxendale, S., Liu, J.Y., Reeves, C., Michalak, Z., and Thom, M. (2016). Hyperphosphorylated tau in patients with refractory epilepsy correlates with cognitive decline: a study of temporal lobe resections. Brain 139, 2441-2455.

Tan, D.C.S., Yao, S., Ittner, A., Bertz, J., Ke, Y.D., Ittner, L.M., and Delerue, F. (2018). Generation of a New Tau Knockout (tauDeltaex1) Line Using CRISPR/Cas9 Genome Editing in Mice. J Alzheimers Dis 62, 571-578.

Tardivel, M., Begard, S., Bousset, L., Dujardin, S., Coens, A., Melki, R., Buee, L., and Colin, M. (2016). Tunneling nanotube (TNT)-mediated neuron-to neuron transfer of pathological Tau protein assemblies. Acta Neuropathol Commun 4, 117.

Temkin, N.R. (2003). Risk factors for posttraumatic seizures in adults. Epilepsia 44, 18-20.

Tepper, K., Biernat, J., Kumar, S., Wegmann, S., Timm, T., Hubschmann, S., Redecke, L., Mandelkow, E.M., Muller, D.J., and Mandelkow, E. (2014). Oligomer formation of tau protein hyperphosphorylated in cells. J Biol Chem 289, 34389-34407.

Thom, M., Liu, J.Y., Thompson, P., Phadke, R., Narkiewicz, M., Martinian, L., Marsdon, D., Koepp, M., Caboclo, L., Catarino, C.B., and Sisodiya, S.M. (2011). Neurofibrillary tangle pathology and Braak staging in chronic epilepsy in relation to traumatic brain injury and hippocampal sclerosis: a post-mortem study. Brain 134, 2969-2981.

Tian, F.F., Zeng, C., Ma, Y.F., Guo, T.H., Chen, J.M., Chen, Y., Cai, X.F., Li, F.R., Wang, X.H., Huang, W.J., and Wang, Y.Z. (2010). Potential roles of Cdk5/p35 and tau protein in hippocampal mossy fiber sprouting in the PTZ kindling model. Clin Lab 56, 127-136.

Tian, H., Davidowitz, E., Lopez, P., Emadi, S., Moe, J., and Sierks, M. (2013). Trimeric tau is toxic to human neuronal cells at low nanomolar concentrations. Int J Cell Biol 2013, 260787.

Trabzuni, D., Wray, S., Vandrovcova, J., Ramasamy, A., Walker, R., Smith, C., Luk, C., Gibbs, J.R., Dillman, A., Hernandez, D.G., *et al*. (2012). MAPT expression and splicing is differentially regulated by brain region: relation to genotype and implication for tauopathies. Hum Mol Genet 21, 4094-4103.

Tran, H.T., Sanchez, L., Esparza, T.J., and Brody, D.L. (2011). Distinct temporal and anatomical distributions of amyloid-beta and tau abnormalities following controlled cortical impact in transgenic mice. PLoS One 6, e25475.

Traver, D., Paw, B.H., Poss, K.D., Penberthy, W.T., Lin, S., and Zon, L.I. (2003). Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. Nat Immunol 4, 1238-1246.

Uhlen, M., Fagerberg, L., Hallstrom, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, A., Kampf, C., Sjostedt, E., Asplund, A., *et al*. (2015). Proteomics. Tissue-based map of the human proteome. Science 347, 1260419.

Uryu, K., Chen, X.H., Martinez, D., Browne, K.D., Johnson, V.E., Graham, D.I., Lee, V.M., Trojanowski, J.Q., and Smith, D.H. (2007). Multiple proteins implicated in neurodegenerative diseases accumulate in axons after brain trauma in humans. Exp Neurol 208, 185-192.

Vartanian, M.G., Cordon, J.J., Kupina, N.C., Schielke, G.P., Posner, A., Raser, K.J., Wang, K.K., and Taylor, C.P. (1996). Phenytoin pretreatment prevents hypoxic-ischemic brain damage in neonatal rats. Brain Res Dev Brain Res 95, 169-175.

Verweij, F.J., Revenu, C., Arras, G., Dingli, F., Loew, D., Pegtel, D.M., Follain, G., Allio, G., Goetz, J.G., Zimmermann, P., *et al*. (2019). Live Tracking of Inter-organ Communication by Endogenous Exosomes In Vivo. Dev Cell 48, 573-589 e574.

Vespa, P.M., Miller, C., McArthur, D., Eliseo, M., Etchepare, M., Hirt, D., Glenn, T.C., Martin, N., and Hovda, D. (2007). Nonconvulsive electrographic seizures after traumatic brain injury result in a delayed, prolonged increase in intracranial pressure and metabolic crisis. Crit Care Med 35, 2830-2836.

Viotti, C. (2016). ER to Golgi-Dependent Protein Secretion: The Conventional Pathway. Methods Mol Biol 1459, 3-29.

Vouillot, L., Thelie, A., and Pollet, N. (2015). Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. G3 (Bethesda) 5, 407-415.

Wagner, U., Utton, M., Gallo, J.M., and Miller, C.C. (1996). Cellular phosphorylation of tau by GSK-3 beta influences tau binding to microtubules and microtubule organisation. J Cell Sci 109 (Pt 6), 1537-1543.

Wainger, B.J., Kiskinis, E., Mellin, C., Wiskow, O., Han, S.S., Sandoe, J., Perez, N.P., Williams, L.A., Lee, S., Boulting, G., *et al*. (2014). Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. Cell Rep 7, 1-11.

Wang, C., Ji, B., Cheng, B., Chen, J., and Bai, B. (2014). Neuroprotection of microRNA in neurological disorders (Review). Biomed Rep 2, 611-619.

Wang, Y., Balaji, V., Kaniyappan, S., Kruger, L., Irsen, S., Tepper, K., Chandupatla, R., Maetzler, W., Schneider, A., Mandelkow, E., and Mandelkow, E.M. (2017). The release and trans-synaptic transmission of Tau via exosomes. Mol Neurodegener 12, 5.

Wang, Y., and Mandelkow, E. (2016). Tau in physiology and pathology. Nat Rev Neurosci 17, 5-21.

Wang, Y., Zhang, Y., Hu, W., Xie, S., Gong, C.X., Iqbal, K., and Liu, F. (2015). Rapid alteration of protein phosphorylation during postmortem: implication in the study of protein phosphorylation. Sci Rep 5, 15709.

Wang, Z., Wang, B., Yang, L., Guo, Q., Aithmitti, N., Songyang, Z., and Zheng, H. (2009). Presynaptic and postsynaptic interaction of the amyloid precursor protein promotes peripheral and central synaptogenesis. J Neurosci 29, 10788-10801.

Ward, S.M., Himmelstein, D.S., Lancia, J.K., and Binder, L.I. (2012). Tau oligomers and tau toxicity in neurodegenerative disease. Biochem Soc Trans 40, 667-671.

Washington, P.M., Villapol, S., and Burns, M.P. (2016). Polypathology and dementia after brain trauma: Does brain injury trigger distinct neurodegenerative diseases, or should they be classified together as traumatic encephalopathy? Exp Neurol 275 Pt 3, 381-388.

Wegmann, S., Bennett, R.E., Delorme, L., Robbins, A.B., Hu, M., McKenzie, D., Kirk, M.J., Schiantarelli, J., Tunio, N., Amaral, A.C., *et al*. (2019). Experimental evidence for the age dependence of tau protein spread in the brain. Sci Adv 5, eaaw6404.

Wegmann, S., Medalsy, I.D., Mandelkow, E., and Muller, D.J. (2013). The fuzzy coat of pathological human Tau fibrils is a two-layered polyelectrolyte brush. Proc Natl Acad Sci U S A 110, E313-321.

Welty, D.F., Schielke, G.P., and Rothstein, J.D. (1995). Potential treatment of amyotrophic lateral sclerosis with gabapentin: a hypothesis. Ann Pharmacother 29, 1164-1167.

Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio), 4th edn (University of Oregon press, Eugene).

White, R.M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., Bourque, C., Dovey, M., Goessling, W., Burns, C.E., and Zon, L.I. (2008). Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell 2, 183-189.

Winter, M.J., Windell, D., Metz, J., Matthews, P., Pinion, J., Brown, J.T., Hetheridge, M.J., Ball, J.S., Owen, S.F., Redfern, W.S., *et al*. (2017). 4-dimensional functional profiling in the convulsant-treated larval zebrafish brain. Sci Rep 7, 6581.

Wischik, C.M., Novak, M., Thogersen, H.C., Edwards, P.C., Runswick, M.J., Jakes, R., Walker, J.E., Milstein, C., Roth, M., and Klug, A. (1988). Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. Proc Natl Acad Sci U S A 85, 4506-4510.

Wittmann, C.W., Wszolek, M.F., Shulman, J.M., Salvaterra, P.M., Lewis, J., Hutton, M., and Feany, M.B. (2001). Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. Science 293, 711-714.

Woerman, A.L., Aoyagi, A., Patel, S., Kazmi, S.A., Lobach, I., Grinberg, L.T., McKee, A.C., Seeley, W.W., Olson, S.H., and Prusiner, S.B. (2016). Tau prions from Alzheimer's disease and chronic traumatic encephalopathy patients propagate in cultured cells. Proc Natl Acad Sci U S A 113, E8187-E8196.

Wolfe, M.S. (2009). Tau mutations in neurodegenerative diseases. J Biol Chem 284, 6021-6025.

Wolfe, M.S. (2012). The role of tau in neurodegenerative diseases and its potential as a therapeutic target. Scientifica (Cairo) 2012, 796024.

Wooten, D.W., Ortiz-Teran, L., Zubcevik, N., Zhang, X., Huang, C., Sepulcre, J., Atassi, N., Johnson, K.A., Zafonte, R.D., and El Fakhri, G. (2019). Multi-Modal Signatures of Tau Pathology, Neuronal Fiber Integrity, and Functional Connectivity in Traumatic Brain Injury. J Neurotrauma 36, 3233-3243.

Wright, L.M., Stein, T.D., Jun, G., Chung, J., McConnell, K., Fiorello, M., Siegel, N., Ness, S., Xia, W., Turner, K.L., and Subramanian, M.L. (2019). Association of Cognitive Function with Amyloid-beta and Tau Proteins in the Vitreous Humor. J Alzheimers Dis 68, 1429-1438.

Wu, J.W., Herman, M., Liu, L., Simoes, S., Acker, C.M., Figueroa, H., Steinberg, J.I., Margittai, M., Kayed, R., Zurzolo, C., *et al*. (2013). Small misfolded Tau species are internalized via bulk endocytosis and anterogradely and retrogradely transported in neurons. J Biol Chem 288, 1856-1870.

Wu, J.W., Hussaini, S.A., Bastille, I.M., Rodriguez, G.A., Mrejeru, A., Rilett, K., Sanders, D.W., Cook, C., Fu, H., Boonen, R.A., *et al*. (2016). Neuronal activity enhances tau propagation and tau pathology in vivo. Nat Neurosci 19, 1085-1092.

Yamada, K., Cirrito, J.R., Stewart, F.R., Jiang, H., Finn, M.B., Holmes, B.B., Binder, L.I., Mandelkow, E.M., Diamond, M.I., Lee, V.M., and Holtzman, D.M. (2011). In vivo microdialysis reveals age-dependent decrease of brain interstitial fluid tau levels in P301S human tau transgenic mice. J Neurosci 31, 13110-13117.

Yamada, K., Holth, J.K., Liao, F., Stewart, F.R., Mahan, T.E., Jiang, H., Cirrito, J.R., Patel, T.K., Hochgrafe, K., Mandelkow, E.M., and Holtzman, D.M. (2014). Neuronal activity regulates extracellular tau in vivo. J Exp Med 211, 387-393.

Yan, X.X., Cai, Y., Shelton, J., Deng, S.H., Luo, X.G., Oddo, S., Laferla, F.M., Cai, H., Rose, G.M., and Patrylo, P.R. (2012). Chronic temporal lobe epilepsy is associated with enhanced Alzheimer-like neuropathology in 3xTg-AD mice. Plos One 7, e48782.

Yang, W.J., Chen, W., Chen, L., Guo, Y.J., Zeng, J.S., Li, G.Y., and Tong, W.S. (2017). Involvement of tau phosphorylation in traumatic brain injury patients. Acta Neurol Scand 135, 622-627.

Yi, S., Liu, Q., Wang, X., Qian, T., Wang, H., Zha, G., Yu, J., Wang, P., Gu, X., Chu, D., and Li, S. (2019). Tau modulates Schwann cell proliferation, migration and differentiation following peripheral nerve injury. J Cell Sci 132.

Yoshida, H., and Goedert, M. (2002). Molecular cloning and functional characterization of chicken brain tau: isoforms with up to five tandem repeats. Biochemistry 41, 15203-15211.

Yoshimura, Y., Ichinose, T., and Yamauchi, T. (2003). Phosphorylation of tau protein to sites found in Alzheimer's disease brain is catalyzed by Ca2+/calmodulin-dependent protein kinase II as demonstrated tandem mass spectrometry. Neurosci Lett 353, 185-188.

Yoshiyama, Y., Higuchi, M., Zhang, B., Huang, S.M., Iwata, N., Saido, T.C., Maeda, J., Suhara, T., Trojanowski, J.Q., and Lee, V.M. (2007). Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron 53, 337-351.

Zanier, E.R., Bertani, I., Sammali, E., Pischiutta, F., Chiaravalloti, M.A., Vegliante, G., Masone, A., Corbelli, A., Smith, D.H., Menon, D.K*., et al*. (2018). Induction of a transmissible tau pathology by traumatic brain injury. Brain 141, 2685-2699.

Zhang, B., Carroll, J., Trojanowski, J.Q., Yao, Y., Iba, M., Potuzak, J.S., Hogan, A.M., Xie, S.X., Ballatore, C., Smith, A.B., 3rd*, et al*. (2012). The microtubule-stabilizing agent, epothilone D, reduces axonal dysfunction, neurotoxicity, cognitive deficits, and Alzheimer-like pathology in an interventional study with aged tau transgenic mice. J Neurosci 32, 3601-3611.

Zheng, P., Shultz, S.R., Hovens, C.M., Velakoulis, D., Jones, N.C., and O'Brien, T.J. (2014). Hyperphosphorylated tau is implicated in acquired epilepsy and neuropsychiatric comorbidities. Mol Neurobiol 49, 1532-1539.

Zhou, L., McInnes, J., Wierda, K., Holt, M., Herrmann, A.G., Jackson, R.J., Wang, Y.C., Swerts, J., Beyens, J., Miskiewicz, K., *et al*. (2017). Tau association with synaptic vesicles causes presynaptic dysfunction. Nat Commun 8, 15295.

Appendix 1: Towards engineering an AD model in zebrafish

A.1 Summary:

Here, we aimed to engineer a new model of AD in zebrafish that would express human amyloid β fragment (Aβ) to offer additional advantages over old models in mimicking the disease, simplifying analysis (particularly at the cellular level), and screening for potential therapeutic measures. Unfortunately, we were unable to create a humanized AD zebrafish model using CRISPR/Cas9 genome editing. However, we were able to successfully isolate three stable *appb* mutant fish that could help uncover new physiological roles of Aβ.

A.2 Introduction:

Alzheimer's disease (AD) is incurable and is most prevalent form of neurodegenerative dementia defined by progressive neuronal loss, which causes cognitive impairment, behavioural disturbance, and psychiatric symptoms (Adlard and Cummings, 2004). In addition to neurofibrillary tangles (NFTs), AD patients develop extracellular amyloid plaques, which primarily constitute amyloid β peptide (Aβ) (Nicolas and Hassan, 2014; Perl, 2010). Aβ is produced from the proteolysis of amyloid precursor protein (APP), a transmembrane glycoprotein (Kang et al., 1987) that tends to play roles in many biological processes, such as synaptogenesis (Wang et al., 2009). However, the full range of APP physiological functions has not been fully explored. Inherited *APP* gene mutations are associated with the familial form of AD (Bekris et al., 2010). Although the generation of several animal models has provided valuable information on the pathogenesis of AD, the actual trigger of neurodegeneration in AD remains unknown. Besides, the transgenic animals used in numerous AD models significantly overexpress mutant APP above physiological levels, which may create an artificial phenotype and undesirable results (Cuello, 2005; Esquerda-Canals et al., 2017; Nilsson et al., 2014). Thus,

there may be a need to re-evaluate and increase understanding of the pathogenesis of AD in new models (humanized models) that could provide more insights into the disease and be adapted easily for therapeutic screening.

The zebrafish expresses two APP paralogs (*appa* and *appb*) that are quite conserved with the human *APP*, particularly the Aβ sequence **(Figure A.1)** and the transmembrane domain (Musa et al., 2001). Our aim is to create zebrafish models of Aβ toxicity using CRISPR/Cas9. Our approach is to use CRISPR to humanize zebrafish *appb*, particularly Aβ sequences, to produce $A\beta_{42}$. One of the advantages of using CRISPR technology is being able to obtain *appb* knockout fish for investigating the physiological functions of APP. In this regard, we will use *appa* homozygous knockout fish ($appa^{ua5005/ua5005}$) generated in our lab to reduce the complexity of the subsequent analysis. We successfully isolated two stable compound *appa* and *appb* knockout fish. However, we were unable to isolate any humanized zebrafish.

A.3 Methods

A.3.1 CRISPR mutagenesis:

To humanize zebrafish, various *appb* CRISPR targets were identified in the region of interest of the *appb* gene (exon 14 and 15) using Geneious software. Two CRISPR targets (listed in table A.1, Figure A.1) were chosen, and oligo-based generation was performed as described previously (Gagnon et al., 2014). Briefly, CRISPR targets, i.e., the site sequence without the PAM motif, SP6 promoter sequences, and a complementary region were ordered as oligonucleotides from (IDT). The oligos were annealed to a constant oligonucleotide encoding the reverse complement of the trans-activating cRNA (tracrRNA) tail. This was followed by DNA polymerization to fill gaps using T4 DNA polymerase (NBE). The fragments were used to

transcribed single guide RNAs (sgRNAs) using an mMachine SP6 kit. The transcribed sgRNAs were precipitated using ammonium acetate. Then, the concentration and quality of the transcribed sgRNAs was checked with the bioanalyzer according to the manufacturer's protocol, aliquoted, and stored at -80°C until used.

A homology repair template comprising a human Aβ sequence encompassing both the Swedish and Indiana mutations: K595N, M596L, and V642F, was ordered as gBlock from IDT (Figure A.1).

For delivery to the zebrafish, the sgRNAs were mixed with the commercially purchased Cas9 nuclease protein (NEB), incubated at 37°C for 5 minutes to assemble the CRISPR complex, and then injected into the zebrafish embryo at the one-cell stage. As a control for successful CRISPR cutting, sgRNAs that target *tyrosinase* (*tyr*) that would result in reduced pigmentation (as published in (Ota et al., 2014)) were included in the mix. At 48 hours post-injection, 2dpf (2 days post-fertilization) zebrafish were screened for reduction in pigmentation from *tyr* CRISPR. Some of the injected animals were sacrificed for high-resolution melting (HRM) analysis as described previously (Fleisch et al., 2013) and sequencing. Subsequently, the most efficient CRISPR was chosen to be injected into one-cell embryos along with a homology-directed repair template designed to humanize the target sequence. The injected fish were grown to adulthood to isolate the germline carrier of either humanized *appb* mutant zebrafish.

Table A 1: Sequences of appb gRNA target sites without the PAM sequence

CRISPR targets	Sequence
***appb* gRNA 1**	GGAGGACATGGGCTCTAATA
***appb* gRNA**	GGGTGCGATCATTGGGCTGA
Tyrosinase	GGTCCAGTCTGGCCCGGCGA

Table A 2: PCR primers for HRM and appb genotyping.

primers for *appb*	Sequence of primers
Forward HRM primer for *appb* CRISPR #1	GTTGAATCAGATGTTCCTCGCGG
Reverse HRM primer for *appb* CRISPR #1	CCCCACCATCAGCCCAATGATC
Forward HRM primer for *appb* CRISPR #2	GCGGAGGACATGGGCTCTAATA
Reverse HRM primer for *appb* CRISPR #2	TACGATGACAGTGGCGATGACC
T7E1 and genotyping forward primer	GAACCTGTAGATGCCCGTCCA
T7E1 and genotyping reverse primer	CTCAATAACTCCATGATGAAT

A.3.2 Genotyping using T7E1 assay

The T7E1 assay was used for easy genotyping of mutant or humanized fish. The T7E1 nuclease cuts heteroduplex DNA strands in which one DNA strand with CRISPR modifications (cuts or inserts) re-anneals to an unmodified DNA strand (Vouillot et al., 2015). The assay was

performed following the manufacturer's protocol with a few modifications. Briefly, PCR primers (listed below in Table A.2) that amplify a 450-bp PCR product around the CRISPR targets were used. PCR products (10–15 μl) were denatured and re-annealed using the thermocycler at the following settings (denaturation at 95°C for 5 mins, re-annealing through temperature decrease at 2°C/second until 4°C). The reannealed primer was then mixed with T7E1 enzyme (NEB) and buffer and incubated at 37°C for 45–60 mins. Undigested PCR products were used as the negative control.

A.4 results and discussion:

***appb* CRISPR Induced Somatic and Germline Mutations in appb**

To ensure that our CRISPR was working before starting the genome editing process, we injected multiple sgRNAs with Cas9 protein into embryos and analyzed their cutting efficiency first. Among the sgRNAs injected, two *appb* sgRNAs (Table A.1) induce somatic mutations in *appb* (Figure A.3). To check for successful CRISPR, we also used a guide RNA (gRNA) targeting the *tyr* gene (published (Ota et al., 2014)), which is associated with the albino phenotype, and I detected significantly reduced pigmentation at 48 hours post-injection (2dpi) in some of the injected animals (Figure A.4). As these fish may have higher chances of *appb* CRISPR working in them as well, we grew them to adulthood and screened them for germline carriers. We identified three germline carriers using both sequencing and T7E1 assay (Figure A.5B, and A.6) and isolated three stable compound *appa*; *appb* knockout. *appb* mutants with assigned allele number ua5013 harbored an in-frame 12-bp deletion at a conserved region (Figure A.6) that we suspect is the region in which Aβ interacts with receptors such as cellular prion protein (PrP). The second *appb* mutants with allele number ua5012 contained a 30-bp

insertion (Figure A.5). *appb* $^{ua5016/+}$ mutants carry a frameshift mutation of 14-bp deletion (Figure A.7). The last two mutations introduce a stop codon. We had success generating heterozygous *appb* yet had difficulty obtaining a homozygous mutant. One possible reason is that complete loss of Aβ is lethal or affects survival to adulthood. Thus, genotyping to identify homozygous larvae and analyzing them immediately rather than when they reach adulthood might be informative. Once some homozygous compound mutants are identified, immunoblotting and transcript analysis will be also performed to evaluate the effect of these mutations of protein production and processing. Another alternative plan is to examine the *appb* mutants on wildtype *appa* genetic background. Unfortunately, we did not identify any humanized zebrafish. This is probably due to the low frequency of the occurrence of the homology-directed repair mechanism after CRISPR cuts in comparison to the end-joining repair mechanism, which is error-prone and often results in deletions and insertions (Devkota, 2018; Hisano et al., 2015). These stable lines could provide valuable insights into the loss of functions of Aβ in the context of AD pathogenesis.

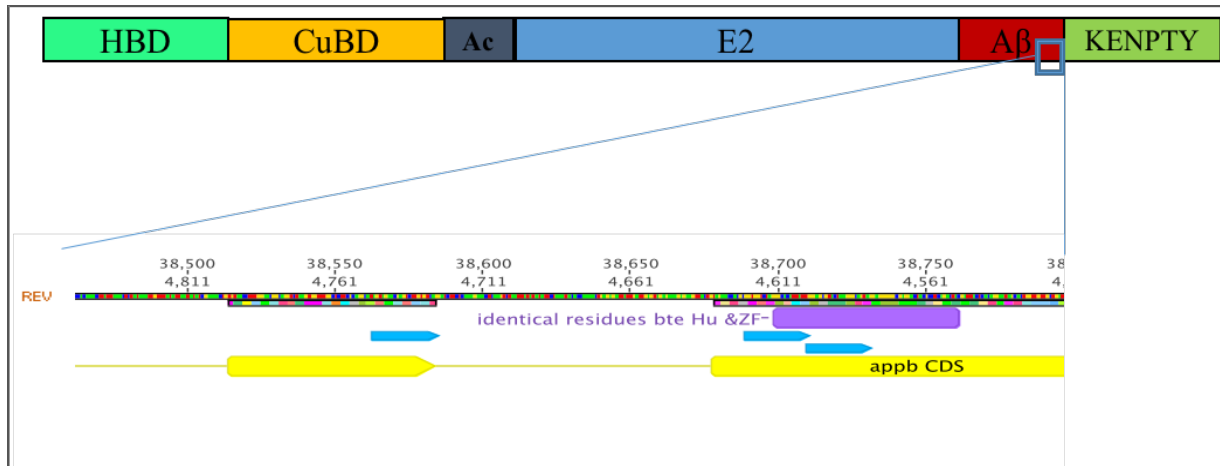


Figure A. 1: Selected CRISPR targets of *appb* on exon 14 and 15.

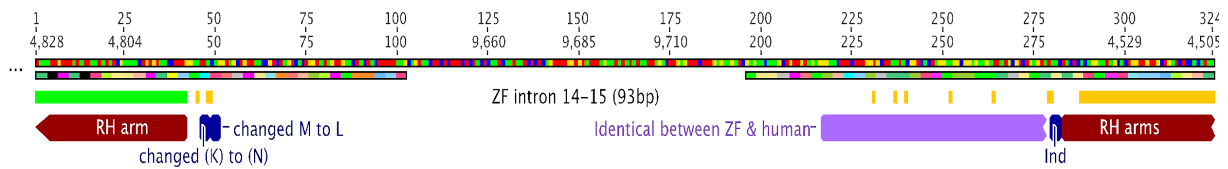


Figure A. 2: The design for the homolog recombination repair template.

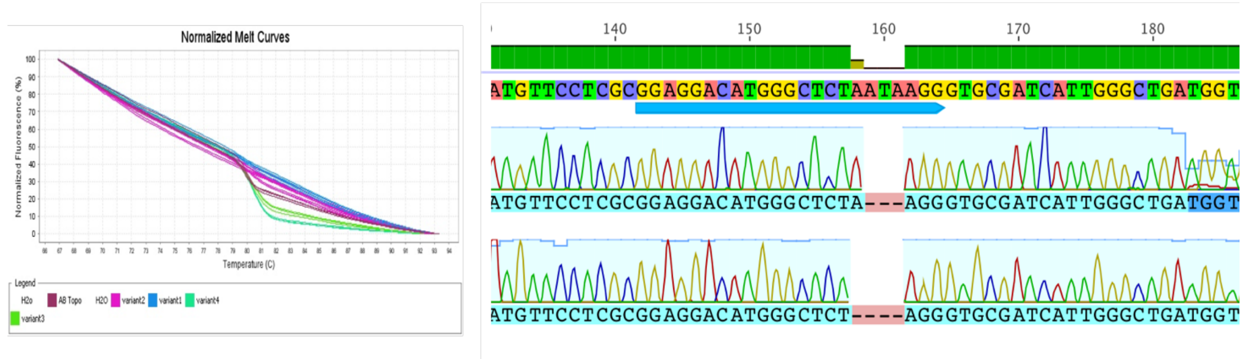


Figure A. 3: HRM melting profile and CRISPR induced mutations in *appb* of injected embryos.

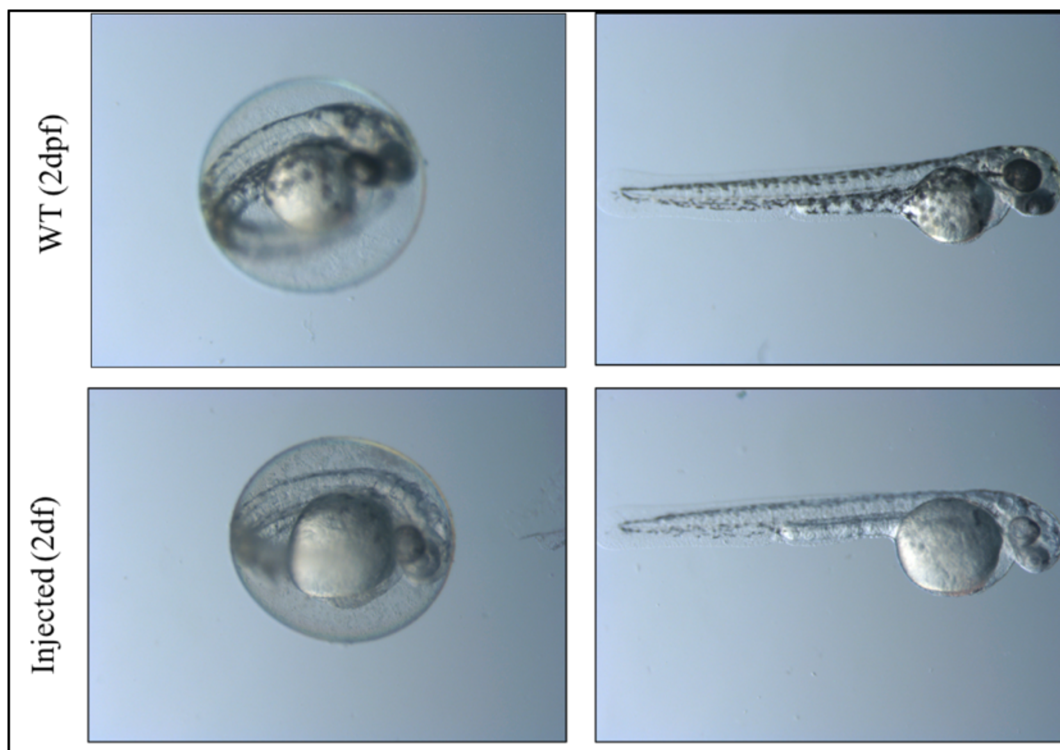


Figure A. 4: Disruption of *tyrosinase* by CRISPR/Cas9 produces mosaic pigmentation phenotype

appb frameshift mutations transmitted through germline

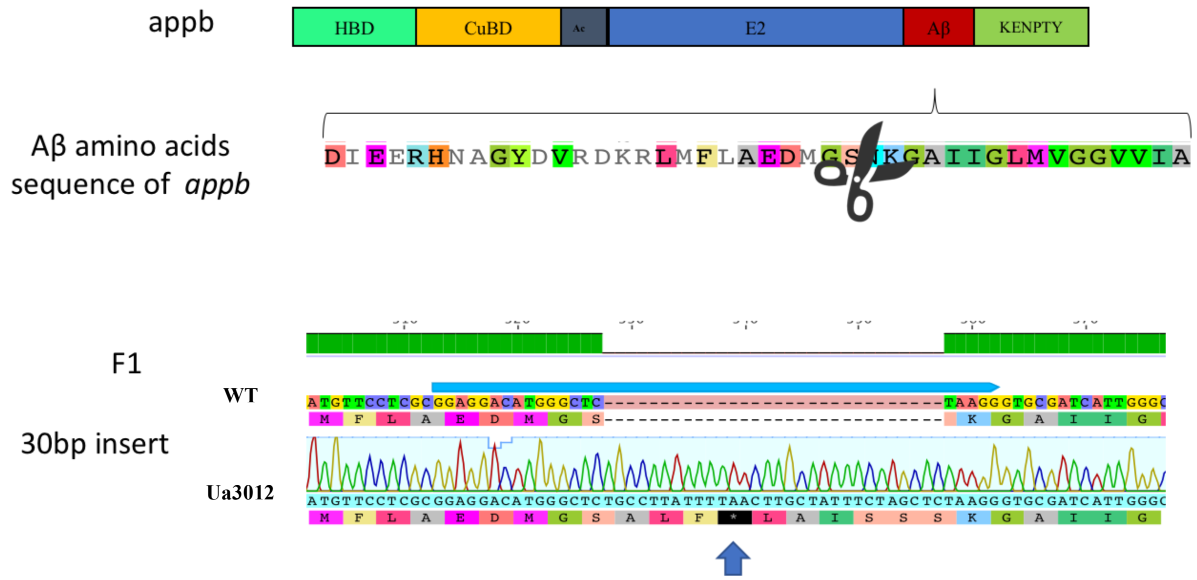


Figure A. 5: A) *appb* insertion of 30bp in an embryo of F1 generation (allele number ua5012).

Arrow points out to a stop codon introduced by the insertion of 30bp fragments).

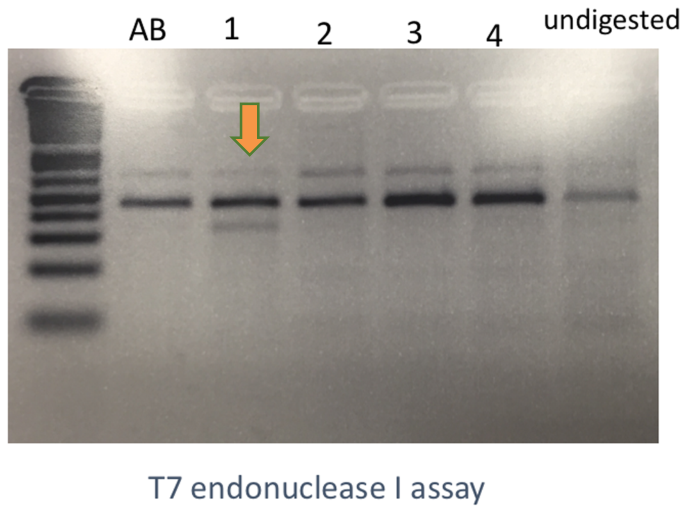


Figure A.5: B) T7EI genotyping for germline carrier of *appb* mutations.

Arrow points to mutants.

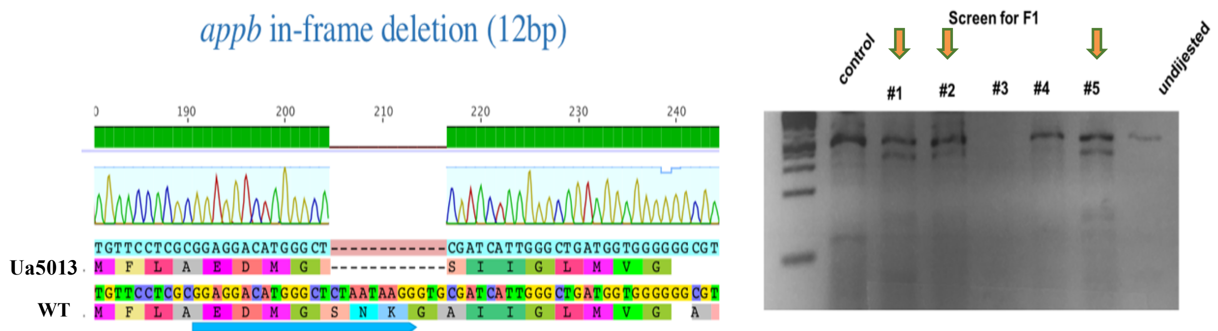


Figure A. 6: *appb* in-frame mutation in adult F1 generation (allele number ua5013). T7EI genotyping for *appb* mutant ua5013.

Arrows points to mutant zebrafish.

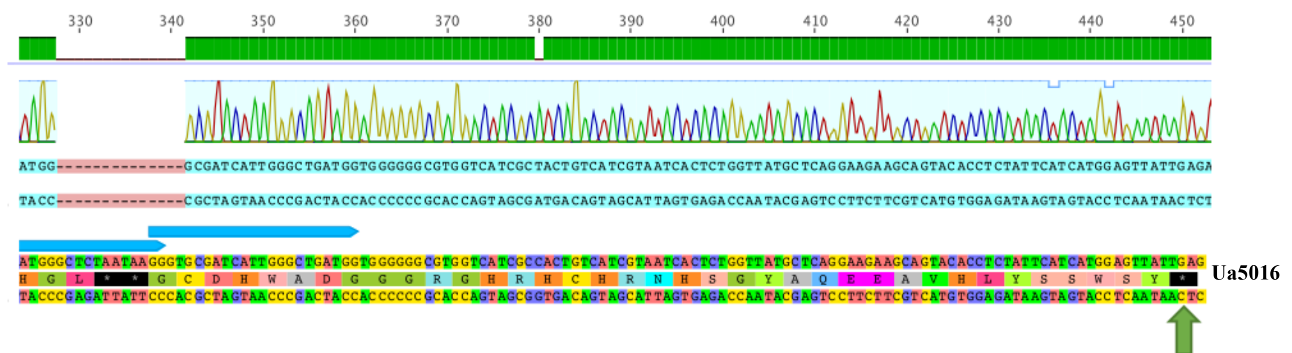


Figure A. 7: *appb* Frame-shift mutations in an embryo of F1 generation (allele number ua5016).

Arrow points out to a stop codon introduced by 14bp deletion.