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Neurodegenerative tauopathies disrupt neuronal function and circadian behaviour in *Drosophila*

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Abstract

Tauopathies are progressive neurodegenerative diseases characterised by the aggregation of hyperphosphorylated tau protein into neurofibrillary tangles (NFTs). To date, the majority of research has focused on NFT formation and neuronal death. However, mounting evidence suggests that clinical symptoms precede aggregate formation and cell loss. Thus, a period of cellular dysfunction precedes cell death. For further understanding, robust animal models of tau-mediated neuronal dysfunction are urgently required. In this thesis, I successfully model the early stages of tauopathies in *Drosophila.*

First, I show tau-mediated neuronal dysfunction (progressive loss of the ERG response and abnormal visual behaviour in the flight simulator system) and neuronal death (progressive loss of the photoreceptor rhabdomeres) are temporally separable in a *Drosophila* eye model. I demonstrate isoform-specific effects of different 4r tau proteins in my model. For example, lGMR>2n4r tau, but not lGMR>0n4r tau, expression causes age-related progressive structural degeneration. I further suggest a role for PAR1 phosphorylation in tau-mediated neuronal dysfunction and show Aβ42 potently exacerbates tau-mediated neuronal dysfunction.

Circadian rhythm disruption is common in tauopathies, such as Alzheimer's disease (AD). I show *Drosophila* expressing human tau, recapitulate many of the features of circadian dysfunction found in AD, including elevated night-time activity, gains in daytime sleep and night-time sleep loss and in LD, and progressive behavioural arrhythmicity in DD. I show that distinct subsets of clock neurons mediate different tau-evoked circadian rhythm disturbances. Tau expression in the PDF neurons, which are considered to be the master pacemaker, prolongs the free-running period and causes hyperactivity, but is insufficient to induce progressive behavioural arrhythmia in DD. All clock neurons is the most clock-restricted domain required to produce behavioural arrhythmicity. The tau species and extent of tau expression affects the circadian behavioural deficits. I find no loss of PDF neurons in flies expressing tau in the clock system. Taken together, these results show in independent neuronal populations tau-mediated neuronal dysfunction is separable from

neuronal death. Collectively, these results suggest circadian dysfunction in AD is not due to neuronal death in the master pacemaker, but neuronal dysfunction throughout the clock system. In this thesis, I present parallel studies that provide a platform for the future dissection of the mechanisms underlying progressive functional and structural degeneration, which bring about clinical symptoms in the early stages of tauopathies.

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Chapter 1: General Introduction

1.1 Tauopathies

Tauopathies are a collection of neurodegenerative diseases characterised neuropathologically by the aggregation of abnormally hyperphosphorylated tau into neurofibrillary tangles (NFTs). At a neuropathological level, tauopathies contain only tau aggregates (frontotemporal dementia (FTD), Picks Disease (PiD), cortical basal degeneration (CBD) and progressive supranuclear palsy (PSP)) or the tau aggregates coexist with amyloid aggregates (Alzheimer's Disease (AD); initially described by Alzheimer (Alzheimer, 1907)) or α-synuclein aggregates (Parkinson's disease (PD)). Tau, which was first discovered in 1975, binds to and stabilises microtubules in a phospho-dependent manner (Weingarten et al., 1975). Tau also promotes microtubule assembly. Mutations in the tau gene have been causally linked to FTD (Spillantini et al., 1998; Ghetti et al., 2015). Tau haplotypes driving slightly higher tau expression are linked to AD (Allen et al., 2014) and PD (Kwok et al., 2004; Tobin et al., 2008; Refenes et al., 2009). Thus, tau abnormalities are sufficient to cause neurodegeneration.

There are a range of tauopathies with overlapping, but distinct, clinical symptoms and neuropathological features. In different tauopathies, the aggregates deposit in distinct brain regions including the hippocampus/entorhinal cortex, cortical regions and mid/hindbrain. The aggregates also consist of different tau proteins. In different tauopathies, the clinical symptoms range from cognitive to motor deficits. However, common to all tauopathies are aggregates of hyperphosphorylated and misfolded tau (Goedert et al., 1989; Weaver et al., 2000). In tauopathies, an increase in tau phosphorylation reduces microtubule binding leading to destabilisation of the microtubule network and axonal transport defects (Mandelkow et al., 2003; Dixit et al., 2008). Detached soluble tau missorts to the somatodendritic compartment and self-aggregates into oligomers and higher order aggregates in a series of steps (Maeda et al., 2007; Sahara et al., 2008). FTD-causing mutations often increase tau phosphorylation and thereby reduce microtubule binding underscoring the toxicity of hyperphosphorylated tau (Iqbal et al., 2009; Gendron and

Petrucelli, 2009). It is currently unclear whether tau-mediated neurodegeneration is due to loss-of-function or toxic gain-of-toxic function (Trojanowski and Lee, 2005; Iqbal et al., 2009).

AD is the most common tauopathy. AD is clinically characterised by cognitive deficits (Burns and Iliffe, 2009). AD is neuropathologically characterised by slow and progressive neurodegeneration and tau aggregates in the cortical regions (Kumar et al., 2015). Mounting evidence suggests neuronal and synaptic dysfunction often precedes neurodegeneration in AD (Palop and Mucke, 2010; Marcello et al., 2012). As well as the cognitive deficits, a substantial proportion of AD patients exhibit circadian rhythm disruption characterised by daytime drowsiness and night-time restlessness (Witting et al., 1990; Satlin et al., 1995; Volicer, 2001). Circadian dysfunction have also been reported in other tauopathies such as FTD (Anderson et al., 2009) and PD (Videnovic and Golombek, 2013; Videnovic and Golombek, 2017). The circadian disturbances appear at an early stage in the disease process and may even precede the onset of cognitive abnormalities. Recent evidence suggests circadian dysfunction may directly influence AD pathogenesis (Maywood et al., 2006; Hastings and Goedert, 2013; Musiek and Holtzan, 2016). Therefore, targeting sleep and circadian alterations provides a promising opportunity to slow down or even halt disease progression. So further understanding of circadian dysfunction in tauopathies may have wide ranging implications that extend far beyond circadian biology. Because, currently there is no effective disease modifying therapies for any tauopathies (Coughlin and Irwin, 2017).

Although tau is best known as a microtubule stabilising protein, mounting evidence has shown that tau has additional physiological roles in the neuron including synaptic plasticity, regulation of genomic stability and cell signalling. This opens up the intriguing possibility that multiple mechanisms mediate tau toxicity in tauopathies. However, despite much research effort both the physiological and pathological roles of tau remain poorly understood (Wang and Mandelkow, 2016; Guo et al., 2017). For further understanding of neuronal dysfunction in tauopathies, robust animal models are needed that recapitulate the early stages of the disease process. The development of such animal models would provide a platform to identify ways to modulate tau-mediated neuronal dysfunction. *Drosophila*

melanogaster are well suited to model tauopathies because of the powerful genetic tools available (Bilen and Bonini et al., 2005; Marsh and Thompson, 2006; Prussing et al., 2013).

1.2 Tau protein structure

Tau is encoded by the MAPT gene, located on chromosome 17 in humans. The tau primary transcript is composed of 16 exons, the majority of which are constitutively expressed, while exons 2, 3 and 10 are alternatively spliced. In the human CNS, alternative splicing produces six tau isoforms that vary in length from 352 to 441 amino acids. Exons 2 and 3 each encode an insert of 29 amino acids in the amino (N)-terminal region of tau. Alternative splicing produces tau isoforms that contain no N-terminal inserts (0n), one N-terminal insert (1n, exon 2) or two N-terminal inserts (2n, exons 2 and 3). Exons 9, 10, 11 and 12 each encode a 30-31 amino acid highly conserved imperfect repeat in the carboxyl (C)-terminal half of tau. These C-terminal repeats comprise the microtubule binding domain of tau. Exon 10 is alternatively spliced producing tau isoforms that contain three (3r) or four (4r) C-terminal repeats (Figure 1.1). Tau expression is developmentally regulated. For example, in the adult human brain all six isoforms are expressed, whereas in the foetal brain only 0n4r tau is expressed (Sergeant et al., 2005; Llorens-Martin et al., 2012). In the cerebral cortex of healthy adults, the ratio of 3r to 4r tau is approximately 1:1 (Buee et al., 2000; Takuma et al., 2003). Differential splicing of exons 2 and 3 results in the amount of the 2n tau isoform (~9%) being much less than the 0n (~37%) and 1n (~54%) tau isoforms in the human brain (Deshpande et al., 2008). Tau expression also varies in different brain regions. For example, the overall amount of tau is much higher in the neocortex than in the white matter and cerebellum (Trabzuni et al., 2012). Splicing of the MAPT gene also varies in different brain regions. For example, On3r tau levels are lower in the cerebellum than in other brain regions (Trabzuni et al., 2012). These differences in tau expression in specific brain regions most likely contribute to the region specific effects of distinct tauopathies.

Biophysical studies have revealed that tau is prototypically a ‘natively unfolded’ protein (Gamblin, 2005). Tau is composed of four domains. First, the N-terminal acidic projection domain, which contains zero, one or two N-terminal inserts. Second, the central region,

which contains the proline-rich domain. Third, the microtubule binding domain in the C-terminal half of tau, which is comprised of three or four C-terminal repeats. And finally, the C-terminal basic tail (Figure 1.1).

The N-terminal projection domain of tau projects away from the microtubule surface and can interact with other cytoskeletal elements, cytoplasmic organelles and the neural plasma membrane (Buee et al., 2000). The N-terminal domain of tau regulates the distance between the axonal microtubules and thereby can increase the axon diameter (Chen et al., 1992). The interaction of the N-terminal domain with other cytoskeletal elements, like microfilaments, regulates the flexibility of microtubule lattices. The extreme N-terminal region of tau plays a role in a signalling cascade that regulates axonal transport (Kanaan et al., 2011). However, the specific function of the N-terminal inserts is not well known. *In vitro,* in the mouse brain different 4r tau isoforms show specific subcellular distributions (Liu and Gotz, 2013). Thus, the N-terminal inserts seem to influence the subcellular distribution of tau. Additionally, the N-terminal inserts also seem to affect tau protein-protein interactions, because different 4r tau isoforms show different protein interaction partners. For example, β-synuclein binds 0n rather than 1n or 2n tau isoforms. In contrast, apolipoprotein A1 only binds to 2n tau isoforms (Liu et al., 2016). Moreover, *in vitro* the N-terminal inserts also affect tau's ability to aggregate. The inclusion of one N-terminal inserts promotes tau aggregation, whereas the inclusion of two N-terminal inserts suppresses tau aggregation (Zhong et al., 2012)

Tau binds through the proline rich domain to SH3-containing proteins, including Fyn, in a phospho-dependent manner (Lee et al., 1998). Microtubule binding is mediated by the microtubule binding domain, located in the C-terminal half of tau. 4r tau isoforms binds more effectively than 3r tau isoforms, which is probably because of variation in the C-terminal repeats (Butner and Kirschner, 1991; Lu and Kosik, 2001). Tauopathies can be grouped according to the tau isoform found in the tau deposits: 4r-tauopathies (including PSP and CBD), 3r–tauopathies (such as PiD), and 3r+4r-tauopathies (such as AD) (Irwin, 2016). The microtubule binding domain (and proline rich domain) also binds DNA and RNA

and lipid rafts (Qi et al., 2015). The function of the C-terminal region has not been established.

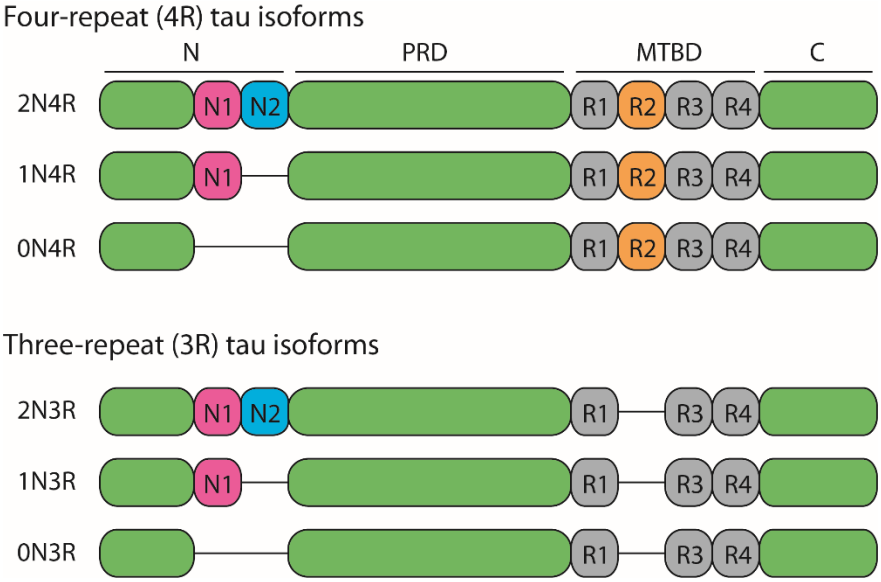


Figure 1.1: Isoforms of tau in the human brain. Six isoforms of tau are produced in the human CNS by alternative splicing of the MAPT gene. Different tau isoforms contain zero, one or two N-terminal inserts and three or four C-terminal repeats. Tau is made up of the N-terminal region, the proline-rich domain (PRD), microtubule binding domain (MTBD) and C-terminal tail.

1.3 Tau phosphorylation

The longest human CNS tau isoform (2n4r, 441aa) contains 85 putative phosphorylation sites (80 serines and threonines in addition to five tyrosine residues) (Hanger et al., 2009). Because of the large number of phosphorylation sites, it is to be expected phosphorylation will have a major effect on tau function. More than 39 of these sites are often phosphorylated in NFTs (Buee et al., 2000). The overall phosphorylation status of tau is tightly regulated by the combined balanced activity of various kinases and photospheres. Phosphorylation levels of tau are dramatically higher (> fourfold) in brains from AD patients than in brains from healthy controls (Kopke et al., 1993). Tau is phosphorylated at certain sites in both normal and AD brains, whereas it is phosphorylated at others in only AD brains (Sergeant et al., 2008). Under pathological conditions, tau phosphorylation increases, which reduces microtubule binding resulting in cytoskeleton destabilisation. For example, tau

phosphorylation at specific residues in the C-terminal-repeats (including Ser262, Ser293, Ser323 and Ser356) substantially reduces tau microtubule binding affinity (Drewers et al., 1995). Detached soluble tau then mislocalises to the somatodendritic compartment and forms aggregates of increasing complexity in a series of steps. Currently, it is unknown the order in which specific residues are phosphorylated in tauopathies. Both *in vitro* and *in vivo* studies have identified several kinases that phosphorylate tau (for a complete list see recent review by Guo et al., 2017). Intriguingly, the majority of these kinases have been shown to phosphorylate tau in both physiological and pathological conditions.

Glycogen synthase kinase (GSK) has been shown to phosphorylate approximately half of the tau phosphorylation sites, with the majority of them being phosphorylated in AD brains (Hanger et al., 2007). The amount and activity of GSK in AD brains correlates well with neurodegeneration and aggregate formation (Rankin et al., 2007). In mice, overexpressing human tau, treatment with a GSK inhibitor reduces tau phosphorylation and rescues neuronal death (Sereno et al., 2009). Tau phosphorylation by the tyrosine kinase Fyn (at Tyr18) has been shown to regulate axonal transport (Cox et al., 2016). In mice, expressing human tau, an increase in overall Tyr phosphorylation coincides with aggregate formation. Thus, overall Tyr phosphorylation levels may contribute to aggregate formation. *In vitro* studies have shown protein phosphatase 1 (PP1), PP2A, PP2B, and PP2C dephosphorylate tau (Avila et al., 2004; Braithwaite et al., 2012). Activity of PP2A the main phosphatase (accounts for the majority of cellular phosphatase activity in the human brain) is reduced by ~50 % in the brain of individuals with AD (Gong et al., 1993; Liu et al., 2005). Tau is also subject to other post-translational modifications (PTMs), including acetylation, which regulate tau function in both physiological and pathological conditions (Min et al., 2010; Cohen et al., 2011; Irwin et al., 2012).

1.4 Physiological function

1.4.1 Microtubule-related axonal transport

Tau is best known as a microtubule associated protein (MAP). In adult neurons, the majority of tau localises to the axons, where it binds with the microtubules, the tracks for the intracellular transport of organelles, proteins and mRNA, through the microtubule binding domain. Thus, tau stabilises the microtubules and promotes microtubule assembly. The ability of tau to bind microtubules is regulated by phosphorylation. The phosphorylation state of tau is highly dynamic and regulated by various kinases and phosphatases. Tau considerably increases microtubule polymerisation and reduces microtubule depolymerisation *in vitro* (Drechsel et al., 1992). In normal neurons, the amount of tau greatly exceeds the amount of tubulin (~tenfold), therefore nearly all tau is microtubule bound in the cell (Iqbal et al., 2009). Tau is also able to bind actin through the N-terminal domain and cross-link microtubules to microfilaments and thereby stabilise, regulate and allow reorganisation of the cytoskeleton. This cross-linking is disrupted in tauopathies (Fulga et al., 2007).

In addition to regulating microtubule dynamics, tau can regulate axonal transport by influencing the function of the motor proteins, dynein and kinesin. Dynein transports cargo in a retrograde direction (towards the cell body), whereas kinesin transports cargo in an anterograde direction (towards the axon terminus). Tau can influence the binding of motor proteins to both microtubules and cargo (Seitz et al., 2002; Vershinin et al., 2007). Additionally, tau controls the release of cargo vesicles from kinesin chains by activating a GSK signalling cascade via its extreme N-terminal domain (Kanaan et al., 2011). Furthermore, tau can facilitate dynein-mediated transport by binding to its cofactor dynactin, and thereby stabilising the interaction of dynein with the microtubules (Magnani et al., 2007). However, because all of these studies were done *in vitro* or in cultured cells and tau knockout mice show no obvious defects in axonal transport (Yuan et al., 2008), it is not clear whether physiological tau influences the function of the motor proteins *in vivo*.

1.4.2 Function of dendritic and nuclear tau

A tiny amount of tau localises to the nucleus. Nuclear tau can bind to DNA through the proline rich and microtubule binding domain (Camero et al., 2014). Tau hyperphosphorylation compromises the ability of tau to enter the nucleus and bind to DNA. Thus, tau hyperphosphorylation substantially reduces both microtubule and DNA binding (Qi et al., 2015). Nuclear tau contributes to DNA protection in both physiological and hypothermic stress conditions (Sultan et al., 2011; Violet et al., 2014). Furthermore, nuclear tau also been reported to be involved in the DNA repair mechanism (Rossi et al., 2013; Violet et al., 2014). Nuclear tau has also been shown to regulate gene expression. Via the proline-rich and microtubule binding domain, tau binds to the AT rich minor grove of DNA causing a conformational change. The altered DNA conformation affects the activity of multi-protein DNA complexes that control gene transcription (Sultan et al., 2011; Rossi et al., 2013; Qi et al., 2015). In support of this, ~20 genes have been identified whose transcription is altered in a tau knockout mice (Gomez de Barreda et al., 2010). Finally, nuclear tau has also been reported to be involved in chromosome stability and processing/silencing of rRNA (Rossi et al., 2013). A tiny amount of tau localises to the dendrites and dendritic spines (Ittner et al., 2010). In cell culture, pharmacological synaptic activation causes endogenous tau to translocate from the dendritic spines into the excitatory postsynaptic compartment. These results suggest a role for dendritic tau in synaptic plasticity, however the precise mechanisms are as of yet unclear (Frandemiche et al., 2014).

1.4.3 Other functions of tau

Tau has been shown to play critical roles in synaptic plasticity and neurogenesis. For example, tau knockout mice show defective long-term depression (LTD) in the hippocampus (Kimura et al., 2014). Concurrently, a different study has shown tau knockout mice show defective long-term potentiation (LTP) in the hippocampus (Ahmed et al., 2014). Tau has been reported to be involved in neurogenesis and neuronal migration (Hong et al., 2010; Fuster-Matanzo et al., 2012). Recent evidence suggests tau regulates the speed of stress

granule formation and trafficking (Vanderweyde et al., 2016). Many studies have noted the role of tau in mediating network hyperactivity in disease models. Knocking out tau rescues the excitotoxicity produced by Aβ in mice overexpressing either human APP or human APP and PS1 (Roberson et al., 2007; Ittner et al., 2010). Similarly, reduction of endogenous tau with antisense oligonucleotides (ASO) suppresses seizures in chemically induced seizure models (DeVos et al., 2013). These studies suggest a role for tau in mediating pathological network hyperactivity, however, it is currently unknown whether tau is involved in regulating physiological network activity. A recent study has shown that tau modulates cellular iron export. The interaction of APP with ferroportin at the cell surface is essential for iron export and tau is involved in the trafficking of APP to the cell surface. In aged tau knockout mice, iron accumulation results in neuronal death in the substantia nigra. In tauopathies, there is an accumulation of iron in brain regions with reduced soluble tau such as the cortex in AD (Lei et al., 2014). A small amount of tau localises to glial cells, but the role of tau in glia is as of yet unknown (Muller et al., 1997). Despite a vast amount of research the physiological role of tau remains poorly understood.

1.5 Pathological function

Currently, the underlying mechanisms of tau-induced neurodegeneration are poorly understood (despite a vast amount of research). Abnormal tau modifications including mis-splicing, various PTMs including phosphorylation and aggregation convert physiological tau species into pathological tau species. In familial tauopathies (<5 % of cases), mutations in the MAPT gene cause tau pathology, whereas in sporadic tauopathies, the cause of tau pathology is unknown. It is unclear whether pathological tau causes neurodegeneration by loss-of-function or toxic gain-of-function (for recent indepth reviews see Wang and Mandelkow, 2016 and Guo et al., 2017).

1.5.1 Tau mutations

Currently, around 80 mutations have been identified in the MAPT gene. These mutations are linked to various tauopathies including FTD, CSP and PSP, but not AD (Kouri et al., 2014;

Coppola et al., 2012). They are either missense or splicing mutations. Missense mutations tend to be located in the C-terminal half of tau (for example P301L, V337M or R406W) and result in tau protein with reduced affinity for microtubules and increased propensity to aggregate (Hong et al., 1998; Barghorn et al., 2000). Splicing mutations tend to promote the inclusion of exon 10 and thereby increase the 4r-3r tau ratio. It is unknown how altering the 3r-4r ratio triggers aggregation; it might be due to the different microtubule binding abilities and phosphorylation susceptibilities of 3r and 4r tau.

1.5.2 Loss–of-function

Even though tau function to a certain extent can be compensated by MAP1A and MAP2, aged (12-month old) tau knockout mice show behavioural impairments. Thus, tau is required for normal functioning of the brain (Lei et al., 2014). Furthermore, dementia lacking distinctive histopathology (DLDH), which is characterised by neuronal loss in the absence of any brain lesions, is caused by reduced tau levels (Zhukareva et al., 2001). Hyperphosphorylation of tau substantially reduces microtubule binding, which leads to cytoskeleton destabilisation. Tau aggregation reduces the amount of soluble functional tau, which also reduces microtubule binding and leads to cytoskeleton destabilisation. Thus, tau loss-of-function due to either hyperphosphorylation or aggregation can lead to a damaged microtubule network. An intact microtubule network is needed for efficient axonal transport. Often in tauopathies, cargo accumulates in both the axons and cell body leading to ‘traffic jams’ (Millecamps and Julien, 2013). Thus, axonal transport defects may contribute to disease pathogenesis. In support of this, one of the earliest abnormalities in several animal models of tauopathies are defects in axonal transport (for review see Ballatore et al., 2007). For example, human tau expression in *Drosophila* larval motor neurons disrupts axonal transport as evidenced by overaccumulation of transport vesicles in the motor neuron axon (Mudher et al., 2004; Chee et al., 2005). Additionally, K3 mice, which express human tau with the K389I mutation, show impaired anterograde axonal transport of specific cargoes, which precedes neuronal loss (Ittner et al., 2008).

Pathological tau disrupts axonal transport by interfering with the function of the motor proteins, dynein and kinesin. Overexpression of tau inhibits access of the motor proteins to the microtubule tracks (Seitz et al., 2002). *In vitro*, tau is capable of reducing the velocity of kinesin-mediated retrograde transport (Dixit et al., 2008). Overexpression of tau reduces the amount of cargo bound to motor proteins by sequestering available motors (Vershinin et al., 2007). Protein levels of the components of the dynein and kinesin complexes are reduced in brains from AD patients compared with brains from healthy controls (Seward et al., 2013). Tau overexpression causes selective inhibition of kinesin-mediated anterograde transport, resulting in an accumulation of cargo, such as mitochondria, endoplasmic reticulum (ER) and synaptic proteins in the cell body. Depletion of mitochondria and ER in the axon produces a decrease in glucose and lipid metabolism and ATP synthesis that results in energy deprivation and oxidative stress, which leads to 'dying back' axonal degeneration. For example, human tau overexpression in the *Drosophila* larval motor neurons causes morphological changes and perturbs synaptic transmission as a result of impaired axonal transport of mitochondria (Chee et al., 2005). Disruption of any of the various processes that tau is involved in, including, neuronal activity, synaptic plasticity, DNA protection and iron export may play a part in the neurodegenerative process.

In post mortem brain tissue from individuals with AD tau in NFTs is always hyperphosphorylated, and tau hyperphosphorylation precedes aggregation (Braak et al., 1994). However, whether tau hyperphosphorylation drives aggregation remains disputed. *In vitro,* hyperphosphorylated tau isolated from AD patient brains forms aggregates (Alonso et al., 2001). However, tau hyperphosphorylation is not necessarily toxic as it occurs during animal hypothermia and anaesthesia-induced hypothermia (Arendt et al., 2003), and may even have a protective effect (Schneider et al., 1999). Therefore, tau hyperphosphorylation is not sufficient to cause aggregation in all circumstances. Furthermore, *in vitro* tau hyperphosphorylation is not necessary for aggregate formation, (Goedert et al., 1996).

The ability of hyperphosphorylated tau to enter the nucleus and bind to DNA is compromised (Camero et al., 2014). The lack of nuclear tau results in stress-induced DNA damage and disrupted heterochromatin organisation, which can lead to cell cycle re-entry,

which is fatal to differentiated neurons (Seward et al., 2013). Loss of tau function in the nucleus can also lead to altered gene expression, which in turn causes altered protein synthesis (Frost et al., 2014; Hernandez-Ortefa et al., 2016). However, it is also possible hyperphosphorylated tau in the nucleus has a toxic gain-of-function. Rod-like tau aggregates have been found in neuronal nuclei in tauopathies such as AD, FTD and HD (Metuzals et al., 1988; Fernandez-Nogales et al., 2014). Although the consequences of harbouring tau aggregates in the nucleus are, as of yet, unidentified.

1.5.3 Neurotoxic toxic gain-of-function

In AD brains, the region distribution of NFTs correlates well with cognitive deficits. So initially the NFTs were thought to cause neurodegeneration. However, recent findings have shown NFT formation is not required for, or sufficient to cause, neurodegeneration. In post mortem brain tissue from AD patients, the number of NFTs greatly exceeds neuron loss, indicating that most neurons die without having formed NFTs (Gomez-Isla et al., 1997). Additionally, neurons containing NFTs in AD can survive for up to 20 years (Morsch et al., 1999). In mice overexpressing human wild-type or mutant tau, neuronal death does not coincide with tau aggregation. Neurons can die without forming aggregates and neurons which contain aggregates do not die and appear functional (Andorfer et al., 2005). Additionally, mice overexpressing human wild-type or mutant show cognitive deficits, impaired synaptic transmission and synapse loss before or without aggregation formation. For example, Tg4510 mice, which overexpress inducible human tau with P301L mutation, develop age-related NFTs, neuronal loss and cognitive abnormalities. Switching off tau expression rescues the cognitive impairments even though the NFTs continue to form (Santacruz et al., 2005). Similarly, mice, which overexpress regulatable human tau with the ΔK280 mutation, show age-dependent aggregate formation, synapse loss, cognitive deficits and loss of LTP. When tau expression is switched off, memory and LTP recover, even though the aggregates remain (Sydow et al., 2011; Van der Jeugd et al., 2012). Furthermore, *Drosophila* overexpressing human wild-type or mutant tau show neuronal loss and behavioural impairments in the absence of aggregate formation (Wittmann et al., 2001; Jackson et al., 2002; Mershin et al., 2004; Chee et al., 2005; Kosmidis et al., 2010). These

studies suggest that tau-mediated neuronal dysfunction and degeneration is caused by a soluble tau species, rather than NFTs. Different isoforms, mutations and PTMs lead to an enormous number of different tau species, which mostly likely contribute to the clinical and neuropathological heterogeneity found in tauopathies (Sanders et al., 2014). It has been proposed that NFT formation is a protective cellular response because the NFTs can scavenge toxic soluble tau species (Alonso Adel et al., 2006). Recent evidence shows that tau oligomers rather than NFTs are the toxic species in tauopathies. Levels of soluble tau oligomers are increased in brains from AD patients compared to brains from healthy controls (Lasagna-Reeves et al., 2012). Additionally, several studies have demonstrated soluble tau oligomers are toxic in cultured mouse neurons (Tian et al., 2013; Tepper et al., 2014).

Hyperphosphorylated tau is able to mediate its toxic effects independent of aggregation. For example, hyperphosphorylated tau interacts with the scaffolding protein, c-Jun N-terminal kinase-interacting protein 1 (JIP1) and impairs formation of the kinesin complex, which thus disrupts axonal transport (Ittner et al., 2009). Additionally, in various animal models of tauopathy, hyperphosphorylated tau promotes neurodegeneration by causing aberrant alignment and accumulation of F-actin filaments (Fulga et al., 2007). Hyperphosphorylation of tau alters its degradation, through both the unfolded protein response (UPR) and autophagosome/lysosomal system. For example, tau phosphorylation at Ser262 and Ser356 interferes with the binding of the ubiquitin ligase, C-terminus of HSP70-interacting-protein (CHIP), which therefore protects tau from proteasome-mediated degradation (Dickey et al., 2007).

Under normal circumstances, only small amounts of tau localise to the dendrites. Mislocalisation of tau to the dendrites occurs at an early stage of the disease process. Tau modifications (PTMs and mutations) and increasing the level of tau can all lead to the mislocalisation of tau into the somatodendritic compartment. For example, transfection of tau in mature hippocampal neurons causes the redistribution of tau into the somatodendritic compartment, which coincides with loss of dendritic spines (Thies and Mandelkow, 2007). In Tg4510 mice, overexpressing human P301L tau, tau mislocalises and

accumulates in the somatodendritic compartment. Overaccumulation of tau in the dendrites interferes with the trafficking and anchoring of NMDA receptors to the post synaptic membrane, resulting in defective synaptic transmission (Hoover et al., 2010). Expression of the LRK2 kinase in adult dopaminergic neurons in *Drosophila* causes degeneration of the dendritic arborizations as a result of hyperphosphorylation and mislocalisation of endogenous tau into the somatodendritic compartment. LRK2-mediated dendritic degeneration is rescued by knockdown of endogenous tau (using RNAi) and enhanced by overexpression of human wild-type tau. Thus, hyperphosphorylation and subsequent mislocalisation of tau is enough to cause dendritic degeneration in *Drosophila* (Lin et al., 2010). Additionally, mislocalisation of tau to the somatodendritic compartment results in a reduction in the amount of soluble functional tau to bind to and stabilise axonal microtubules. The mechanisms of tau missorting are not well understood.

Recent evidence has demonstrated dendritic tau is involved in amyloid-beta (Aβ)-induced neurotoxicity. For example, in cultured hippocampal neurons Aβ oligomers cause the mislocalisation of endogenous tau into the somatodendritic compartment, which leads to dendritic spine loss (Zempel et al., 2010). Dendritic tau recruits the FYN kinase to the postsynaptic compartment, where it phosphorylates the NDMA receptor subunit, NR2B/GluNRB, causing increased calcium conductance and thereby enhancing Aβ excitotoxicity (Roberson et al., 2007; Ittner et al., 2010). Tau also mediates Aβ-induced cell cycle re-entry. Exposure of cultured wild-type neurons to Aβ oligomers causes cell cycle re-entry and activation of various kinases. In contrast, in cultured tau knockout neurons, Aβ oligomers are unable to trigger cell cycle re-entry (Seward et al., 2013).

1.6 Modelling neurodegenerative disease in Drosophila

Drosophila have been used to study biological questions for well over a 100 years. As such, *Drosophila* have contributed to our understanding of a wide number of processes including circadian rhythms, learning and memory and synaptic transmission (for a comprehensive review see Bellen et al., 2010 and Pandey and Nichols, 2011). The conservation of fundamental physiological processes between humans and *Drosophila*, and remarkably high

degree of conservation between the human and *Drosophila* genome (the majority of disease-linked genes have *Drosophila* orthologues), suggests we may be able to use *Drosophila* to investigate the underpinnings of mammalian disease (Bier, 2005). During the last decade, several *Drosophila* models of neurodegenerative disease (including AD, PD and HD) have been created (reviewed in Marsh and Thompson, 2006 and Iijima-Ando and Iijiama, 2010). It is possible to study circadian rhythms in *Drosophila* at both a cellular and circuit level. In *Drosophila* it is possible to assess the consequences of manipulating small groups of neurons or even individual neurons. Moreover, the anatomy and physiology of *Drosophila* are relatively well understood. *Drosophila* have a fully sequenced and highly annotated genome, ~13,600 protein coding genes located in only four chromosomes (Adams et al., 2000). There is less genetic redundancy in *Drosophila* than in humans. *Drosophila* are easy and inexpensive to maintain.

Drosophila have been used to study circadian rhythms for nearly five decades (Konopka and Benzer, 1971). In *Drosophila*, circadian rhythms and sleep are studied by recording locomotor activity using the DAM system (TriKinetics, Walthman, MA). In the DAM system, individual flies are placed in glass tubes which are bisected by an infrared beam. When a fly is active it breaks the infrared beam and this activity is recorded. The DAM system is placed in light and temperature controlled incubators and activity is measured over several days (Chiu et al., 2010). In rodents, there are several different systems to monitor locomotor activity, including wheels, cages with infrared beams and piezoelectric systems, which may differentially influence circadian rhythms and sleep (Siepka and Takahashi, 2005; Bains et al., 2018). Additionally, the genetic background of transgenic mice has a profound effect on circadian behaviour and sleep (Banks et al., 2015). Thus, it is difficult to directly compare different studies because you don't know if differences are real or contributed to by differing methodology or genetic background. Genetic background also has a major influence on sleep in *Drosophila* (Zimmerman et al., 2012). However, it is much quicker and easier to outcross into a common genetic background in *Drosophila* than in mice. *Drosophila* better recapitulate the sleep-wake cycle in humans than rodents. Mice are nocturnal and have fragmented sleep, whereas, *Drosophila* are diurnal and have a period of consolidated sleep during the night. This is particularly useful when modelling human neurodegenerative

diseases, like AD, which are characterised by circadian rhythm and sleep disruption, in addition to cognitive deficits. It is possible to combine the tools to study circadian behaviour and model neurodegenerative disease in *Drosophila* to shed light on the circadian dysfunction in human tauopathies, such as AD (Chen et al., 2014; Long et al., 2014). There is a panoply of tools to monitor neuronal function and death in *Drosophila* (for review see Ugur et al., 2016). *Drosophila* have a short lifespan (2-3 months) and so are well suited to studying late-onset progressive neurodegenerative diseases.

The main advantage of *Drosophila* is the extensive genetic toolkit (Adams and Sekelsky, 2002). In *Drosophila* genetics, the Swiss army knife is the binary Gal4-UAS system. Gal4 is a yeast transcription factor that binds to Upstream Activation Sequences (UAS). Gal4 activates UAS-transgenes expression in all cells it is expressed in. Therefore, to produce tissue specific UAS-transgene expression, Gal4 is placed under the control of a tissue-specific promoter. There is a large collection of Gal4 drivers that express in single cells, groups of cells or entire tissues (Brandt and Perrimon, 1993). Frequently used are the GMR driver, inducing pan-retina expression, and Elav driver, inducing pan-neuronal expression (Brand and Perrimon, 1993). It is also possible to use the Gal4/UAS system to coexpress various different UAS-transgenes in the same tissue. It is also possible to further restrict UAS-transgene expression to a smaller number of cells by using the Gal4 repressor, Gal80 (Suster et al., 2004). Both Gal4 and Gal80 are placed under the control of different tissue-specific promoters whose expression partially overlaps. Gal4 is only able to activate UAS-transgene expression in cells in which it is expressed in but Gal80 is not.

Most commonly used Gal4 drivers activate UAS-transgene expression during development. When modelling adult-onset neurodegenerative disease it is often necessary to restrict expression to adulthood. Modifications of the Gal4/UAS system allows for combined spatial and temporal control of UAS transgene expression. The TARGET system (McGuire et al., 2003) uses a temperature-sensitive variant of the Gal4 repressor, Gal80 ($Gal80^{ts}$). At 18 °C (permissive temperature), $Gal80^{ts}$ is active and inhibit Gal4 activity. Whereas, after shifting the flies to 29-30 °C (restrictive temperature), $Gal80^{ts}$ becomes inactive and permits Gal4 driven UAS-transgene expression. The GeneSwitch (GS) system uses a RU486 sensitive

variant of Gal4 (Osterwalder et al., 2001). In the absence of RU458, RU486 sensitive Gal4 is unable to activate UAS-transgene expression. When RU458 is present it binds to RU458 sensitive Gal4 and activates UAS-transgene expression. To activate RU458 sensitive Gal4 driven UAS-transgene expression, RU458 is added to fly food. *Drosophila* are amenable to *in vivo* (genetic or pharmacological) medium to high-throughput enhancer/suppressor screens because of two main reasons. First, it is simple and inexpensive to set up 100s or even 1000s of fly crossings. Second, several robust readouts of neuronal degeneration and dysfunction exist in *Drosophila* (Ugur et al., 2016).

1.7 Drosophila models of tauopathy

1.7.1 Neuronal toxicity

Tauopathies are neuropathologically characterised by brain deposits of aberrantly phosphorylated and misfolded wild-type or mutant tau. Consequently, to create Drosophila models of tauopathies the Gal4/UAS system has been used to overexpress wild-type or FTD-linked mutant tau in particular neurons (or glia) in larval or adult *Drosophila*. The effects of overexpressing tau can be examined by studying neuronal death and neuronal function (for review see Papanikolopoulou and Skoulakis, 2011; Cowan et al., 2011; Gistelinck at al., 2012). William et al. (2000) was the first anatomical study to show that tau expression causes neurotoxicity in *Drosophila*. The authors showed expression of bovine tau fused with GFP in larval sensory neurons causes axonal degeneration.

Subsequently, many *Drosophila* models of human tauopathy were then created by transgenetically expressing human wild-type or FTD-linked mutant (R406W/V337M) tau. Human wild-type tau (0n4r isoform) expression throughout the CNS causes progressive age-related neurodegeneration and vacuolisation in neurons throughout the brain (Wittmann et al., 2001; Nishimura et al., 2004). Tau-expressing flies also die a lot sooner than controls. This most likely represents bona fide degeneration, because it only seems to affect adult terminally differentiated neurons, because no neurodegeneration or vacuolisation was found in newborn adult flies. Interestingly, vacuolisation is not commonly observed in either

AD patients or in AD mouse models, however neurodegeneration in *Drosophila* is often accompanied by vacuolisation. Vacuolisation probably reflects the rapid tempo of neurodegeneration in flies. There is evidence of apoptosis in tau-expressing flies, as indicated by the presence of TUNEL-positive cells. Tau-mediated neurodegeneration coincides with a substantial increase in tau phosphorylation (including at the AT8 and AT100 epitopes). No aggregates were detectable immunohistochemically in fly neurons, not even in old flies. Thus, aggregates are not needed to cause neuronal death in this model. Therefore, it appears soluble hyperphosphorylated tau species are intrinsically toxic. Thus, *Drosophila* models of tauopathy recapitulate the early pre-aggregate events of tau-associated neurodegeneration. FTD-linked mutant tau precipitates a more severe pathological phenotype than wild-type tau. Increasing tau dosage also enhances the severity of the pathological phenotype. Tau expression throughout the CNS preferentially targets cholinergic neurons. This model successfully recapitulates many features of human tauopathies, such as AD.

Tau expression throughout the CNS also dramatically affects the mushroom bodies (MBs). The MBs are composed of ~2,000 neurons and play an essential role in learning and memory (functionally analogous to the hippocampus) (for review see Heisenberg, 2003). Pan-neuronal tau expression (0n4r or 2n4r isoform) throughout development results in nearly complete ablation of the MBs (other brain structures are unaffected) (Kosmidis et al., 2010). This was due to the toxic effect of hyperphosphorylated tau on the MB neuroblasts that develop into MB neurons. Therefore, it does not reflect bona fide degeneration because tau interferes with the development of MB neurons rather than causes the loss of extant adult MB neurons. Tau-mediated MB ablation requires tau phosphorylation. Expression of mutant tau with a reduced ability to be phosphorylated does not cause MB ablation. In contrast, expression of pseudophosphorylated tau has a more dramatic effect on the MBs than wild-type tau. The selective loss of MB neurons in flies pan-neuronally expressing tau, demonstrates the differential susceptibility of different neuronal types to tau pathology. 2n4r tau produces a more severe effect on the MB neuroblasts than 0n4r tau. Whereas, FTD-linked mutant tau produces a weaker effect on the MB neuroblasts than wild-type tau, indicating isoform-specific effects (Kosmidis et al., 2010; Papanikolopoulou et al., 2010).

Glial cells are commonly affected in tauopathies (Ikeda et al., 1995; Berry et al., 2001; Ballatore et al., 2007). Inducible expression of human tau in adult glia results in neurodegeneration in both neurons and glia and decreased longevity. Tau becomes hyperphosphorylated at the AT8 and AT100 epitopes (via the JAK/STAT signalling pathway) and, in contrast to neurons, forms fibrillar glial aggregates, reminiscent of NFTs, and triggers apoptosis. However, switching-off tau expression results in a reduction in the number of apoptotic cells, without any concurrent change in the number of glial tangles. These results further suggest that soluble hyperphosphorylated tau species (rather than glial aggregates) mediate tau-linked neurodegeneration in *Drosophila* (Colodner and Feany, 2010).

Tau expression in fly eye causes retinal toxicity, which manifests as a rough eye phenotype (REP), characterised by a reduced eye size and disrupted ommatidial organisation. The REP is also associated with degeneration and vacuolisation in the optic lobe. The adult fly retina is composed of ~800 identical units called ommatidia, arranged in a hexangular array. Each ommatidia contains eight photoreceptor neurons and twelve accessory cells (Ready et al., 1976; Wolff and Ready, 1991). Expression of various tau isoforms and proteins using GMR-Gal4, which is expressed throughout the eye, causes a REP (Jackson et al., 2002; Grammenoudi et al., 2008). However, an important note is that the *Drosophila* eye surface is formed during late larval and pupal stages in development (Moses et al., 1991; Ellis et al., 1993). This combined with the fact that GMR-Gal4 is expressed during development, means that a REP in newborn adult flies is probably caused by tau-dependent toxicity during development (similar to the toxic effects of tau on the MB neuroblasts), rather than bona fide degeneration.

Whether tau phosphorylation is required for tau toxicity has been extensively studied using the REP (Jackson et al., 2002; Nishimura et al., 2004; Steinhilb et al., 2007; Chatterjee et al., 2009; Ambegaokar and Jackson, 2010). Jackson et al. (2002) demonstrated coexpression of the Shaggy (SGG, the fly homology of GSK3β) kinase enhanced the REP and precipitated the formation of large NFT-like aggregates in the optic lobe. To date no other study in *Drosophila* has reported NFT formation apart from in glia. This study linked tau phosphorylation with neurotoxicity. Chatterjee et al. (2009) provided further evidence

though a series of elegant experiments that tau phosphorylation is critical in tau-mediated toxicity. They showed coexpression of the PAR1 kinase with human tau increases its phosphorylation at Ser262/Ser356 (12E site) and exacerbates the REP. In contrast, tau that is unable to be phosphorylated at the PAR1 phosphorylation sites (2n4r tau^{S2A}; which has alanine substitutions at Ser262/Ser356) fails to cause a REP. Another study has shown that expression of a phosphomimetic tau mutant (0n4r tau^{E14}) causes a more severe REP, whilst a nonphosphorylatable mutant (0n4r tau^{AP}) fails to cause a REP (Fulga et al., 2007). The REP is frequently used in unbiased high-throughput enhancer/suppressor screens, because it is easily quantifiable and provides a robust readout of tau neurotoxicity. Several independent screens have successfully identified numerous modifiers of tau neurotoxicity, a number of which, perhaps unsurprisingly, were kinases (including SGG and PAR1) and phosphatases (such as PP2A). However, several other novel modifiers have been identified, including cytoskeleton proteins, molecular cochaperones, ion transporting ATPases, transcription cofactors and RNA binding proteins (Shulman and Feany, 2003; Blard et al., 2006; Blard et al., 2007; Ambegaokar and Jackson, 2011).

1.7.2 Neuronal dysfunction

Several *Drosophila* models have shown tau expression causes substantial neurotoxicity, however other *Drosophila* models have shown behavioural abnormalities without any apparent neuronal death. Cognitive deficits are a cardinal symptom of most tauopathies (Irwin, 2016). Additionally, the cognitive deficits may first appear prior to overt NFT formation and neuronal death (Oddo et al., 2003a; Oddo et al., 2003b). Mershin et al. (2004) investigated the effects of tau overexpression in adult MB neurons in *Drosophila*. The MBs are required for learning and memory in *Drosophila*. Human wild-type tau (0n4r or 2n4r isoform) or *Drosophila* tau expressed in adult MB neurons precipitates olfactory associative learning and memory deficits before or in the absence of obvious neurodegeneration (Kosmidis et al., 2010). Neurodegeneration was detected in no flies up to 45-days of age and in only a few flies over 45-days of age. Because the authors restricted tau expression to adulthood they were able to circumvent the toxic effects of tau on MB neuroblasts during development. Therefore, the associative learning and memory deficits represent tau-

dependent neuronal dysfunction, because they are not due to tau-mediated toxicity on the MB neuroblasts. No aggregates were detectable (neither biochemically nor immunohistochemically) in the tau-expressing flies. Thus, these results suggest soluble tau species mediate tau-dependent neuronal dysfunction in *Drosophila*. FTD-linked mutant tau precipitates a milder effect than wild-type tau on associative learning (Grammenoudi et al., 2008). Similar findings were reported by Papanikolopoulou et al. (2010). The authors showed expression of human WT tau (0n4r or 2n4r isoform) in the adult CNS (using the TARGET system) impairs associative learning without affecting MB morphology. Collectively, these results suggest the impaired olfactory learning is due to toxic gain-of-function effects caused by tau overaccumulation in the adult MB neurons. Similar findings reported in a mice model of tauopathy. Mice overexpressing human wild-type tau (2n4r isoform) display cognitive impairments without any overt neuronal loss (Kimura et al., 2007). Collectively, these results show tau expression in the MBs produces temporally separable dysfunction and degeneration. Tau expression in MB neuroblasts is toxic, whereas tau expression in adult MB neurons causes neuronal dysfunction without neuronal death.

Tau expression in the larval motor neurons also leads to neuronal dysfunction (Mudher et al., 2004; Chee et al., 2005; Ubhi et al., 2007). Mudher et al. (2004) showed expression of human wild-type tau (0n3r isoform) in larval motor neurons disrupts neuromuscular junction morphology and axonal transport, which causes obvious defects in larval locomotor function. Similarly, Chee et al. (2005) showed human wild-type tau (0n4r isoform) or *Drosophila* tau expression disrupts endo/exocytosis, resulting in dramatically reduced synaptic potentials. The synaptic transmission perturbations are due to axonal transport defects, resulting in a depletion of mitochondria in the presynaptic terminal. Behavioural abnormalities coincide with an increase in tau phosphorylation. Tau phosphorylation by SGG is important for tau-linked axonal transport defects. Reducing tau phosphorylation by LiCl treatment (SGG inhibitor) mostly rescued the behavioural impairments. In contrast, increasing tau phosphorylation by coexppression of SGG enhanced the behavioural impairments, as it does for retinal toxicity, suggesting a common pathogenic mechanism (Mudher et al., 2004). The authors found no detectable aggregates or cell loss in any of these models. Thus, these results show tau-mediated dysfunction in the larval

neuromuscular junction (NMJ) in *Drosophila* is caused by soluble hyperphosphorylated tau species. Collectively, this evidence from both the adult brain and larval neuromuscular junction suggests that tau-mediated neuronal dysfunction is separable from toxicity.

1.8 Circadian rhythms

Circadian rhythms are ~24 hour rhythms in behaviour and physiology. They are entrained by environmental cues (e.g. light and temperature) but persist or free run without these cues. Circadian rhythms are not passively driven by environmental cycles but generated by an endogenous timekeeping mechanism, called the circadian clock. The circadian clock allows organisms to adapt to and prepare for changes in the environment (e.g. the best time to find food or a mate) (Hut et al., 2012). The importance of the circadian clock is underscored by its ubiquity, clocks are present in organisms ranging from prokaryotes to mammals. In all organisms, circadian systems are composed of three parts: the timekeeper, input pathways (e.g. from the eye) and output pathways (e.g. to the motor centres). Circadian rhythms regulate a vast number of functions in the body, including sleep and wakefulness, body temperature and hormone production. Sleep-wake cycle and circadian rhythm disturbances often compromise health. Many studies have shown damage to the circadian system leads to accelerated aging (Kondratova and Kondratov, 2012). Furthermore, other studies in both mice and humans have shown sleep and circadian disturbances lead to neurodegenerative pathology, including AD (Kang et al., 2009). Finally, in humans the breakdown of daily circadian rhythms, induced by shift work or jetlag for example, is a risk factor for the development of cardiovascular disease, cancer and metabolic syndromes (Kondratov and Antoch, 2007; Takahashi et al., 2008; Sahar and Sassone-Corsi, 2009; Bass and Takahashi, 2010).

1.9 Molecular circadian clock

In all organisms, circadian rhythms at a cellular and molecular level are controlled by interlocking autoregulatory transcription/translation feedback loops (TTLs) (Figure 1.2) (for extensive review see Ozkaya and Rosato, 2012 and Hardin and Panda, 2013). In mammals, in

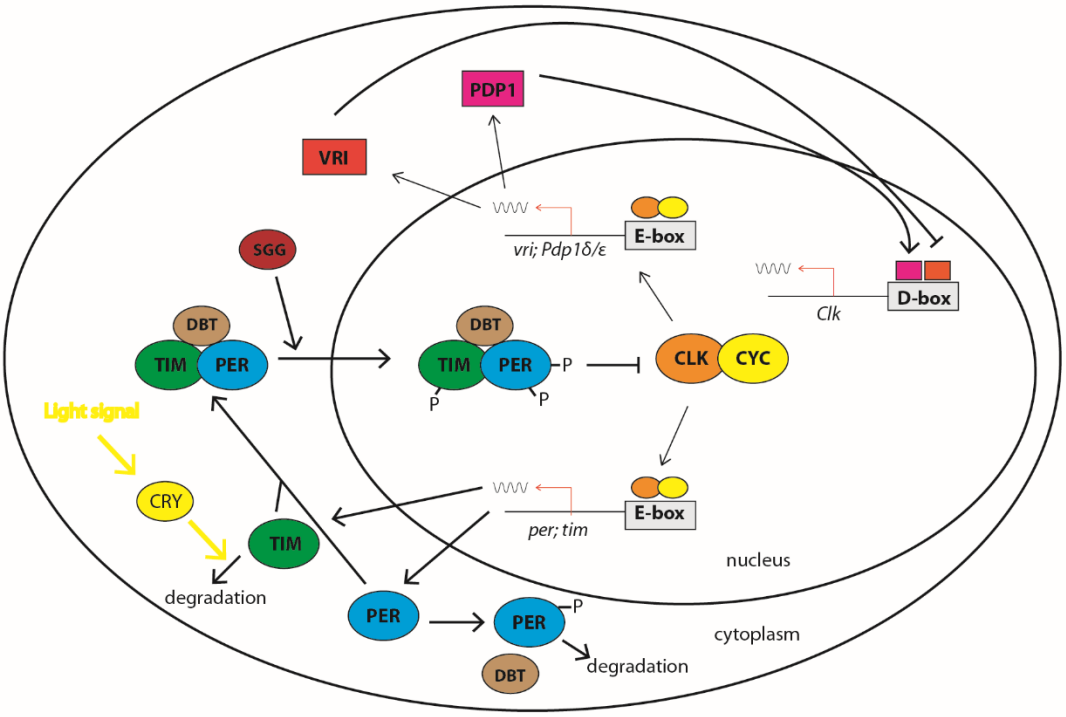
the main TTL basic helix-loop-helix (bHLH)-PAS transcription factors, CLOCK (CLK) and BMAL1 heterodimerise and activate transcription of 100s of genes by binding E-boxes in their promoters, including *period 1-3 (mPer)*, *cryptochrome 1*-2 (*mCry*). *mPer* and *mCry* are translated in the cytoplasm. PER and CRY heterodimerise. PER/CRY heterodimers accumulate in the cytoplasm. Following a delay, PER/CRY heterodimers enter the nucleus and inhibit CLK/BMAL1-mediated transcription. After another delay, PER/CRY heterodimers are degraded, which allows CLK/BMAL1 heterodimers to bind E-boxes and start a new cycle of transcription (Gekakis et al., 1998; Zheng et al., 1999; Shearman et al., 2000; Lee et al., 2001). The timing of the nuclear entry of PER/CRY heterodimers is regulated by PER phosphorylation by glucose synthase kinase (GSK3) and casein kinase II (CKII). The stability of PER/CRY heterodimers is regulated by PER phosphorylation by casein kinase Iε (CKI) and NEMO. PER is degraded via the proteasome following phosphorylation by CKI and ubiquitination by β-TrCP. In the SCN (master pacemaker), light from the retinohypothalamic tract (RHT) entrains the clock by activating transcription of *mPer* genes (Hankins et al., 2008).

In *Drosophila*, in the main TTL bHLH-PAS transcription factors, CLOCK (CLK) and CYCLE (CYC) heterodimerise and activate transcription of a vast number of genes by binding E-boxes in their promoters, including *period (per)* and *timeless* (*tim*). *Per* and *tim* are translated in the cytoplasm. PER and TIM heterodimerise. PER/TIM heterodimers accumulate in the cytoplasm. Following a delay, PER/TIM heterodimers enter the nucleus and inhibit CLK/CYC-mediated transcription. After another delay, PER/TIM heterodimers are degraded, which allows CLK/CYC heterodimers to bind E-boxes and start a new cycle of transcription (Allada et al., 1998; Darlington 1998; Rutila et al., 1998) . The timing of the nuclear entry of PER/CRY heterodimers is regulated by PER phosphorylation by SHAGGY (SGG) and casein kinase II (CKII). The stability of PER/CRY heterodimers is regulated by PER phosphorylation by DOUBLETIME (DBT) and NEMO. PER is degraded via the proteasome following phosphorylation by DBT and ubiquitination by SLIMB. At a cellular level, *Drosophila* entrain the clock using the blue-light sensitive photoreceptor, CRYTOCHROME (CRY). Light-activated CRY promotes TIM degradation via the proteasome (which affects the stability of PER) (Koh et al., 2006; Peschel et al.., 2009).

A second interlocked TTL drives the cyclic expression of B*mal1* in mammals and C*lk* in *Drosophila.* In mammals, nuclear hormone receptors ROR α/β/γ and REVERB α/B bind Rev response elements (RREs) in the *Bmal1* promoter to activate and repress *Bmal1* transcription, respectively (Guillaumond et al., 2005). In *Drosophila*, bZIP transcription factors PDP1 δ/Ɛ and VRILLE (VRI) bind D boxes in the *Clk* promotor to activate and repress *Clk* transcription, respectively (Cyran et al., 2003). Additionally, CLK/BMAL1 complexes in mammals and CLK/CYC complexes in *Drosophila* activate transcription of *Dec1-2* and *clockwork orange* (*cwo*), respectively. DEC1-2 and CWO feedback to repress the activity of BMAL1 and CLK, respectively, by binding E boxes in their promoters (Honma et al., 2002; Kadener et al., 2007; Lin et al., 2007).

A

Drosophila



B

Mammals

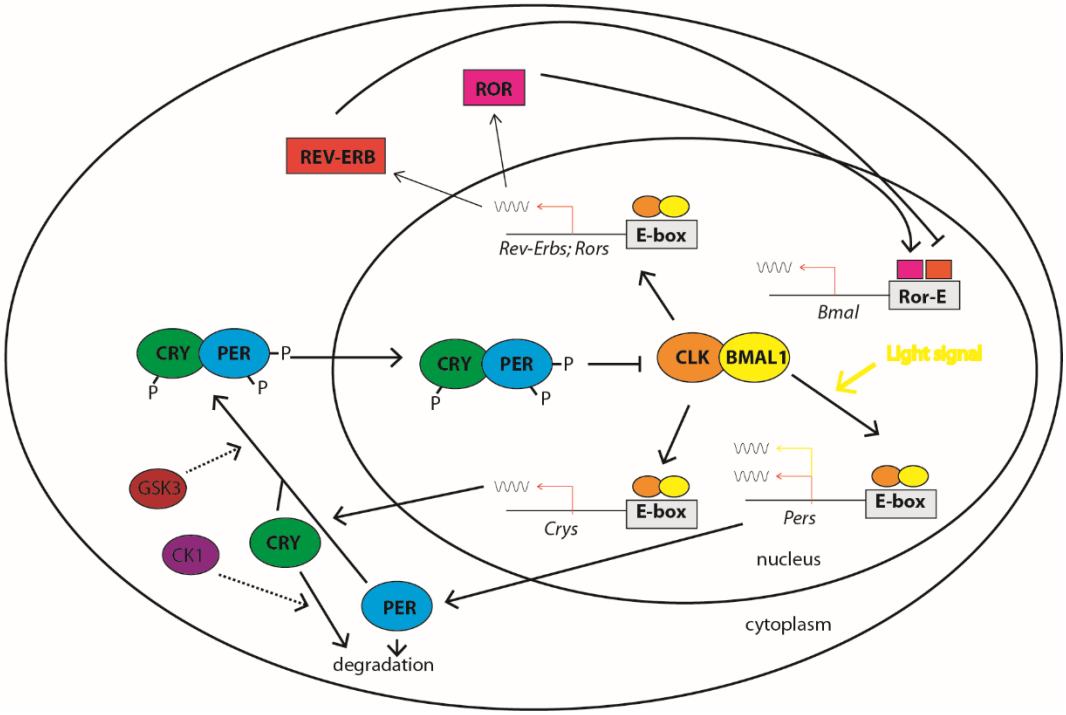


Figure 1.2: Circadian molecular clock in *Drosophila* (A) and mammals (B). (A) In the main TTL, CLK/CYC heterodimers activate transcription of *per* and *tim* genes by binding to E boxes in their promoters. *Per* and *tim* are translated in the cytoplasm. PER and TIM form heterodimers. PER/TIM heterodimers accumulate in the cytoplasm and following a delay, enter the nucleus, and repress CLK/CYC-mediated transcription. CLK/CLK heterodimers also activate transcription of *Pdp1 δ/Ɛ* and *vri* genes by binding to E-boxes in their promoters. *Clk* expression is regulated by PDP1 δ/Ɛ and VRI which bind to a D-box in the *Clk* promoter. CLK and CYC are phosphorylated by DBT and SGG, which modulates the timing of nuclear entry and stability of CLK/CLK heterodimers. At the cellular level, the clock is entrained by the photoreceptor, CRY. Light-activated CRY promotes TIM degradation. (B) In the main TTL, BMAL1 and CLK heterodimers activate transcription of m*Per* and m*Cry* genes by binding to E-boxes in their promoters. *mPer* and *mCry* are translated in the cytoplasm. PER and CRY form heterodimers. PER/CRY heterodimers accumulate in the cytoplasm and following a delay, enter the nucleus, and repress CLK/BMAL1-mediated transcription. PER/CRY heterodimers also activate transcription of *Ror α/β/γ* and *RevErb α/β* genes by binding to E-boxes in their promoters. *Bmal1* expression is regulated by ROR and REVERB which bind to a Ror-E in the *Bmal1* promoter. PER and CRY are phosphorylated by GSK3 and CKI, which modulates the timing of nuclear entry and stability of PER/CRY heterodimers. In the SCN at the cellular level, the clock is entrained by light from the RHT, which activates transcription of *mPer* genes. Modified from Zordan and Sandrelli, 2015.

1.10 Circadian clock at an organismal level

In mammals, the vast majority of cells express a molecular clock. All the molecular clocks must be synchronised with each other and the environment for the circadian system to function properly. In mammals, the master pacemaker, the suprachiasmatic nucleus (SCN) is responsible for synchronising all the circadian oscillators. All neurons produce ~24 h oscillations in gene expression patterns. However, only SCN neurons receive photic input from the RHT. SCN neurons are electrically coupled (via gap junctions) to one another. They also generate rhythmic neuronal activity. Thus, SCN neurons entrain to light-dark (LD) cycles and then synchronise all the circadian oscillators in the body via various neuronal and humoral pathways. Furthermore, in constant darkness (DD) the SCN neurons, as a result of being coupled to one another, provide a circadian signal. This is required for normal behavioural and physiological rhythms in free-running conditions. The SCN is found in the hypothalamus just above the optic chiasma. The SCN is divided into several anatomical subdivisions that express distinct neuropeptides and receptors. The arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) neuropeptides which are released from

distinct subsets of SCN neurons, paracrinely synchronise the molecular oscillations of other cells (Welsh et al., 2010).

Compared to mammals in which all neurons in the brain express a molecular clock, only ~150 neurons express a molecular clock in *Drosophila*. These neurons make up a neural network that controls circadian behaviour. The ~150 neurons are subdivided into nine groups based on their anatomical position. There are five groups of lateral neurons and four groups of dorsal neurons. The ~150 neurons are comprised of eight large ventral lateral neurons (lLNvs), eight small ventral lateral neurons (sLNvs), two 5^{th} sLNvs, ~eight lateral posterior neurons (LPNs), 12 dorsal lateral neurons (LNds), four anterior DN1 neurons (DN1as), ~32 posterior DN1 neurons (DN1ps), four DN2 neurons (DN2s), and ~80 DN3 neurons (DN3s) (Figure 1.3). There is heterogeneity between groups and within groups, similar to the mammalian SCN. Distinct subsets of the ~150 neurons have different roles in circadian behaviour, driving activity at specific points in the LD cycle (reviewed in Allada and Chung, 2010; Peschel and Helfrich Forster, 2011; Muraro et al., 2013).

In LD conditions, flies show two circadian clock-controlled peaks of activity, a morning activity peak around the time of lights-on and an evening activity peak around the time of lights-off. Flies anticipate the light transitions by gradually increasing their locomotor activity in advance of both lights-on and lights–off. Flies also exhibit non-circadian startle responses, substantial transient bursts of locomotor activity in response to the light transitions. In constant darkness (DD), the morning and evening activity peaks gradually merge into a single activity peak that reoccurs at roughly the same time every day (period ~24 hours). This single activity peak in DD is normally slightly in advance of the evening activity peak in LD conditions (Figure 1.4).

Molecular oscillations in the ~150 clock neurons are synchronised (Yoshii et al., 2009; Roberts et al., 2015). The clock cells exhibit cycling neuronal activity, peaks and troughs in neuronal activity occur at different times for distinct clock neurons (Cao and Nitabach, 2008; Sheeba et al., 2008; Flourakis et al., 2015). The sLNvs, which control the morning activity

peak, are called morning (M) cells and the LNds and DNs, which control the evening activity peak, are called evening (E) cells (Grima et al., 2004; Stoleru et al., 2004).

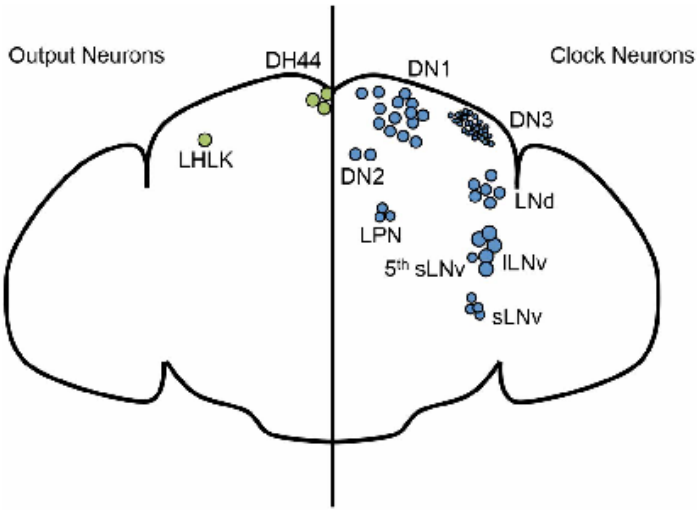


Figure 1.3: Clock neurons and output neurons in the *Drosophila* brain. ~150 neurons express a molecular clock in the fly brain (blue circles). These ~150 neurons are divided into several groups based on their anatomical position. Distinct subsets of the ~150 neurons have different functional roles in circadian behaviour. All the LNvs except the 5th sLNv express the neuropeptide pigment dispersing factor (PDF), which paracrinely synchronises the molecular oscillations of clock neurons. DH44 and LHLK cells (green circles) do not express a molecular clock but exhibit cyclic neuronal activity, suggesting clock input. DH44 and LHLK cells express the neuropeptides Diuretic hormone 44 (DH44) and leuokinin (LK), respectively. DH44 and LK are critical regulators of robust activity rhythms in DD. Reproduced from Dubowy and Sehgal (2017).

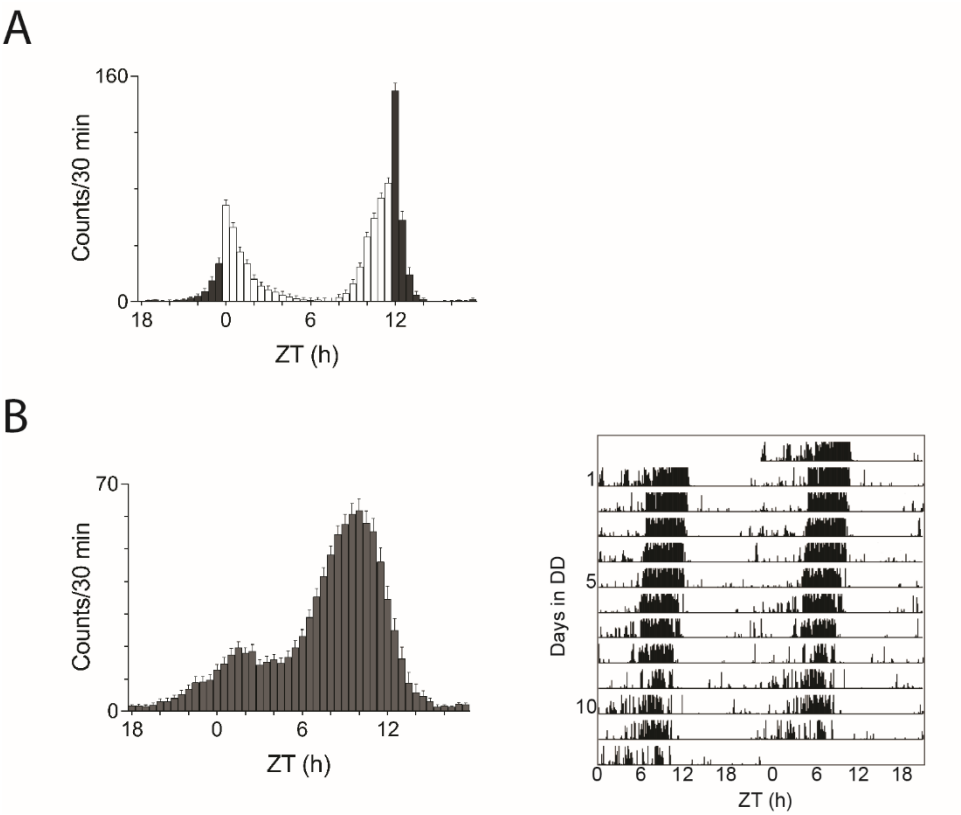


Figure 1.4: Locomotor activity of a group of wild-type male flies in LD (A) or DD conditions (B) at 25 °C. (A) Average daily activity histogram for three days of 12/12 h LD cycles with abrupt LD transitions. Flies exhibit a morning activity peak around the time of lights-on (ZT0) and an evening activity peak around the time of lights-off (ZT12). Flies anticipate lights-on and -off by gradually increasing their locomotor activity in advance of these transitions. Flies also exhibit non-circadian startle responses, transient bursts of locomotor activity in response to the light transitions. (B) Average daily activity histogram and double-plotted actogram for twelve days of constant darkness (DD). Flies exhibit a single peak of activity that reoccurs at roughly the same time each day. This activity peak is slightly in advance of the evening activity peak in LD conditions.

The neuropeptide pigment dispersing factor (PDF), is expressed by all the LNvs, except the 5th sLNv (~16 neurons). Ablation of the PDF neurons (using *hid)* or genetic deficiency in PDF or its receptors (*pdf*^{01} and *pdfr*, respectively) affects the timing of the activity peaks and anticipation in LD conditions and causes a rapid loss of behavioural rhythms in DD conditions. The loss of behavioural rhythms coincides with reduced amplitude and desynchronicity of molecular rhythms throughout the ~150 clock neurons (Renn et al., 1999; Lin et al., 2004; Mertens et al., 2005). Thus, PDF acts in both PDF and non-PDF clock neurons. Mechanistically how PDF alters the timing of molecular clocks is not well

established (Shafer et al., 2008). However, PDF has the ability either slow down or speed up molecular clocks (Yoshii et al., 2009). PDF neurons act on the LNds and a small number of the DN1s (E-cells) to affect the evening activity peak in LD and free-running period (Lear et al., 2009). Despite the fact both sLNvs and lLNvs express PDF, the sLNvs seem especially important for normal free-running rhythms. The sLNvs show strong molecular rhythms that persist for several days in DD (Stoleru et al., 2005). Speeding up the molecular clock (by overexpression of SGG) in only the sLNvs also speeds up the molecular clock in various other groups of clock neurons, including the LNds, DN1s and DN3s, and alters behavioural rhythms (Stoleru et al., 2005). The ability of the sLNvs to control behavioural and molecular rhythms is PDF dependent (Yao and Shafer, 2014). Because of the sLNvs ability to control the timings of both behavioural and molecular rhythms in free-running conditions, they are considered to be the master pacemaker in *Drosophila* (equivalent to the mammalian SCN). However, recent data indicates the entire clock network works collectively to generate strong activity rhythms in DD (Dissel et al., 2014; Yao and Shafer, 2014).

How does the central clock talk to downstream brain regions to drive robust circadian locomotor behaviour? The DN1 neurons are connected to the sLNvs via synapses. DN1s play an essential role in generating robust behavioural rhythms in constant darkness. DN1s maintain rhythmic neuronal activity in DD conditions, despite a dampened molecular clock (Yoshii et al., 2009; Roberts et al., 2015). DN1s are connected via synapses to specific neurons in the pars intercerebralis (PI) (functionally analogous to the hypothalamus). These cells in the PI do not express molecular clocks, but exhibit rhythmic neuronal activity, suggesting that they receive time-of-day information from the clock system. Thus, DN1s connect the central clock with output neurons. PI cells secrete several neuropeptides, including Diuretic hormone 44 (DH44) (*Drosophila* homology of corticotrophin releasing hormone). DH44 is critical for the generation of strong behavioural rhythms in DD (Cavanaugh et al., 2014). DH44 receptors are expressed in various different brain regions, including the lateral protocerebrum. The lateral protocerebrum has been associated with stress-induced locomotor activity (Zhao et al., 2010). Moreover, stress glucocorticoids show circadian production and act to synchronise molecular clocks (Cavanaugh et al., 2014). Furthermore, two neurons in the lateral horn (LH) produce the neuropeptide, leucokinin

(LK), which is necessary for the generation of robust behavioural rhythms. The LK-positive LH neurons exhibit rhythmic neuronal activity, suggesting that they receive time-of-day information from the clock system (most likely the sLNvs) (Cavey et al., 2016).

A small subset of glia exhibit rhythmic expression of PER (Ewer et al., 1992). The close proximity of the PER-positive glial cells to the sLNvs indicates that they may interact to regulate activity rhythms (Helfrich-Forster, 1995; Suh and Jackson, 2007). It has been proposed glia regulate PDF transport and release (Ng et al., 2011). These studies suggest glia-to-neuron signalling plays an important role in circadian behaviour. A similar role for glial cells in circadian behaviour in mammals has been suggested (Slat et al., 2013).

1.11 Sleep in Drosophila

Drosophila has been used to study sleep for nearly 20 years (Hendricks et al., 2000; Shaw et al., 2000). During sleep, flies exhibit reduced activity/movement, sensory responsiveness and brain activity, and an increased arousal threshold. It is widely accepted that the underlying mechanisms of mammalian and *Drosophila* sleep are relatively similar (see for review Cirelli, 2009). Like circadian behaviour, sleep is studied using the DAM system. A fly is characterised as being asleep when it does not break the beam for at least five minutes. Both behaviourally and electrophysiologically fly sleep intensity varies over the course of 24 hours. In laboratory LD conditions, wild-type flies have a midday nap and period of consolidated sleep at night (Figure 1.5).

Both the circadian and homeostatic systems regulate sleep (for review see Cirelli and Bushey, 2008). Serotonergic and GABAergic signalling promotes sleep, whereas dopaminergic and octopaminergic signalling promotes arousal and wakefulness. Multiple brain regions have been shown to play a role in controlling sleep in *Drosophila* including the MBs (which harbours both wake- and sleep-promoting neurons), dorsal fan-shaped body, pars intercerebralis (PI) (which harbours both wake- and sleep-promoting neurons) and pars lateralis (PL).

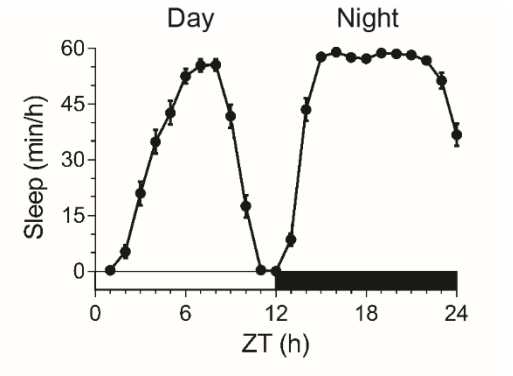


Figure 1.5: Sleep of a group of wild-type male flies in LD conditions at 25 °C Average daily sleep profile for three days of 12/12 h LD cycles with abrupt LD transitions. Flies have a siesta in the middle of the day and a period of consolidated sleep at night.

Some clock genes seem to have a role in sleep regulation. Compared to wild-type flies, sleep is reduced in C*lk* and *Cyc* mutant flies. Whereas, compared to wildtype flies, sleep is normal in *tim* mutant flies (Hendricks et al., 2000; Shaw et al., 2002; Hendricks et al., 2003). The clock circuitry also seems to play a role in sleep regulation. Distinct clock neurons promote sleep or wakefulness at specific times of the day. The lLNvs promote wakefulness at specific times of the day. The lLNvs neuronal activity fluctuates throughout the day and is reduced during the night (Cao and Nitabach, 2008; Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008; Liu et al., 2014). Changes in neuronal activity are mediated by clock driven expression of wide awake, which interacts with the GABA-A receptor, Rdl, and thereby modulates inhibitory currents in the lLNvs (Liu et al., 2014). Activation of lLNvs disrupts nightime sleep, whereas silencing them increases night-time sleep (Sheeba et al., 2008; Parisky et al., 2008; Shang et al., 2008). lLNvs neurons also mediate their wake prompting effects via PDF expression. PDF has wide ranging effects in *Drosophila* because several brain areas express the PDF receptor. Of note, is the ellipsoid body (EB), which is involved in locomotion control (Parisky et al., 2008). The role of lLNvs in controlling sleep is independent from their role in the circadian system, since lLNvs maintain robust behavioural and molecular rhythms, despite genetic manipulations that affect sleep behaviour (Sheeba et al., 2008; Shang et al., 2013; Gmeiner et al., 2013). In contrast, the sLNvs are sleep-prompting neurons. The sLNVs promote sleep during the night via the expression of the neuropeptide short neuropeptide F (sNPF) (Shang et al., 2013). lLNvs express sNPF receptors, which are important for sLNv-lLNv coordination (Shang et al., 2013). The DN1 neurons promote wake in advance of lights-on (ZT21-24) via expression of the neuropeptide Diuretic hormone 31 (DH31). Knockdown of DH31 increases sleep, whereas

overexpression of DH31 decreases sleep specifically at the end of the night. The DN1 neurons also promote wake in advance of lights-on in a PDF-dependent manner. Expression of tethered PDF in DN1s also reduces sleep specifically during ZT21-24 (Kunst et al., 2014). Intriguingly, the DN1 neurons also promote sleep at different times of the day via independent mechanisms. Because activating the DN1 neurons increases daytime sleep, while silencing them decreases night-time sleep (Guo et al., 2016).

1.12 Circadian and sleep disturbances in tauopathies

Memory deficits are the most common symptom of AD. However, the majority of AD patients also exhibit disturbances in the sleep-wake cycle and circadian rhythms. A substantial portion of AD patients display progressive disruption of the sleep-wake cycle, typified by increased daytime naps, reduced and fragmented nightime sleep and increased night-time wakefulness (for review see Musiek et al., 2015). Sleep disturbances are one of the earliest symptoms (prodrome) of AD and may even precede cognitive abnormalities. Similar fragmentation of the sleep-wake cycle has also been reported in FTD (Anderson et al., 2009). Additionally, in AD patients daily rhythms of activity and core body temperature show reduced amplitude and marked phase shifts. AD patients show reduced daytime activity (active phase) and increased night-time activity (inactive phase) compared to healthy controls. Furthermore, peaks in activity and core body temperature occur approximately four hours later in AD patients compared to healthy controls (Satlin et al., 1995; Volicer et al., 2001; Harper et al., 2001; Tranah et al., 2011). Similar disruption in daily activity and body temperature rhythms has also been reported in FTD (Harper et al., 2001). Furthermore, many individuals with late/moderate AD display sundowning syndrome, which is an obvious increase in behavioural symptoms in the late afternoon/early evening (Volicer et al., 2001). Moreover, there is a strong link between sleep disorders and PD. For example, the vast majority of individuals diagnosed with a REM sleep behaviour disorder will subsequently develop tauopathies associated with tau and α-synuclein pathology (Boeve et al., 2011; Bjørnarå et al., 2018). Disruption of sleep and circadian rhythms pose a difficult challenge for caregivers and are a major factor leading to the institutionalisation of AD

patients and increased healthcare cost (the current global cost for caring for AD patients exceeds $800 billion annually see UsAgainstAlzheimer's website).

Circadian dysfunction is traditionally regarded as a downstream effect of tauopathies, but recent evidence has suggested it may in fact contribute to the disease process. For example, disrupted daily activity rhythms in cognitively normal adults increase the risk of developing AD in later life (Tranah et al., 2011). Furthermore, three independent studies have identified separate polymorphisms in the *Clk* gene that are a risk factor for AD (Chen et al. 2013; Chen et al., 2013; Yang et al., 2013). Collectively, these studies have established a link between the circadian clock and neurodegeneration. These studies open up the exciting possibility that treatment of the circadian and sleep disturbances in the early stages of tauopathies, such as AD, may slow down the progression of the disease or even stop it. Thus, a current priority is to develop therapeutics to target circadian misalignment and sleep disruption in AD. However, in spite of their serious consequences, sleep and circadian disturbances in tauopathies, such as AD, remain enigmatic.

It is not known whether sleep and circadian disturbances in tauopathies are caused by damage to the central clock or by blocking its communication with output neurons. Several studies have suggested that degeneration of the SCN causes circadian dysfunction in AD. For example, there is substantial atrophy of the SCN in post mortem brains of AD patients (Swaab et al., 1985; Swaab et al., 1988; Zhou et al., 1995). There is a dramatic decrease in the number of VIP- and AVP-expressing neurons in the SCN of AD patients when compared with healthy controls. There is also a reduction in mRNA and protein levels of AVP and VIP in the brains of AD patients compared with healthy controls (Stopa et al., 1999; Wu et al., 2007; Harper et al., 2008). However, amyloid plagues are not often found in the SCN ruling them out as the cause of neurodegeneration (Stopa et al., 1999). This suggests that that soluble Aβ and tau species may cause circadian dysfunction. In support of this, Tate et al. (1992) showed grafting of PC12 cells overexpressing disease linked variants of APP (but not control PC12 cells) in the SCN of adult rats causes behavioural arrhythmicity.

However, other studies have suggested central clock output failure may cause circadian dysfunction. For example, clock gene expression in the pineal gland (downstream target of the SCN) is similar in post mortem brain tissue from AD patients and in rats in which the pineal gland has been derived of SCN control (Wu et al., 2006). Moreover, clock gene expression in various different clocks in post mortem brain tissue for AD patients is rhythmic, but there are marked phase shifts between the clock, suggesting changes in their relative synchronisation. The authors suggested that this failure to successfully synchronise the clocks is due to blocked communication between the clocks at a synaptic level (Cermakian et al., 2011).

1.13 Animal models of AD-linked circadian and sleep disturbances

1.13.1 β-amyloidosis

To understand the mechanisms underlying the circadian and sleep disturbances in tauopathies, such as AD, and to try to develop ways to ameliorate these problems robust animal models are needed. Mouse models of β-amyloidosis have been created by a variety of different ways. One option is to express wild-type or mutant APP (amyloid β precursor protein). A second option is to coexpress wild-type or mutant APP and wild-type or mutant PS1 (presenilin 1). PS1 is a secretase that proteolytically cleaves APP to produce Aβ peptides. These mice models develop similar neuropathological and behavioural features to those observed in human AD. However, it is important to note that no one AD mice model fully recapitulates all the features of human AD.

Mice overexpressing disease-linked variants of APP exhibit abnormalities in daily activity rhythms in LD conditions. PDAPP mice overexpressing human APP with the London mutation ($717_{V\rightarrow F}$), show increased cage activity during the active dark phase but not the inactive light phase pre-amyloid plaques (3-5 months old) but not post-amyloid plaques (22 months old) (Huitron Resendiz et al., 2002) . Similar results reported in two studies from the Peter Deyn laboratory. APP23 mice, which overexpress human APP carrying the Swedish double mutation (K670N, M671L), are hyperactive during the active dark phase up to 6

months of age (Van Dam et al., 2003). Cognitive deficits precede amyloid deposition in APP23 mice. APP23 mice begin to develop amyloid plaques in the cortex and hippocampus by 6 months of age. Subsequently, Vloeberghs et al. (2004) reported in APP23 mice an age-dependent increase in cage activity during the second half of the active dark phase. This resembles sundowning found in AD patients. In 3 month old mice, there are only minor differences in cage activity, whereas 6 and 12 month old mice exhibit a bimodal night-time activity pattern.

However, Wisor et al. (2005) reported in Tg2576 mice, which overexpress human APP with the Swedish double mutation (K670N, M671L), no differences in wheel running activity up to 22 months of age. Amyloid deposits are present in Tg2576 mice by 5-7 months of age. Cognitive impairments coincide with amyloid deposition in Tg2576 mice. In contrast, Gorman and Yellon (2010) reported in Tg2576 mice an age-independent increase in cage activity during the active dark phase (5-22 month old). Additionally, Gil-Bea et al. (2007) reported an increase in spontaneous activity in Tg2576 mice. Ambree et al. (2006) reported similar findings in TgCRND8 mice overexpressing human APP with the Swedish (K670N/M671L) and Indiana (V717F) mutations. TgCRND8 mice develop amyloid plagues in the cortex and hippocampus by 3 months of age. TgCRND8 mice are hyperactive both pre- and post-amyloid plaques (1-4 month old) (Ambree et al., 2006). Collectively, these studies show APP mice exhibit an age-independent increase in activity, particularly during the active dark phase.

Studies on sleep in APP mice have yielded confusing and contradictory findings. Huitron Resendiz et al. (2002) reported 3-5 month old PDAPP mice exhibit a reduction in sleep during the inactive light phase but not during the active dark phase. Whereas, 22 month old PDAPP mice exhibit a reduction in wakefulness and increase in sleep during the inactive light phase and an increase in wakefulness and reduction in sleep during the active dark phase. In contrast, Wisor et al. (2005) reported no difference in the amount of overall sleep in Tg2576 mice up to 17 months of age. Contradictory findings were reported by Zhang et al. (2005). The authors report Tg2576 mice exhibit an age-dependent reduction in daily sleep, sleep is normal in 2 month old mice but reduced in 6 and 12 month old mice. Additionally, Tg2576

mice exhibit age-related abnormalities in a sleep electroencephalogram (EEG) (Wisor et al., 2005; Zhang et al., 2005). Inconsistencies in findings in APP mouse models could be attributed to differences in the level of APP expression, different APP constructs, sex differences, or differences in the method used to measure activity (either wheel running or cage activity).

Similarly, mice expressing disease linked-variants of APP and PS1 also exhibit abnormalities in sleep and daily activity rhythms in LD conditions. APP/PS1 knock-in mice (uses endogenous promoters to prevent supraphysiological levels of APP/PS1) exhibit a two hour phase delay in wakefulness (Duncan et al., 2012), reminiscent of the phase delays in activity and body temperature rhythms found in AD patients (Satlin et al., 1995; Harper et al., 2001; Volicer et al., 2001). APP/PS1 knock-in mice also show a reduction in endogenous *mPer* mRNA oscillations (but no change in VIP or AVP expression). Roh et al. (2012) reported in $APP^{swe}/PS1^{\delta E9}$ mice, which express APP with the Swedish mutation and exon 9 deleted PS1 mutation an age-related fragmentation of the sleep-wake cycle. Cognitive deficits and amyloid deposits appear in $APP^{swe}/PS1^{\delta E9}$ mice at about 6-9 months of age. In 3 month old mice, the sleep-wake cycle is normal, whereas in 6 and 9 month old mice, there is a progressive increase in wakefulness and decease in sleep during the inactive light phase. Additionally, Bano Otalora et al. (2012) reported an increase in activity specifically during the second half of the inactive light phase in $APP^{swe}/PS1^{\delta E9}$ mice. However, $APP^{swe}/PS1^{\delta E9}$ mice display normal daily body temperature rhythms (Bano Otalora et al., 2012). Taken together, these studies show APP/PS1 mice exhibit an increase in activity and decrease in sleep during the inactive light phase, reminiscent of human AD.

Only minor abnormalities in activity rhythms in constant darkness have been reported in APP and APP/PS1 mice. Wisor et al. (2005) reported 5-22 month old Tg2576 mice display a longer period (~0.25 h) in wheel running activity. However, Gorman and Yellon (2010) reported Tg2576 mice up to 22 months of age display a normal period. Similarly, Bano Otalora. (2012) reported $APP^{swe}/PS1^{\delta E9}$ mice exhibit a normal period. Inconsistences in the findings could be attributed to differences in methodology (wheel running/cage activity or length of recording), genetic background, or sex.

3xTg-AD mice, which overexpress disease linked variants of APP (Swedish), PS1 (M146V) and tau (P301), show increased activity during inactive light phase and reduced percentage of overall activity during the active dark phase both pre- and post-amyloid plaques. The alterations in activity rhythms coincide with loss of AVP- and VIP- containing cells in the SCN (Sterniczuk et al., 2010). This suggests that damage to the central clock may cause the circadian dysfunction; a notion, which is supported by post mortem studies on brain tissue from AD patients (Swaab et al., 1985; Zhou et al., 1995; Swaab et al., 1998). As with AD patients and APP/PS1 mice, 3xTg-AD mice exhibit an increase in activity during the typically inactive phase. Additionally, Knight et al. (2013) reported oscillations in daily body temperature rhythms are phase advanced in 3xTg-AD mice. Sterniczuk et al. (2010) reported 3xTg-AD mice display a shorter period (~0.25 h) in free-running conditions both pre- and post-amyloid plaques.

Drosophila models, in which Aβ42 or aggregate-prone Aβ42arc (E226 mutation) are expressed directly in the nervous system, have been developed as a complement to β-amyloidosis mice models. Pan-neuronal expression (using Elav-Gal4) of Aβ42 or Aβ42 arc causes progressive age-dependent behavioural arrhythmia in LD and DD conditions. However, despite the behavioural arrhythmia, the central clock remains intact as indicated by normal endogenous *per* mRNA and PER protein oscillations and normal morphology of central clock neurons (Chen et al., 2014; Long et al., 2014). Thus, these studies suggest that circadian dysfunction is not caused by damage to the central clock, but by blocking its communication with output neurons.

Tabuchi et al. (2015) reported ubiquitous Aβ42 expression during adulthood (using daughterlessGS) reduces night-time sleep, but daytime sleep is unaffected. Whereas, ubiquitous Aβ42arc expression during adulthood reduces daytime sleep and reduces and fragments night-time sleep. Reduced and fragmented sleep at night is characteristic of AD and FTD (Harper et al., 2001; for review see Musiek et al. 2015). The authors also showed altering sleep affects Aβ deposition. Reducing sleep increases the Aβ burden, whereas increasing sleep reduces the Aβ burden. Additionally, sleep deprivation via Aβ42 expression markedly increases neuronal excitability in the LNvs (Tabuchi et al., 2015). In contrast,

Gerstner et al. (2017) demonstrated pan-neuronal Aβ42 expression during adulthood (using elavGS) reduces and fragments daytime sleep, but night-time sleep is unaffected. There is also a reduction in night-time activity, but daytime activity is unaffected. Inconsistencies in the findings could be attributed to differences in the expression system (ubiquitous or limited to the nervous system), duration of expression, or sex differences. The authors also showed pan-neuronal Aβ42 expression (using Elav-Gal4) reduces and fragments both daytime and night-time sleep and increases night-time activity (but daytime activity unaffected). The sleep and activity abnormalities are already present in 2-3 day old flies, whereas learning and memory abnormalities first appear in 6-7 day old flies. Therefore, the circadian and sleep disruption precedes the onset of the cognitive deficits, consistent with findings in AD mice models. Additionally, Dissel et al. (2017) showed ubiquitous APP and BACE (secretase that proteolytically cleaves APPL to produce Aβ peptides) expression during adulthood (using daughterlessGS) reduces and fragments night-time sleep (but daytime sleep is unaffected). Collectively, these studies demonstrate that Aβ42 expression (either ubiquitous or limited to the nervous system) affects activity and sleep in LD conditions.

1.13.2 Tauopathy

In contrast to the large number of studies on circadian and sleep disturbances in mouse and *Drosophila* models of β-amyloidosis, very few studies have investigated whether circadian dysfunction exists in animal models of tauopathy. Circadian abnormalities have been reported in daily activity and body temperature rhythms in 3xTg-AD mice (which overexpress APP^{swe}, $PS1^{M146V}$, and tau^{P301L}). However, it is not possible to assess the specific contribution of tau to the circadian dysfunction, because β-amyloidosis is also present (Sterniczuk et al., 2010; Knight et al., 2013). The behavioural characterisation of early transgenic tau mice was hindered by brainstem and spinal cord pathology, resulting in motor deficits. But more recently, the use of region specific promoters (i.e. CaMKII) mostly overcame this problem and allowed in-depth assessment of behaviour. However, to date daily activity rhythms in LD and DD conditions have been characterised in only two mouse models of tauopathy (Koss et al., 2016; Stevanovic et al., 2017)

Tg4510 mice which overexpress FTD-linked mutant P301L tau (0n4r isoform), under the control of the forebrain specific CaMKII promoter, exhibit an increase in activity during the inactive light phase (but activity during the active dark phase is unaffected) at an age when NFTs and cognitive deficits are present (Stevanovic et al., 2107). Similar to AD patients and 3xTg-AD mice, Tg4510 mice exhibit an increase in the percentage of total daily activity, occurring during the inactive phase. In free running conditions, Tg4510 mice display a long period (~1 h) in wheel running activity. No change in overall daily activity in Tg4510 mice in DD conditions. Behavioural abnormalities in Tg4510 mice coincide with the appearance of hyperphosphorylated pathological tau in the SCN (and hippocampus) and disruption of cyclic expression of PER (but not BMAL1) in the hypothalamus and hippocampus. Thus, tau expression impacts the clock at a molecular level (Stevanovic et al., 2017). In contrast, knock-in PLB2 mice, which express FTD-linked mutant P301L and R406W tau (2n4r isoform) under the control of the CaMKII promoter, exhibit a reduction in activity during both the inactive light phase and active dark phase. However, they exhibit increased wakefulness and reduced sleep during the inactive light phase. Thus, they are more awake during the inactive light phase despite reduced activity. Additionally, PLB2 mice exhibit abnormalities in a sleep EEG (Koss et al., 2016).

To date no studies have described in detail circadian behavioural abnormalities in a *Drosophila* model of tauopathy. Recently, Dissel et al. (2017) showed in *Drosophila* ubiquitous expression of tau during adulthood (using daughterlessGS) causes a reduction in daytime sleep (but night-time sleep is unaffected), opposite to what has been reported in AD (Bonanni et al., 2005; Musiek et al., 2015).

1.14 Aims of thesis

Chapter 2

- Investigate whether different 4r tau isoforms and proteins have differential ability to cause neuronal dysfunction and neuronal death.
- Study whether tau-mediated neuronal dysfunction is separable from neuronal death.
- Test whether adult-onset tau expression causes functional and structural degeneration.
- Analyse whether tau phosphorylation plays a causative role in tau-mediated neuronal dysfunction.
- Test whether coexppression of Aβ42 with tau synergistically enhances tau-mediated neuronal dysfunction.

Chapter 3

- Develop a fly model of tauopathy that recapitulates the circadian rhythm disruption in AD i.e. increased daytime sleep, reduced night-time sleep and reduced amplitude circadian rhythms with phase shifts.
- Investigate whether the circadian rhythm disturbances are due to dysfunction or degeneration in the central pacemaker or in neurons downstream of the central pacemaker.
- Test whether adult-onset tau expression is capable of causing circadian dysfunction.
- Examine whether tau abnormalities in glial cells plays a role in AD-linked circadian dysfunction.

Chapter 4

- Measure the limit of the spatial resolution of *Drosophila* motion vision through optomotor behaviour in a flight simulator system.

Chapter 2: Neuronal dysfunction is separable from neuronal death in a *Drosophila* model of tauopathy

2.1 Introduction

Tauopathies are a group of neurodegenerative diseases including Alzheimer's disease (AD), Frontotemporal dementia (FTD) and Parkinson's disease (PD). Depending upon its phosphorylation status, tau binds to and stabilises microtubules. In tauopathies, tau is hyperphosphorylated and aggregates into neurofibrillary tangles (NFTs). Mutations in tau lead to familial FTD; indicating, tau abnormalities give rise to neurodegeneration (Kouri et al., 2014; Coppola et al., 2012). Hyperphosphorylated tau has reduced binding affinity for microtubules, which leads to the breakdown of the microtubule network. Recent evidence shows tau also regulates neuronal activity and synaptic plasticity (Wang and Mandelkow, 2016; Guo et al., 2017).

Tauopathies are clinically characterised by cognitive decline. The presence of NFTs does not correlate well with cognitive decline found in individuals with AD. Instead, analysis of AD brains has revealed synapse loss correlates far better with cognitive decline. Memory deficits in AD patients coincide with a reduction in the density of presynaptic glutamatergic boutons. However, neuron death fails to account for the substantial synapse loss, thus synapses must be lost prior to death (reviewed in Arendt, 2009). Furthermore, levels of various synaptic proteins are dramatically reduced in brains from AD patients (Masliah et al., 2001; Marksteiner et al., 2002). Similarly, analysis of PD brains has shown dopaminergic terminal dysfunction precedes nigral cell death (Kordower et al., 2013). Therefore, the early clinical symptoms in tauopathies, such as AD and PD, precede NFT formation and overt neuronal death. Currently, much effort is being directed at identifying affected individuals earlier, which would allow therapeutic intervention before irreparable neuronal damage.

Therefore, animal models of tau-mediated neuronal dysfunction are required. Mice models, overexpressing human tau, show depletion of synaptic proteins, memory deficits, loss of dendritic spines and impaired synaptic transmission, prior to or in the absence of aggregate

formation and cell death (Schindowski et al., 2006; Kimura et al. 2007). Switching of tau expression, in mice overexpressing human tau, rescues the cognitive deficits although aggregates continue to form (Santacruz et al., 2005). Thus, overaccumulation of soluble tau is capable of causing neuronal dysfunction in the absence of neurodegeneration and NFT formation. However, the mechanisms of tau-mediated neuronal dysfunction remain poorly understood. Hoover et al. (2010) showed in Tg4510 mice, which overexpress human P301L tau under the control of the forebrain specific CaMKII promotor, tau mislocalises and overaccumulates in the somatodendritic compartment. Dendritic tau interferes with NMDA receptor trafficking and anchoring to the postsynaptic membrane, resulting in impairments in long-term potentiation (LTP) in the hippocampus (CA1-CA3 synapses). Whereas, other studies have shown tau induces neuronal dysfunction by reducing the probability of neurotransmitter release from the presynaptic terminal (Yoshiyama et al., 2007; Polydoro et al., 2009; Tai et al., 2012).

Drosophila are a valuable tool for studying neurodegenerative disease (for review see Lu and Vogel, 2009). Overexpression of human wild-type (WT) or FTD-linked mutant (R406W/V337M/P301L) tau in the developing *Drosophila* brain is associated with neurotoxicity (Wittmann et al., 2001). Similarly, overexpression of tau in the developing *Drosophila* eye is also associated with neurotoxicity, which manifests as a rough eye phenotype (REP) in adult flies (Jackson et al., 2002). In other *Drosophila* models, tau expression is associated with behavioural abnormalities in the absence of neuronal death (Mudher et al., 2004; Chee et al., 2005, Ubhi et al., 2007; Talmat-Amar et al., 2011). Mudher et al. (2004) showed overexpression of human tau in larval motor neurons disrupts neuromuscular junction (NMJ) morphology and axonal transport, which causes severe defects in locomotor function. Additionally, Chee et al. (2005) showed overexpression of human or *Drosophila* tau in larval motor neurons causes a reduction in synaptic boutons and perturbs synaptic transmission. Several studies have shown overexpression of human or *Drosophila* tau in adult mushroom body (MB) neurons impairs associative learning and memory (Mershin et al., 2004; Kosmidis et al., 2010; Papanikolopoulou et al., 2010). No neurodegeneration or aggregates were found in any of these models. Therefore, *Drosophila*

models of tauopathy faithfully recapitulate the neuronal dysfunction, observed in the early stages of AD.

Spatiotemporally regulated alternative splicing of the MAPT gene creates six tau isoforms in the human brain. These contain three or four carboxyl (C)-terminal inserts and zero to two amino (N)-terminal inserts. The C-terminal inserts comprise the microtubule binding domain of tau. In humans, 3r and 4r tau isoforms are normally expressed in the brain at a ratio of 1:1 (Goedert et al., 1989). FTD-causing mutations increase the ratio of 4r to 3r tau isoforms in the brain. The function of the N-terminal inserts is not well known. Differential splicing results in 2n tau being relatively sparse compared to the 0n and 1n tau isoforms. 0n, 1n and 2n tau isoforms are expressed at an approximate ratio of 27:54:9 in the human brain (Goedert and Jakes, 1990). Mutant and wild-type tau are associated with different diseases with distinct pathology, symptomology and molecular aetiology (reviewed in Guo et al., 2017). *In vitro,* different tau proteins exhibit different phosphorylation susceptibilities (Goedert and Jakes, 1990; Goedert et al., 1992; Buee et al., 2000), microtubule binding capabilities (Kanaan et al., 2011; Matsumoto et al., 2015), subcellular distribution (Liu and Gotz, 2013; Paholikova et al., 2015) and interaction partners (Liu et al., 2016).

It is not known whether different tau isoforms precipitate equivalent results when studied in insolation. Several studies have investigated the toxic effects of different tau proteins in the developing *Drosophila* visual system (Jackson et al., 2002; Grammenoudi et al., 2008; Chatterjee et al., 2009) and MB neuroblasts (Kosmidis et al., 2010; Papanikoloulou et al., 2010). These studies have shown that specific tau isoforms have differential effects in different neuronal populations. Other studies have investigated the ability of different tau proteins to cause associative learning and memory deficits when expressed in adult MB neurons (Mershin et al. 2004; Kosmidis et al., 2010; Papanikolopoulou et al., 2010), and axonal transport defects when expressed in larval motor neurons (Mudher et al., 2004; Chee et al., 2005; Ubhi et al., 2007; Talmat-Amar et al., 2011). These studies show isoform-specific effects in tau-mediated neuronal dysfunction in different neuronal types and at different life stages. However, to date no study has compared the ability of different 4r tau isoforms to induce neuronal dysfunction in the same neuronal population.

In this study, we develop a model overexpressing human tau in the *Drosophila* retina. In our model, tau is expressed at a low enough level, as to not produce a REP. Therefore, this provides a powerful system to examine age-dependent changes in function and structure. We further restrict tau expression to adulthood; this allows us to compare the effects of tau in adult neurons versus developing neurons. In this study, we investigate whether different human 4r tau proteins have a differential ability to cause functional and structural degeneration in the fly eye. We further investigate whether tau-mediated neuronal dysfunction is separable from neuronal death.

In this study, we demonstrate isoform-specific differences in tau-mediated neuronal dysfunction and neuronal death. We show human 0n4r tau causes age-related progressive functional degeneration without structural degeneration. On the other hand, human 2n4r tau causes progressive functional degeneration, which precedes structural degeneration, when expressed in the developing *Drosophila* eye. In contrast, mutant (R406W) and wild-type tau (0n4r isoform) precipitate similar effects in our model. 2n4r tau expression in the adult *Drosophila* retina causes progressive neuronal dysfunction without neuronal death. Thus, adult tau-mediated toxicity requires a neurodevelopmental component. Collectively, these results demonstrate that tau-mediated neuronal dysfunction is independent from neuronal death. We further show phosphodeficient S2A tau fails to cause dysfunction; this suggests tau phosphorylation plays a role in tau-mediated neuronal dysfunction. Therefore, S2A inhibitors may be a valid therapeutic strategy. Finally, we show human Aβ42 expression does not produce neuronal dysfunction, but coexpression of Aβ42 with 2n4r tau^{WT} potently exacerbates tau-mediated neuronal dysfunction. These results suggest tau abnormalities are required for Aβ42-mediated neuronal dysfunction.

2.2 Materials and Methods

2.2.1 Fly strains

All flies were reared on standard food (containing 0.8 % agar, 1.0 % soy flour, 8.0 % medium cornmeal, 8.0 % malt extract, 1.8 % yeast, 4.0 % molasses, 2.5 % 10% Nipagin in absolute ethanol and 0.4 % propionic acid) in a 12 h/12 h light/dark (LD) cycle at 25 °C, except for animals carrying tub-Gal80^{ts} which were reared at 18 °C. To obtain flies expressing human tau or Aβ in the *Drosophila* eye we used longGMR-Gal4 (pan-retina) or Rh1-Gal4 (R1-R6 photoreceptors) to drive expression of UAS-tau or UAS-Aβ. Whereas, to obtain flies expressing tau pan-neuronally we used Elav-Gal4 to drive expression of UAS-tau. The UAS-0n4r tau^{WT} transgene expresses the full-length (383 amino acids) 0n4r isoform of human wild-type tau. The UAS-0n4r tau^{R406W} transgene expresses the full-length 0n4r isoform of human R406W tau (Wittmann et al., 2001). The UAS-2n4r tau^{WT}1 and UAS-2n4r tau^{WT}2 transgenes express the full-length (441 amino acids) 2n4r isoform of human wild-type tau. The UAS-2n4r tau^{S2A}1 and UAS-2n4r tau^{S2A}2 transgenes express the full-length 2n4r isoform of human wild-type with mutations at the two PAR1 phosphorylation sites (S262A and S356A). The UAS-2n4r tau^{S11A} transgene expresses the full-length 2n4r isoform of human wild-type with mutations at 11 GSK/sgg phosphorylation sites (S46A, T50A, S119A, T202A, T212A, S214A, T231A, S235A, S396A and S404A) (Chatterjee et al., 2009). The UAS-Aβ42 transgene expresses the 42 amino acid fragment of human APP (Iijima et al., 2004).

Tau expression in the adult retina was achieved by using the TARGET system: temperature sensitive tub-Gal80^{ts} combined with longGMR-Gal4. At 18 °C (permissive temperature), Gal80^{ts} is active and represses the transcriptional activity of Gal4. After adult flies are shifted to 30 °C (restrictive temperature), Gal80^{ts} becomes inactive and no longer represses Gal4 activity (McGuire et al., 2003, McGuire et al., 2004). All transgenic lines were backcrossed against Canton-S for five generations to reduce the variation from genetic background. ninaE (Rh1)-eGFP was used to visualise the photoreceptor rhabdomeres in live flies (Pichaud and Desplan, 2001). The various UAS-tau lines used were previously generated by P-element mediated transgenesis. Therefore, they are susceptible to position effects which alter

expression levels and activity of transgenes. A summary of stocks used, including full genotype, source and references, can be found in Table 2.1.

Stock	Full genotype	Source	References
Canton-S	+; +; +	In house	
UAS-0n4r tau^{WT}	w[*]; +; P{UAS-Hsap\MAPT.WT}	Mel Feany (Harvard Medical School, US)	Wittmann et al., 2001
UAS-0n4r tau^{R406W}	w[*]; +; P{UAS-Hsap\MAPT.R406W}	Mel Feany (Harvard Medical School, US)	Wittmann et al., 2001
UAS-2n4r tau^{WT}1	w[1118]; P{w[+mC]=UAS-Tau.wt}1.13; +	Bloomington stock centre (#51362)	Chatterjee et al., 2009
UAS-2n4r tau^{WT}2	w[1118]; +; P{w[+mC]=UAS-Tau.wt}7B	Bloomington stock centre (#51363)	Chatterjee et al., 2009
UAS-2n4r tau^{S2A}1	y[1] w[1118]; P{w[+mC]=UAS-TauS2A}1.13; +	Bloomington stock centre (#51364)	Chatterjee et al., 2009
UAS-2n4r tau^{S2A}2	w[1118]; P{w[+mC]=UAS-TauS2A}1.62; +	Bloomington stock centre (#51365)	Chatterjee et al., 2009
UAS-2n4r tau^{S11A}	w[1118]; P{w[+mC]=UAS-TauS11A}1.2; +	Bloomington stock centre (#51366)	Chatterjee et al., 2009
UAS-Aβ42	w[*]; P{w[+mC]=UAS-Abeta1-42.G}3	Bloomington stock centre (#32037)	Iijima et al., 2004
tub-Gal80^{ts}	w[*]; +; P{w[+mC]=tubP-GAL80[ts]}2/TM2	Bloomington stock centre (#7017)	McGuire et al., 2003; McGuire et al., 2004
Elav-Gal4	P{w[+mW.hs]=GawB}elav[c155]; +; +	Bloomington stock centre (#458)	Luo et al., 1994
Rh1-Gal4	w[*]; P{ry[+t7.2]=rh1-Gal4}2/Cyo; +	Bloomington stock centre (#68385)	Mollereau et al., 2000
lGMR-Gal4	w[*]; P}w[+mC]=longGMR-Gal4}2; +	Bloomington stock centre (#8605)	Freeman et al., 1996; Wernet et al., 2003
ninaE(Rh1)-eGFP	w[*]; P{w[+mC]=ninaE-EGFP.p}2; MKRS/TM2	Bloomington stock centre (#7460)	Pichard and Desplan, 2001
ElavGS-Gal4	y[1] w[*]; +; P[w[+mC]=elav-Switch.O}GSG301	Bloomington stock centre (#43642)	Osterwalder et al., 2001
tim-Gal4	y[1] w[*]; P{w[+mC]=GAL4-tim.E}62; +	R. Stanewsky (University of Munster, Germany)	Kaneko and Hall, 2000
Pdf-Gal4	w[*]; P{w[+mC]=Pdf-Gal4.P2.4}2; +	R. Stanewsky (University of Munster, Germany)	Renn et al., 1999
Pdf-Gal80	w[*]; P{Pdf-GAL80.S}96A; +	C. Forster (University of Wurzburg, Germany)	Stoleru et al., 2004
repo-Gal80	w[*]; +; P{repo-GAL80.L}N18	S. Schirmeier (University of Munster, Germany)	Awasaki et al., 2008

Table 2.1: Stocks used in Chapters 2 and 3.

2.2.2 Corneal neutralisation

Flies were anesthetized using CO_2 and placed in a 35 mm petri dish, half-filled with 1 % molten agarose (~55 °C). The agarose was allowed to solidify and covered with ice-cold water (4 °C). The petri dish was kept on ice until imaging (Pichaud and Desplan, 2001). Eyes photoreceptor rhabdomeres were imaged using an upright confocal fluorescent microscope (Olympus Fluoview FV1000), equipped with a 40x water immersion long-distance objective. Images were obtained by opening the pinhole significantly wider than is usually

recommended for the objective. Images were analysed using ImageJ and Adobe Photoshop. Photoreceptor rhabdomeres were visually quantified.

2.2.3 ERGs

Flies were immobilised with low melting point wax inside a copper cone. ERGs were recorded using ~10 MΩ glass microelectrodes filled with Ringer (containing in nM: 120NaCl, 5KCl, 10TES, 1.5CaCl2, 4MgCl2 and 30 sucrose) inserted in the distal retina. A similar reference electrode was inserted into the ocellus. All flies tested were female. All flies were dark-adapted for two minutes prior to recording. We used a high-power white LED driven by an OptoLED power supply as a light source, mounted on a cardan arm system. The stimulus was composed of a one second light pulse preceded by 0.2 seconds of darkness and followed by one second of darkness. The 2.2 second stimulus was repeated ~20 times. The mean ± SEM of all ERG responses for each genotype/age is shown. Two of the three main components of an ERG were quantified; the ON transient and photoreceptor-dependent depolarization (Heisenberg, 1971). For each genotype and age, the mean and SEM were calculated. For each genotype and age, at least eight flies were tested. Biosyst run in Matlab was used for stimulus generation and data acquisition. Origin and GraphPad Prism were used for data analysis and graphing.

2.2.4 Flight simulator system

In all experiments, 1- to 3-day-old female flies were used. Flies were reared in bottles at a low population density to prevent overcrowding and maximise size. Under cold-anaesthesia (lasting <three minutes), a triangle-shaped copper-wire hook (diameter ~0.5 mm) was glued (Loctite UV glue) to both a fly's head and thorax. Flies were prepared in the late afternoon (four-six pm). They were then left overnight to recover individually in small moist chambers with sugar. To study *Drosophila*'s optomotor behaviour we used a bespoke flight simulator system (Wardill et al., 2012). *Drosophila* were connected to a small metal clamp by their small triangular hooks. The small metal clamp was then connected to the torque meter (Tang & Guo, 2001). A fly, connected to the torque meter was placed in the centre of a

circular arena. Homogenous illumination of the arena was provided by a ring–shaped light tube (Philips, 350-900 nm). The arena forms a continuous (360°) scene of alternative black and white vertical stripes around the fly. The arena is rotatable by the use of a stepping motor. We used an eight second stimulus which consisted of two seconds of counterclockwise rotation and two seconds of clockwise rotation separated by two seconds when the arena was still – preceded and concluded by one second when the arena was still. The eight second stimulus was repeated ~20 times. When presented with scene rotations, flies attempt to follow them producing yaw torque responses (optomotor response). At least 10 flies per genotype were tested. All experiments were performed in a dark room at 23 °C.

2.2.5 Statistical analysis

Statistical analysis was performed in Graph Pad Prism 7. Data distribution was normal (Kolmogorov-Smirnov normality test). All graphs show individual datapoints with mean ± SEM. Data were analysed by a two-tailed Student's t-test if there were two groups, 1-way ANOVA if there were more than two groups with a single factor (genotype) and 2-way ANOVA if there were more than two groups with two factors (genotype and age) followed by post-hoc tests. Multiple comparisons after ANOVA were performed by a Tukey HSD test. P levels are indicated as ns $p>0.05$, *$p<0.05$, **$p<0.001$ or ***$p<0.0001$. In all graphs, asterisks (in black) show differences between the experimental genotypes and the control genotype of the same age. Asterisks (in black) above horizontal lines show differences between experimental genotypes of the same age. Numbers symbols (in red) show differences between 5-, 10- or 30-day old flies and 1-day old flies of the same genotype. Graphs were created in Graph Pad Prism 7. Sample sizes are reported in figures. Sample sizes were not predetermined by any statistical test. However, sample sizes are similar to those used in previous publications (Gonzalez-Bellido et al., 2009; Chouhan et al., 2016; Hindle et al., 2017; Nippe et al., 2017).

2.3 Results

2.3.1 0n4r tau expression in the retina causes neuronal dysfunction

The transgenic models we used in this study used the binary Gal4/UAS system, in which cDNA encoding different human tau isoforms is placed downstream of UAS sequences. Using a Gal4 driver it is possible to drive UAS-tau expression in a tissue specific manner. The shortGMR-Gal4 (sGMR-Gal4) driver is strongly expressed in all retinal cell types during development (Freeman, 1996). However, expression of Gal4 in the developing eye under the control of the sGMR promotor causes developmental defects; sGMR-Gal4 expression causes a REP in newborn adult flies when reared at ≥23 °C (Kramer and Staveley, 2003). We therefore selected the longGMR-Gal4 (lGMR-Gal4) driver, which has the same expression pattern as the more commonly used sGMR-Gal4 but lower expression levels (Freeman, 1996; Wernet et al., 2003). To examine the toxic effects of different tau proteins in the eye we used both functional and structural assays. We used electroretinograms (ERGs) and visual behaviour in the flight system, to examine function (Heisenberg, 1971; Wang and Montell, 2007), and we used the corneal neutralisation technique, quantified by counting the number of photoreceptor rhabdomeres, to examine structure (Pichaud and Desplan, 2001).

UAS-tau transgenic lines, which express full-length human wild-type or R406W tau (0n4r isoform), were crossed to the lGMR-Gal4 driver (Wittmann et al, 2001). Both wild-type and R406W tau (0n4r isoform) cause adult-onset progressive neurodegeneration and premature death, when expressed in the developing *Drosophila* brain (using Elav-Gal4), and a REP, when expressed in the developing *Drosophila* eye (using sGMR-Gal4). Previous studies have reported R406W tau is more toxic than wild-type tau when expressed in either the developing *Drosophila* brain or visual system (Wittmann et al., 2001; Jackson et al., 2002; Nishimura et al., 2004; Khurana et al., 2006).

The external appearance of lGMR>0n4r tau^{WT} and lGMR>0n4r tau^{R406W} eyes was normal (data not shown). To assess functionality, ERGs were measured to repeated one second light

pulses with an electrode inserted into the distal retina. lGMR-Gal4 control flies exhibited a strong rapid sustained photoreceptor-dependent depolarization (~10 mV) in response to a light stimulus, as well as second order neurons response in the optic lobe, detectable as ON and OFF peaks (transients) before and after photoreceptor-dependent depolarization (indicated in Figure 2.1A).

We found normal depolarization in young lGMR>0n4r tau^{WT} flies. There were no significant differences in depolarization in 1- and 5-day old lGMR>0n4r tau^{WT} flies compared with age-matched Gal4 control flies, indicating normal photoreceptor function in lGMR>0n4r tau^{WT} flies early in life. However, in aged animals, the situation had changed dramatically. lGMR>0n4r tau^{WT} flies showed an age-related progressive reduction in depolarization. Quantification of the depolarization revealed that it was moderately reduced (~20 %) in 10-day old lGMR>0n4r tau^{WT} flies and severely reduced (~60 %) in 30-day old lGMR>0n4r tau^{WT} flies, compared with age-matched controls. lGMR>0n4r tau^{WT} flies showed a significant age-related decline in depolarization. Depolarization in 10- and 30-day old lGMR>0n4r tau^{WT} flies was significantly smaller than in 1-day old lGMR>0n4r tau^{WT} flies. In contrast, depolarization was stable in Gal4 control flies up to 30-days of age. We found no significant differences in depolarization between age groups in control flies (Figure 2.1A,B).

We observed normal ON transients in young lGMR>0n4r tau^{WT} flies. No significant differences in ON transients were seen in 1- and 5-day old lGMR>0n4r tau^{WT} flies with respect to age-matched controls, indicating normal synaptic transmission in lGMR>0n4r tau^{WT} flies up to 5-days of age. With aging, there was a rapid and progressive loss of ON transients in lGMR>0n4r tau^{WT} flies. We found a severe reduction in ON transients in 10-day old lGMR>0n4r tau^{WT} flies (~1 mV compared to ~3.5 mV in control flies), indicating compromised synaptic transmission. 10-day old lGMR>0n4r tau^{WT} flies exhibited a wide range of ON transients: two flies displayed responses in the wild-type range, 15 flies displayed small transients and 15 flies displayed no transients. We found no 30-day old lGMR>0n4r tau^{WT} flies exhibited an ON transient, indicating a total block of synaptic transmission. lGMR>0n4r tau^{WT} flies showed a significant reduction in ON transients as a function of age. ON transients were significantly smaller in 10- and 30-day old lGMR>0n4r

tau^{WT} flies compared to 1-day old lGMR>0n4r tau^{WT} flies. Whereas, no significant age-related differences in ON transients were seen in control flies (Figure 2.1A,C).

Similarily, we also found normal depolarization in lGMR>0n4r tau^{R406W} flies up to 5-days of age. With aging, there was a progressive reduction in depolarization in lGMR>0n4r tau^{R406W} flies. We found 10-day old lGMR>0n4r tau^{R406W} flies showed a ~40 % reduction in depolarization and 30-day old lGMR>0n4r tau^{R406W} flies showed a ~80 % reduction in depolarization, relative to age-matched controls. lGMR>0n4r tau^{R406W} flies showed a significant age-related reduction in depolarization. Depolarization was significantly smaller in 10- and 30-day old lGMR>0n4r tau^{R406W} flies compared to 1-day old lGMR>0n4r tau^{R406W} flies (Figure 2.1A,B). We observed normal ON transients in 1-day old lGMR>0n4r tau^{R406W} flies. With aging, there was a rapid and progressive loss of ON transients in lGMR>0n4r tau^{R406W} flies. A severe reduction in ON transients was seen in 5-day old lGMR>0n4r tau^{R406W} flies (~2 mV compared to ~3.5 mV in control flies). 5-day old lGMR>0n4r tau^{R406W} flies exhibited a wide range of ON transients: seven flies displayed responses in the wild-type range, nine flies displayed small transients and one fly displayed no transient. We found nearly a complete lack of ON transients in 10-day old lGMR>0n4r tau^{R406W} flies, 17 out of 20 flies recorded from had no ON transient. Furthermore, we found a complete abolishment of ON transients in 30-day old lGMR>0n4r tau^{R406W} flies. lGMR>0n4r tau^{R406W} flies showed a significant age-related decrease in ON transients. ON transients in 5-, 10- and 30-day old lGMR>0n4r tau^{R406W} flies were significantly smaller than in 1-day old lGMR>0n4r tau^{R406W} flies (Figure 2.1A,C) .

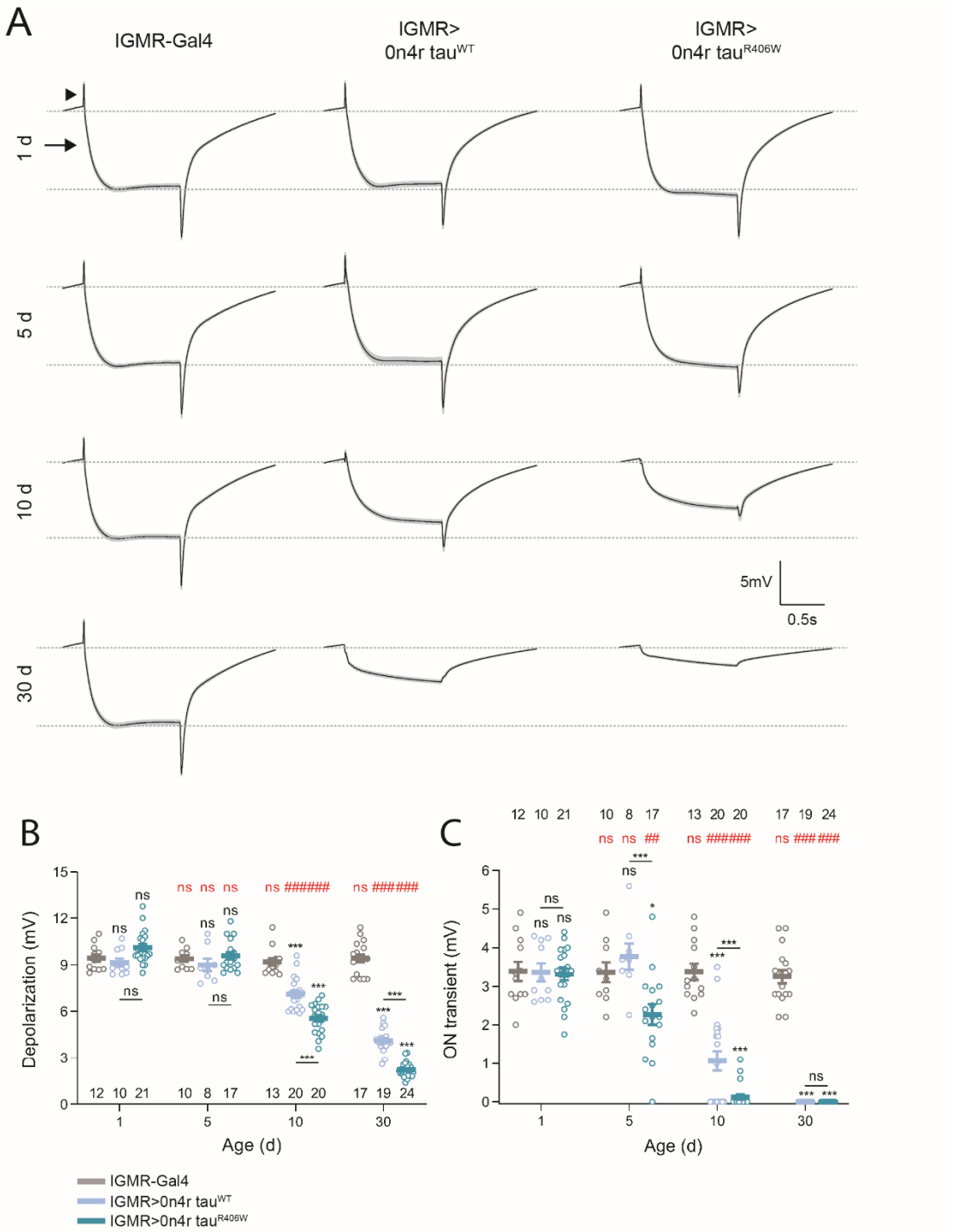

A
IGMR-Gal4
IGMR>
0n4r tau^WT
IGMR>
0n4r tau^R406W
1 d
5 d
10 d
30 d
5mV
0.5s
B
Depolarization (mV)
Age (d)
C
ON transient (mV)
Age (d)
IGMR-Gal4
IGMR>0n4r tau^WT
IGMR>0n4r tau^R406W

Figure 2.1: 0n4r tau expression in the retina causes neuronal dysfunction. (A) Measuring retinal function using ERGs in control and tau-expressing flies at various ages. ERG recordings in response to a one second light pulse are shown from 1-, 5-, 10- and 30-day-old lGMR-Gal4, lGMR>0n4r tau^{WT} and lGMR>0n4r tau^{R406W} eyes. Normal depolarization (arrow) and ON transient (arrowhead) indicated, which reflect the phototransduction cascade and synaptic transmission, respectively. Normal depolarization in 1- and 5-day old 0n4r tau-expressing flies. Age-related progressive reduction in depolarization in 0n4r tau-expressing flies. Moderate reduction in depolarization in 10-day old 0n4r tau-expressing flies and severe reduction in depolarization in 30-day old 0n4r tau-expressing flies. Normal ON transients in 1-day old 0n4r tau-expressing flies. Age-related rapid and progressive loss of ON transients in 0n4r tau-expressing flies. Tiny ON transients in 10-day old 0n4r tau-expressing flies and no ON transients in 30-day old 0n4r tau-expressing flies. ERGs are stable in control flies up to 30-days of age. Each trace represents the mean response from >8 flies from the mean of ~15 responses per fly. (B) Quantification of ERG depolarization. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on depolarization, with a significant interaction between factors ($p<0.0001$). (C) Quantification of ERG ON transients. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on ON transients, with a significant interaction between factors ($p<0.0001$). Graphs show individual datapoints with mean ± SEM. n = 8-24 flies per genotype/age. ns $p>0.05$,**$p<0.001$, ***$p<0.0001$. Data were normally distributed. Asterisks (in black) show comparisons with the control genotype of the same age, asterisks (in black) above horizontal lines show comparisons between experimental genotypes of the same age and number symbols (in red) show comparisons with 1-day old flies of the same genotype by post-hoc Tukey HSD tests.

Thus, we found a similar but more severe phenotype in lGMR>0n4r tau^{R406W} flies compared to lGMR>0n4r tau^{WT} flies. There was a more dramatic age-related reduction in depolarization in lGMR>0n4r tau^{R406W} flies compared to lGMR>0n4r tau^{WT} flies. Depolarization in 1- and 5-day old lGMR>0n4r tau^{R406W} flies did not differ significantly from age-matched lGMR>0n4r tau^{WT} flies. On the other hand, depolarization in 10- and 30-day old lGMR>0n4r tau^{R406W} flies was significantly smaller than in age-matched lGMR>0n4r tau^{WT} flies. Additionally, there was an accelerated loss of ON transients in lGMR>0n4r tau^{R406W} flies compared with lGMR>0n4r tau^{WT} flies. We found ON transients were significantly smaller in 5-day old lGMR>0n4r tau^{R406W} flies compared to age-matched controls. Whereas, ON transients in 5-day old lGMR>0n4r tau^{WT} flies did not significantly differ from age-matched controls. We found a significant reduction in ON transients between the 1- and 5- day old age groups in lGMR>0n4r tau^{R406W} flies, but not in lGMR>0n4r tau^{WT} flies. These transgenic UAS-0n4r tau lines were generated by P-element mediated transgenesis where constructs are inserted at random into the Drosophila genome.

Therefore, positional effects on transgene activity and expression may account for the stronger phenotype in lGMR>0n4r tau^{R406W} flies compared to lGMR>0n4r tau^{WT} flies.

We also tested lGMR>0n4r tau^{R406W} flies' sight behaviourally. In concordance with the ERG data, young (1- to 3-day old) tethered flying lGMR>0n4r tau^{R406W} flies in the flight simulator system successfully followed scene rotations, generating reflex-like turns (optomotor responses) to the left or the right (Figure 2.5A). When we quantified the optomotor responses (min-to-max) for lGMR>0n4rtau^{R406W} flies, they did not differ significantly from Gal4 control flies (Figure 2.5B). It is not possible to reproducibly test visual behaviour in older (≥10 day old) flies in the flight simulator system, because although they can be motivated to fly by airflow they fail to do so for long enough to collect sufficient data. Taken together, these results demonstrate intact retinal function in both lGMR>0n4r tau^{WT} and lGMR>0n4r tau^{R406W} flies at a young age, as indicated by the normal ERG responses and visual behaviour in the flight simulator system in <5-day old 0n4r tau-expressing flies. However, retinal function is severely compromised in aged flies, as indicated by the progressive reduction in ERG depolarization, which coincides with the rapid and progressive loss of ERG ON transients, in >5-day old 0n4r tau-expressing flies.

2.3.2 No neuronal death in 0n4r tau-expressing retina

Next, we investigated whether neuronal dysfunction in 0n4r tau-expressing eyes coincides with neuronal death. To achieve this we assessed the survival of photoreceptor rhabdomeres in living adult flies at various ages. Rhabdomeres are the expanded apical domain of photoreceptors, which consist of many (~30,000) microvilli, which contain the light sensing apparatus (Harris et al., 1976). For this, we used the quantitative cornea neutralisation technique, in which one visualises the photoreceptor rhabdomeres directly though the cornea of a living water covered anesthetised fly with an immersion objective (Franceschini and Kirschfeld, 1971). The six outer photoreceptors (R1-R6) are detected based on the expression of eGFP using a Rh1(ninaE)-eGFP transgene (Pichaud and Desplan, 2001).

First, to ensure the presence of eGFP in the photoreceptors did not have an adverse effect on function we recorded ERGs from lGMR-Gal4; Rh1-eGFP flies. Depolarization and ON transients in lGMR-Gal4; Rh1-eGFP flies did not differ significantly from lGMR-Gal4 flies (Supplementary Figure 1). Typically in *Drosophila* eyes, ommatidia are regularly spaced and within each ommatidium rhabdomeres are arranged in a precise asymmetric trapezoidal pattern. In the eyes of lGMR>0n4r tau^{WT} and lGMR>0n4r tau^{R406W} flies the spatial organisation of rhabdomeres appeared normal. Furthermore, rhabdomere morphology (both size and shape) seemed to be similar in 0n4r-tau expressing and control flies (Figure 2.2A). Thus, lGMR>0n4r tau expression does not have a toxic effect on photoreceptor development.

We found no evidence of structural degeneration in either lGMR>0n4r tau^{WT} or lGMR>0n4r tau^{R406W} flies up to 30-days of age. All photoreceptor rhabdomeres were present in the eyes of lGMR>0n4r tau^{WT} and lGMR>0n4r tau^{R406W} flies up to 30-days of age. The number of rhabdomeres per ommatidium in lGMR>0n4r tau^{WT} and lGMR>0n4r tau^{R460W} flies did not significantly differ from age-matched controls in all age groups. We found no significant age-related differences in the number of rhabdomeres per ommatidium within each genotype (Figure 2.2A,B). We also quantified the percentage of ommatidia with a complete set of rhabdomeres. The percentage of ommatidia with a full complement of rhabdomeres was 97 % in control flies, 95 % in lGMR>0n4r tau^{WT} flies and 94 % in lGMR>0n4r tau^{R460W} flies at 1-day of age. Similarily, in 30-day old flies, the percentage of ommatidia with a complete set of rhabdomeres was 92 % in lGMR>0n4r tau^{WT} flies, 93 % in lGMR>0n4r tau^{R406W} flies and 95 % in control flies (Table 2.2). Collectively, these results show that 0n4r tau-expressing eyes lose their responsiveness to light without loss of their photoreceptor rhabdomeres. This evidence supports the notion that tau-mediated neuronal dysfunction is separable from neuronal death.

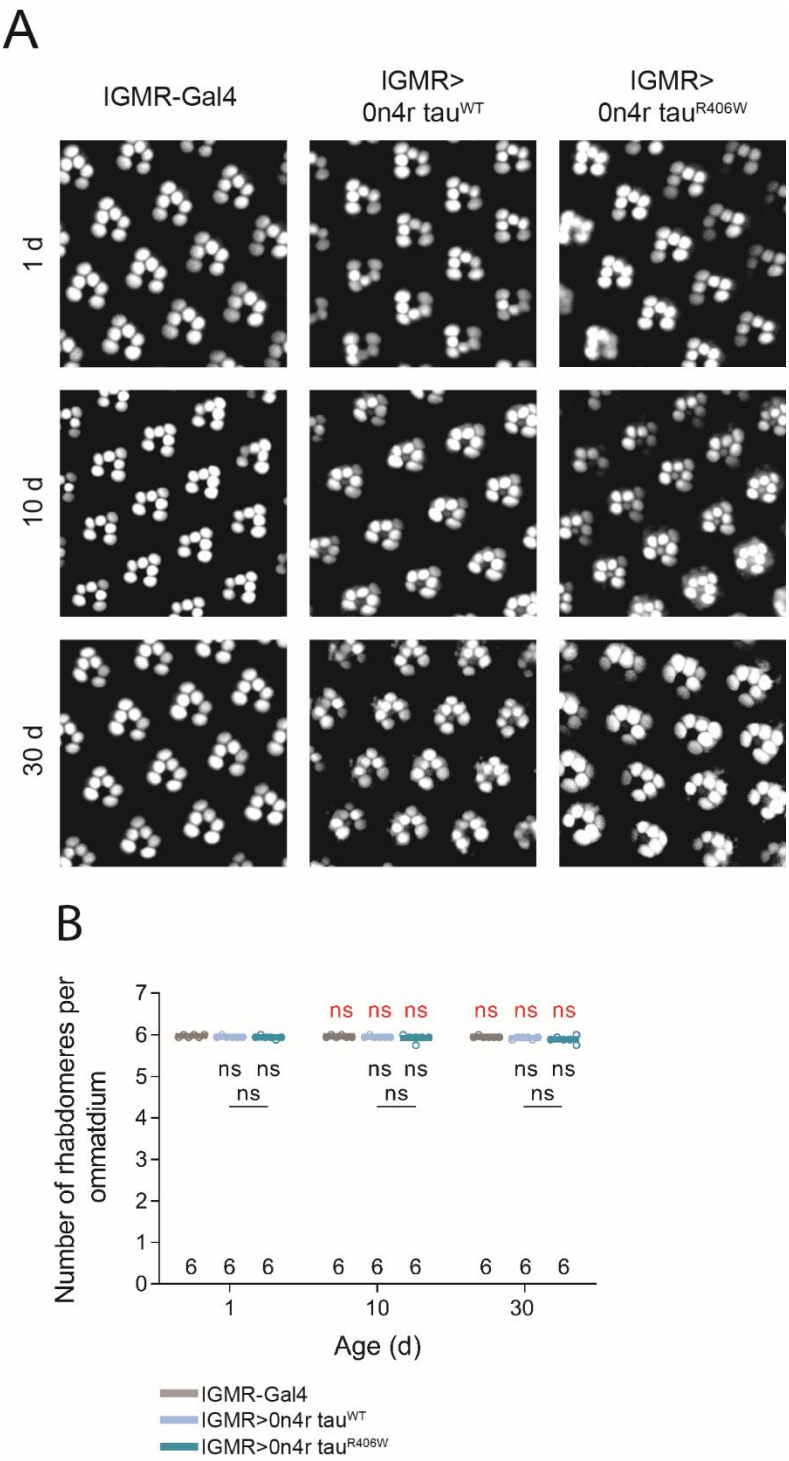

A
IGMR-Gal4
IGMR>
0n4r tauWT
IGMR>
0n4r tauR406W
1 d
10 d
30 d
B
Number of rhabdomeres per ommatdium
7
6
5
4
3
2
1
0
ns ns ns
ns ns
ns
6 6 6
1
10
30
Age (d)
IGMR-Gal4
IGMR>0n4r tauWT
IGMR>0n4r tauR406W

Figure 2.2: No neuronal death in 0n4r tau-expressing retina. Water immersion microscopy images of Rh1-eGFP fluorescence in the eyes of 1-, 10- and 30-day-old lGMR-Gal4, lGMR>0n4r tau^{WT} and lGMR>0n4r tau^{R406W} flies. Outer (R1-R6) photoreceptor rhabdomeres are arranged in an asymmetric trapezoidal pattern. Normal number and arrangement of rhabdomeres in the eyes of lGMR>0n4r tau^{WT} and lGMR>0n4r ^{R406W} flies. No apparent age-related differences in the number of rhabdomeres per ommatidium. All photoreceptor rhabdomeres are visible in the eyes of control, lGMR>0n4r tau^{WT} and lGMR>0n4r tau^{R406W} flies up to 30-days of age. (B) Quantification of the number of photoreceptor rhabdomeres per ommatidium. 2-way ANOVA showed no significant effect of genotype (p=0.2954) and age (p=0.1063) on the number of photoreceptor rhabdomeres per ommatidium, with no significant interaction between factors (p=0.9346). Graphs show individual datapoints with mean ± SEM. n = 6 flies per genotype/age from ~20 ommatidia per fly. ns p>0.05. Data were normally distributed. Asterisks (in black) show comparisons with the control genotype of the same age, asterisks (in black) above horizontal lines show comparisons between experimental genotypes of the same age and number symbols (in red) show comparisons with 1-day old flies of the same genotype by post-hoc Tukey HSD tests.

2.3.3 2n4r tau expression in the retina causes temporally separable neuronal dysfunction and death

Previous studies have compared the toxicity of 3r and 4r tau isoforms in different neuronal types (Grammenoudi et al., 2008; Kosmidis et al., 2010; Papanikoloulou et al., 2010), but to date no study has described in detail the consequences of expressing different 4r tau isoforms in the same neuronal population. Therefore, next we sought to investigate whether lGMR>2n4r tau expression precipitates equivalent results with lGMR>0n4r tau expression. These isoforms differ in the number of N-terminal inserts they carry in their N-terminal domain; 0n4r tau carries no N-terminal inserts, whereas 2n4r tau carries two N-terminal inserts. The role of the N-terminal inserts is not well understood. *In vitro,* different 4r tau isoforms exhibit different phosphorylation susceptibility (Goedert and Jakes, 1990; Goedert et al., 1992; Buee et al., 2000), microtubule binding affinity (Kanaan et al., 2011; Matsumoto et al., 2015), subcellular distribution (Liu and Gotz, 2013; Paholikova et al., 2015) and interaction partners (Liu et al., 2016).

We monitored age-related changes in function and structure in flies expressing the full-length 2n4r isoform of human wild-type tau under the control of lGMR-Gal4. We used two

independent UAS-2n4r tau^{WT} transgenic lines, which were previously generated by P-element mediated transgenesis (Chatterjee et al., 2009). We found a small increase in depolarization in 1-day old lGMR>2n4r tau^{WT}1 flies compared with age-matched Gal4 control flies. With aging, there was a progressive reduction in depolarization in lGMR>2n4r tau^{WT}1 flies consistent with progressive photoreceptor dysfunction. Quantification of the depolarization revealed a ~10 % increase in 1-day old lGMR>2n4rtau^{WT}1 flies, a ~40 % decrease in 10-day old lGMR>2n4rtau^{WT}1 and a ~70 % decrease in 30-day old lGMR>2n4r tau^{WT}1 flies, compared to age-matched controls (Figure 2.3A,B). The lGMR>2n4r tau^{WT}1 flies showed a dramatic decline in depolarization as a consequence of aging. Depolarization was significantly smaller in 5-, 10- and 30-day old lGMR-2n4r tau^{WT}1 flies, compared to 1-day old lGMR>2n4r tau^{WT}1 flies. Alongside the increase in depolarization, 1-day old lGMR>2n4r tau^{WT}1 flies exhibited severe synaptic transmission defects, as indicated by the minute ON transients (~0.2 mV compared to ~3.4 mV in controls flies): seven flies displayed no ON transient and eight flies displayed tiny ON transients. No aged (5-, 10- and 30-day old) lGMR>2n4r tau^{WT}1 flies displayed ON transients, indicating a total block of synaptic transmission. We found no significant differences between age groups in lGMR>2n4r tau^{WT}1 flies, as a result of the minute ON transients in 1-day old lGMR>2n4r tau^{WT}1 flies (Figure 2.3A,C).

Similarly, we found a small increase in depolarization in 1- and 5-day old lGMR>2n4r tau^{WT}2 flies compared with age-matched controls. A weak reduction in depolarization became apparent after 10 days and progressively worsened by 30 days in lGMR>2n4r tau^{WT}2 flies. Quantification of the depolarization revealed a ~10 % increase in 1-day old lGMR>2n4rtau^{WT}2 flies, a ~15 % increase in 5-day old lGMR>2n4r tau^{WT}2 flies, a ~10 % decrease in 10-day old lGMR>2n4rtau^{WT}2 and a ~60 % decrease in 30-day old lGMR>2n4rtau^{WT}2 flies, compared to age-matched control flies. lGMR>2n4r tau^{WT}2 flies showed a significant age-related reduction in depolarization. No significant differences in depolarization were seen in lGMR>2n4r tau^{WT}2 flies between the 5- and 1-day old age groups. On the other hand, depolarization was significantly smaller in 10- and 30-day old lGMR>2n4r tau^{WT}2 flies, compared to 1-day old lGMR>2n4r tau^{WT}2 flies (Figure 2.3A,B). We observed small ON transients in 1-day old lGMR>2n4r tau^{WT}2 flies (~1.3 mV compared to

~3.4 mV in controls), only four out of 18 flies tested displayed no ON transient. No ON transients were found in aged (>5-day old) lGMR>2n4r tau^{WT}2 flies. lGMR>2n4r tau^{WT}2 flies showed a small, but significant, age-related reduction in ON transients. ON transients were significantly smaller in 5-, 10- and 30-day old lGMR>2n4r tau^{WT}2 flies, compared to 1-day old lGMR>2n4r tau^{WT}2 flies (Figure 2.3A,C). Thus, in 2n4r tau-expressing retina, the loss of ERG ON transients precedes the reduction in ERG depolarization, suggesting synaptic transmission is more sensitive to lGMR>2n4r tau^{WT} expression than cell body function.

Thus, we found lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 expression produced a similar reduction in depolarization as a function of age. No significant differences in depolarization were seen between lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 flies at 10- and 30-days of age. However, the decline in depolarization began at an earlier age in lGMR>2n4r tau^{WT}1 flies compared to lGMR>2n4 tau^{WT}2 flies. Between the 1- and 5-day old age groups, we found a significant reduction in depolarization in lGMR>2n4r tau^{WT}1 flies, but not in lGMR>2n4r tau^{WT}2 flies. In 1-day old flies, there was a more dramatic reduction in ON transients in lGMR>2n4r tau^{WT}1 flies, than in lGMR>2n4r tau^{WT}2 flies, compared to age-matched controls. A highly significant increase in ON transients was seen in 1-day old lGMR>2n4r tau^{WT}2 flies compared to age-matched lGMR>2n4r tau^{WT}1 flies. However, it is possible the differences in phenotype strength between lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 flies could be related to the positional effects of different transgenes.

Next, we examined visual behaviour in young (1- to 3-day old) lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 flies in the flight simulator system. In the flight simulator system, tethered flying lGMR> 2n4r tau^{WT}1 flies failed to follow the scene rotations. Flight behaviour appeared to be random and not coupled to the optomotor stimuli. Optomotor responses in lGMR> 2n4r tau^{WT}1 flies were substantially smaller than in Gal4 control flies. In contrast, lGMR>2n4r tau^{WT}2 flies seemed to follow the scene rotations to some extent. However, they also displayed much smaller optomotor responses than in control flies. Optomotor responses in lGMR>2n4r tau^{WT}2 flies were larger than in lGMR> 2n4r tau^{WT}1 flies, albeit differences fell short of significance (Figure 2.5A,B). These results support the ERG data, in

young flies (<5-days old) larger ON transients in lGMR>2n4r tau^{WT}2 flies than in lGMR>2n4r tau^{WT}1 flies (Figure 2.3A,C).

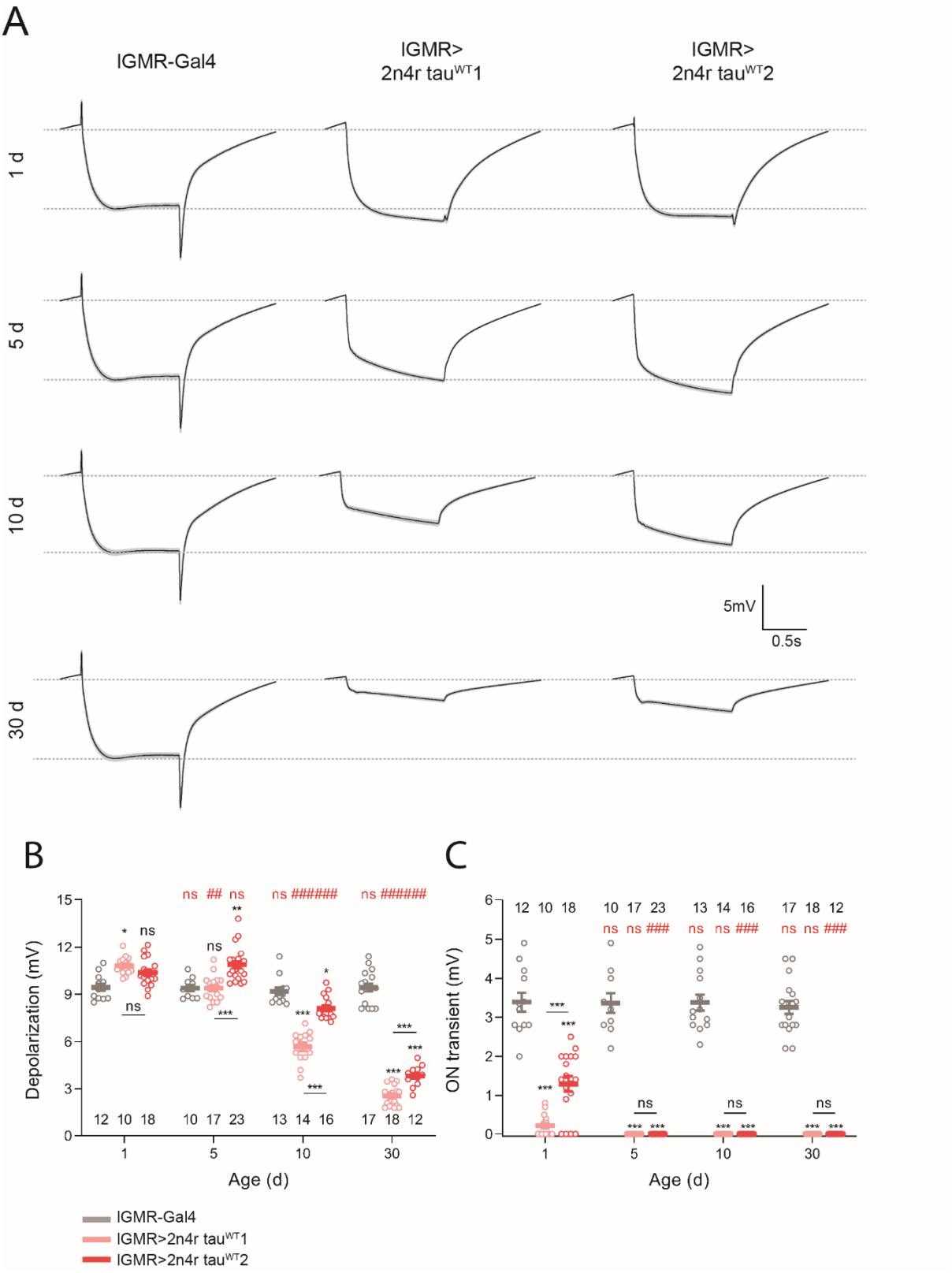


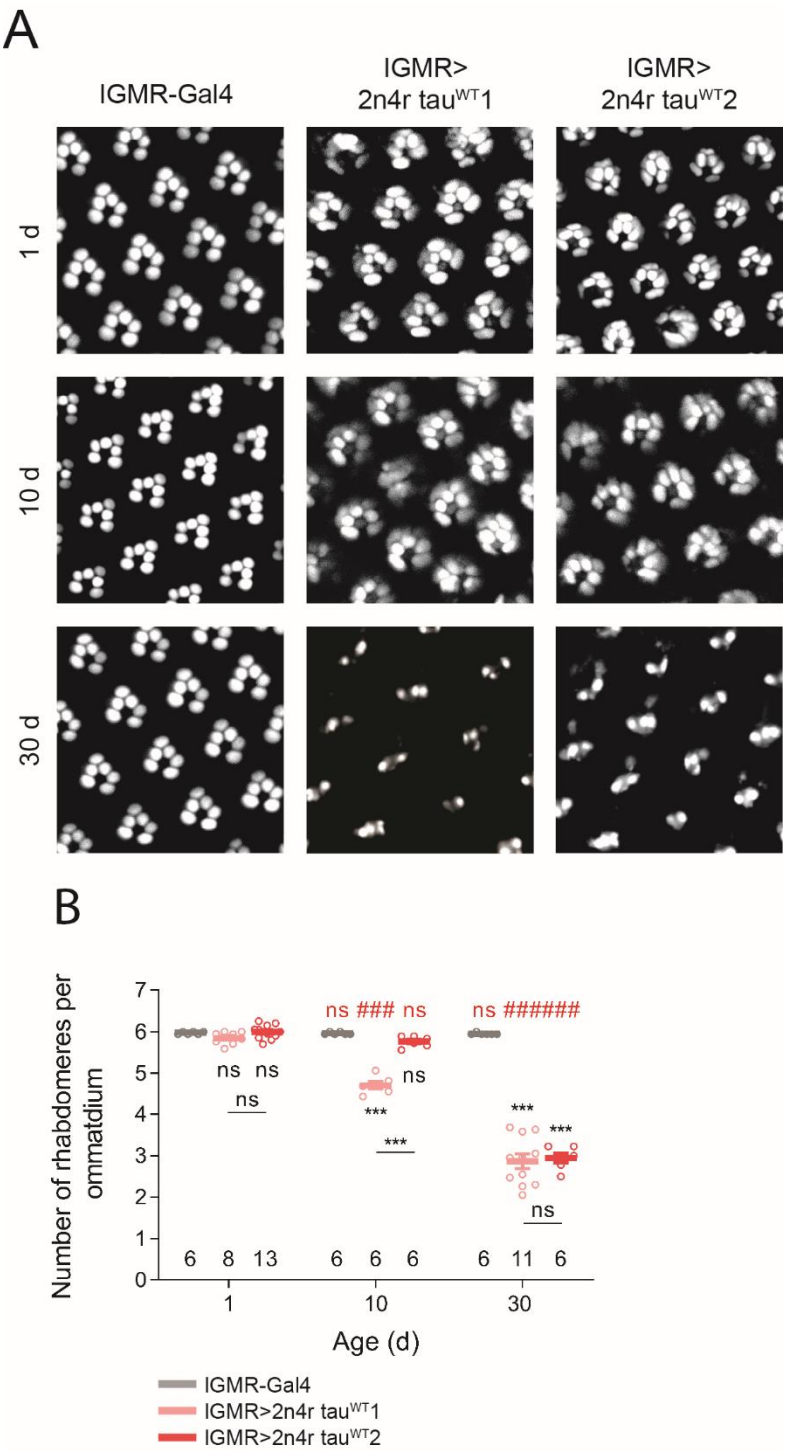
Figure 2.3: 2n4r tau expression in the retina causes neuronal dysfunction. (A) ERG recordings in response to a one second light pulse are shown for 1-, 5-, 10- and 30-day-old lGMR-Gal4, lGMR-Gal4>2n4r tau^{WT}1 and lGMR-Gal4>2n4r tau^{WT}2 eyes. Moderate increase in depolarization in 1-day old 2n4r tau-expressing flies. Lack of ON transients in 1-day old lGMR-Gal4>2n4r tau^{WT}1 flies. Small ON transients in 1-day old lGMR-Gal4>2n4r tau^{WT}2 flies. Moderate increase in depolarization in 5-day old lGMR-Gal4>2n4r tau^{WT}2 flies. Lack of ON transients in 5-day old lGMR>2n4r tau^{WT}2 flies. Age-related progressive reduction in depolarization in 2n4r tau-expressing flies. Moderate reduction in depolarization in 10-day old 2n4r tau-expressing flies and severe reduction in depolarization in 30-day old 2n4r tau-expressing flies. Each trace represents the mean response from >10 flies from the mean of ~15 responses per fly. (B) Quantification of ERG depolarization. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on depolarization, with a significant interaction between factors ($p<0.0001$). (C) Quantification of ERG ON transients. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on ON transients, with a significant interaction between factors ($p<0.0001$). Graphs show individual datapoints with mean ± SEM. n = 10-23 flies per genotype/age. ns $p>0.05$, *$p<0.05$, **$p<0.001$, ***$p<0.0001$. Data were normally distributed. Asterisks (in black) show comparisons with the control genotype of the same age, asterisks (in black) above horizontal lines show comparisons between experimental genotypes of the same age and number symbols (in red) show comparisons with 1-day old flies of the same genotype by post-hoc Tukey HSD tests.

In order to assess if functional degeneration was associated with structural degeneration, we assessed photoreceptor rhabdomere structure in the eyes of 2n4r tau-expressing flies using the quantitative corneal neutralisation technique. In the eyes of 1-day old lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 flies the ommatidia appeared to contain a complete set of rhabdomeres (Figure 2.4A). No significant differences in the number of rhabdomeres per ommatidium were seen between 1-day old 2n4r tau-expressing and control flies (Figure 2.4B). However, we found mild disorganisation in the trapezoidal pattern of rhabdomeres in the eyes of 1-day old 2n4r tau-expressing flies. Additionally, some of the rhabdomeres were abnormal in shape, they appeared rectangular or oblong rather than circular. Also, some of the rhabdomeres were abnormal in size, some appeared to be smaller than in controls, whereas others appeared to be larger than in controls. Additionally, a few of the rhabdomeres appeared split. Finally, a small number of ommatidia had supernumerary outer photoreceptors (Figure 2.4A). Thus, 2n4r tau^{WT} expression during development had a mild toxic effect on photoreceptor development.

As the lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 flies aged, there was a progressive loss of photoreceptor rhabdomeres. In lGMR>2n4r tau^{WT}1 flies a small reduction in the number of rhabdomeres per ommatidium was seen in 10-day old flies, and a large reduction in the number of rhabdomeres per ommatidium was seen in 30-day old flies, compared to age-matched controls. Similarily, a large reduction in the number of rhabdomeres per ommatidium was seen in 30-day old lGMR>2n4r tau^{WT}2 flies compared to age-matched controls. The number of rhabdomeres per ommatidium in 10-day old lGMR>2n4r tau^{WT}2 flies did not significantly differ from age-matched controls. By 30-days of age, ommatidia in the eyes of 2n4r-tau expressing flies contained on average half the normal number of rhabdomeres. Unlike in controls flies, there was a highly significant age-related reduction in the number of rhabdomeres per ommatidium in lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 flies. In lGMR>2n4r tau^{WT}1 flies, the number of rhabdomeres per ommatidium was significantly smaller in 10- and 30-day old flies compared to 1-day old flies. Likewise, in lGMR>2n4r tau^{WT}2 flies the number of rhabdomeres per ommatidium was significantly smaller in 30-day old flies compared to 1-day old flies (Figure 2.4B). We also quantified the percentage of ommatidia that contained a full complement of rhabdomeres. In 1-day old flies, the percentage of ommatidia with a complete set of rhabdomeres was 84 % in lGMR>lGMR>2n4r tau^{WT}1 flies, 93 % in lGMR>2n4r tau^{WT}2 flies and 97 % in control flies. On the other hand, in 10-day old flies, the fraction of ommatidia which had a complete set of rhabdomeres was only 25 % in lGMR>2n4r tau^{WT}1 flies and 84 % in lGMR>2n4r tau^{WT}2 flies, compared to 96 % in control flies. By 30-day of age, <1 % of ommatidia had a full complement of rhabdomeres in lGMR>2n4r tau^{WT}1 and lGMR>2n4r ^{WT}2 flies, compared to 95 % in control flies (Table 2.2).

lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 expression produced a similar phenotype in 30-day old flies. In 30-day old flies no significant differences in the number of rhabdomeres per ommatidium were seen between lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 flies. However, there was an accelerated loss of photoreceptor rhabdomeres in lGMR>2n4r tau^{WT}1 flies compared to lGMR>2n4r tau^{WT}2 flies. In 10-day flies, there were significantly fewer rhabdomeres per ommatidium in lGMR>2n4r tau^{WT}1 flies, but not in lGMR>2n4r tau^{WT}2 flies, compared to age-matched controls. Additionally, between the 1- and 10-day old age

groups, there was a significant loss of rhabdomeres in lGMR>2n4r $tau^{WT}1$ flies, but not in lGMR>2n4r $tau^{WT}2$ flies. We found synaptic transmission defects without detectable photoreceptor rhabdomere loss in 1-day old 2n4r tau-expressing flies (Figure 2.3A,C and Figure 2.4A,B). Collectively, these results show lGMR>2n4r tau expression, unlike lGMR>0n4r tau expression, is sufficient to cause age-related progressive structural degeneration. However, it is unclear whether it is bona fide degeneration, because it is not possible to determine whether adult structural degeneration is contributed to by tau-dependent toxicity during development. These findings show that 2n4r tau expression in our model causes temporary separable functional and structural degeneration.


A
IGMR-Gal4
IGMR>
2n4r tau^WT1
IGMR>
2n4r tau^WT2
1 d
10 d
30 d
B
Number of rhabdomeres per ommatidium
ns ns
ns
ns ### ns
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ns ######
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1
10
30
Age (d)
IGMR-Gal4
IGMR>2n4r tau^WT1
IGMR>2n4r tau^WT2

Figure 2.4: Neuronal death in 2n4r tau-expressing retina. Water immersion microscopy images of Rh1-eGFP fluorescence in the eyes of 1-, 10- and 30-day-old lGMR-Gal4, lGMR>2n4r $tau^{WT}1$ and lGMR>2n4r $tau^{WT}2$ flies. Outer (R1-R6) photoreceptor rhabdomeres are arranged in an asymmetric trapezoidal pattern. A normal number of rhabdomeres is seen in the eyes of 1-day 2n4r tau-expressing flies. However, in 2n4r-tau expressing flies we see mild disruption in the pattern of their rhabdomere spatial organisation. Additionally, some of the rhabdomeres are slightly deformed in lGMR>2n4r tau^{WT} flies. With aging, we find a progressive loss of photoreceptors rhabdomeres in 2n4r tau^{WT}-expressing flies. A dramatic loss of rhabdomeres is seen in 30-day old lGMR>2n4r tau^{WT} flies. (B) Quantification of the number of photoreceptor rhabdomeres per ommatidium. 2-way ANOVA shows a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on the number of rhabdomeres per ommatidium, with a significant interaction between factors ($p<0.0001$). Graphs show individual datapoints with mean ± SEM. n = 6-13 flies per genotype/age from ~20 ommatidia per fly. ns $p>0.05$, *$p<0.05$, **$p<0.001$, ***$p<0.0001$. Data were normally distributed. Asterisks (in black) show comparisons with the control genotype of the same age, asterisks (in black) above horizontal lines show comparisons between experimental genotypes of the same age and number symbols (in red) show comparisons with 1-day old flies of the same genotype by post-hoc Tukey HSD tests.

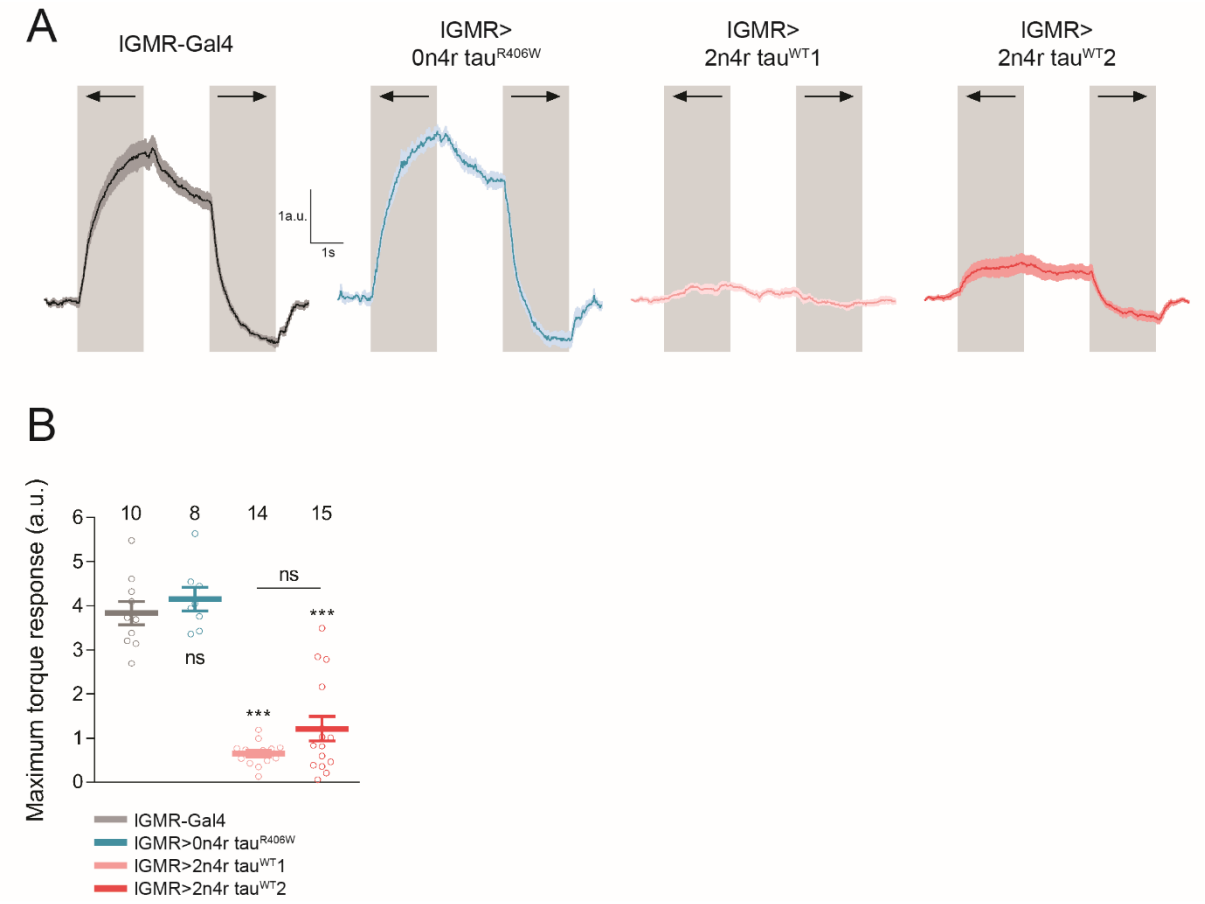


Figure 2.5: Visual behaviour in a flight-simulator system of tau-expressing flies. (A) Optomotor responses (yaw torque) of tethered flying lGMR-Gal4, lGMR>0n4r tau^{R406W}, lGMR>2n4r tau^{WT}1 and lGMR>2n4r tau^{WT}2 flies to counterclockwise and clockwise (arrows) scene rotations recorded in a *Drosophila* flight simulator system. lGMR-Gal4 and lGMR>0n4r tau^{R406W} flies robustly follow the scene rotations. lGMR>2n4r tau^{WT}1 flies fail to follow the scene rotations. lGMR>2n4r tau^{WT}2 flies partially follow the scene rotations. (B) Quantification of the maximum torque response. One-way ANOVA shows a significant effect of genotype ($p<0.0001$) on the maximum torque response. Graphs show individual datapoints with mean ± SEM. n = 8-15 flies per genotype. ns $p>0.05$, *$p<0.05$, **$p<0.001$, ***$p<0.0001$. Data were normally distributed. Asterisks show comparisons with the control genotype and asterisks above horizontal lines show comparisons between experimental genotypes by post-hoc Tukey HSD tests.

Elav>2n4r tau^{WT}1 expression caused a reduced and rough eye (Figure 2.6A). A rough eye has been indirectly associated with photoreceptor viability (Feany et al., 1996; Wittmann et al., 2001; Jackson et al., 2002; Chatterjee et al., 2009). However, ERG recordings from these eyes in 30-day old flies revealed normal depolarization and ON transients, indicating adult photoreceptor function is preserved in Elav>2n4r tau^{WT}1 flies (Figure 2.6B,C). Taken

together, these results demonstrate that tau-dependent toxicity during development is independent from tau-mediated neuronal dysfunction in adult photoreceptors.

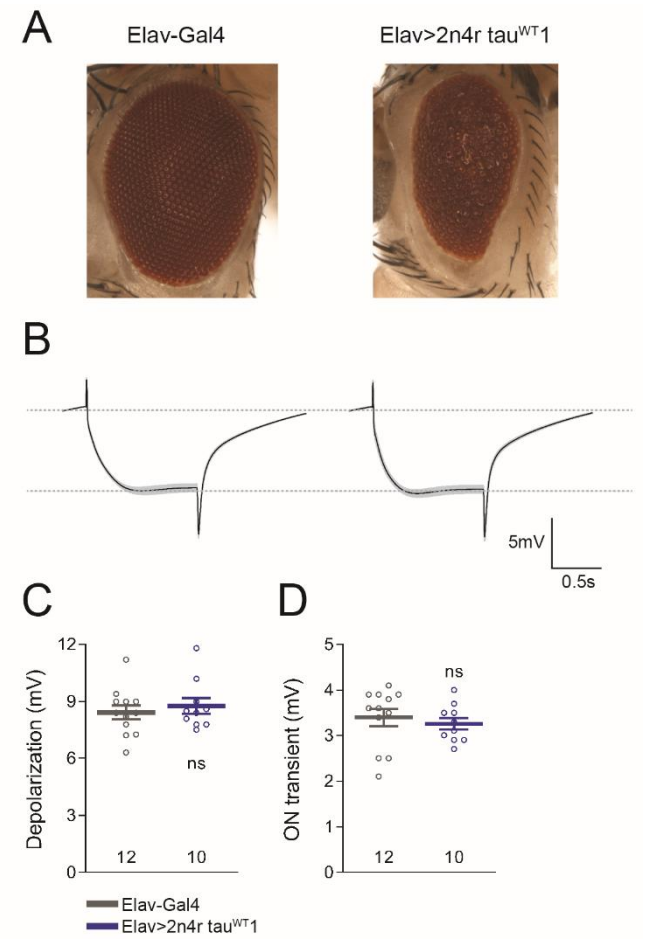


Figure 2.6: Elav>2n4r tau^{WT} expression is toxic during development but does not compromise adult photoreceptor function. (A) Eye pictures of Elav-Gal4 and Elav>2n4r tau^{WT}1 flies. (B) ERG recordings in response to a one second light pulse are shown for 30-day old Elav-Gal4 and Elav>2n4r tau^{WT}1 flies. Normal depolarization and ON transients in Elav>2n4r tau^{WT}1 flies. (C) No differences in depolarization are found in Elav>2n4r tau^{WT}1 flies relative to controls. (D) No differences in ON transients are found in Elav>2n4r tau^{WT}1 flies relative to controls. Graphs show individual datapoints with mean ± SEM. n = 10-12 flies per genotype. Data were normally distributed. ns p>0.05; two-tailed Student's t-test.

2.3.4 Adult-onset 2n4r tau^{WT} expression causes neuronal dysfunction but not neuronal death

The problem with the lGMR-Gal4 driver is it induces expression of tau from early developmental stages (Freeman et al., 1996). We have shown that 2n4r tau^{WT} expression during development has both functional and structural effects in the eyes of newborn adult flies (Figure 2.3 and Figure 2.4). Therefore, it is not possible to determine whether 2n4r tau^{WT}-evoked neuronal dysfunction and neuronal death in adult fly eyes, is related to development defects. Previous studies on whether adult-onset tau expression in the *Drosophila* eye, is sufficient to cause neuronal death have yielded a confusing and contradictory picture (Chouhan et al., 2016; Gorsky et al., 2016; Malmanche et al., 2017).

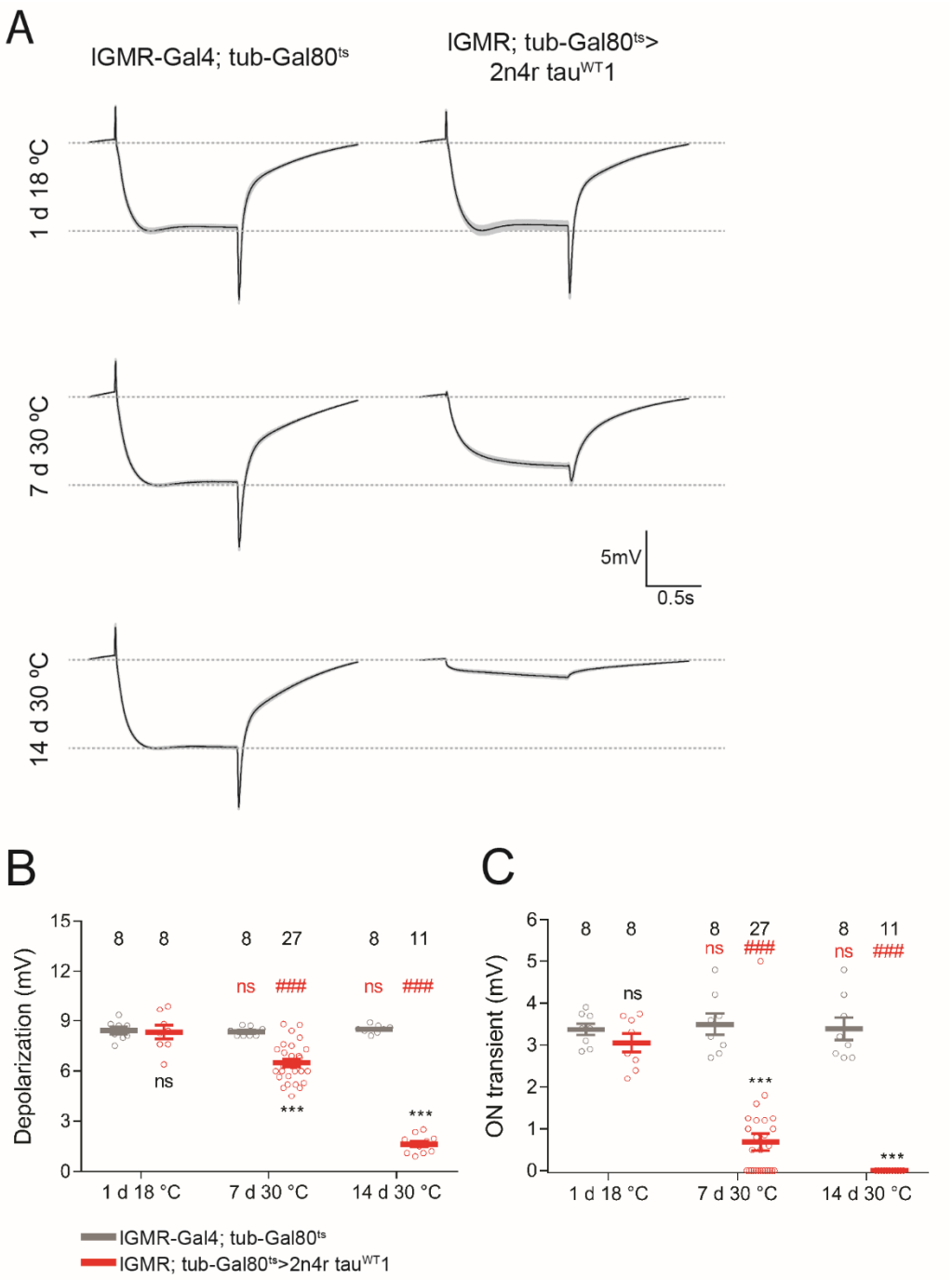
Therefore, it remains unclear whether 4r tau-mediated toxicity in adult flies requires a neurodevelopmental component. Surprisingly, whether tau expression in the adult *Drosophila* retina precipitates neuronal dysfunction has as of yet not been investigated. To answer these questions we developed a model expressing tau throughout the adult fly retina using the TARGET system (McGuire et al., 2003), to circumvent any developmental defects due to tau expression during development and therefore analyse the toxic effects exclusively related to tau expression during adulthood.

lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 and lGMR; tub-Gal80^{ts} control flies raised at 18 °C had comparable ERGs. We found no significant differences in depolarization and ON transients between lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 and control flies raised at 18 °C (Figure 2.7A,B,C). Thus, retinal function was preserved in lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies raised at 18 °C. So, there was no leaky 2n4r tau^{WT} expression during development. However, it was a different story after shifting the flies to 30 °C. They exhibited an age-related progressive reduction in depolarization, indicating progressive photoreceptor dysfunction.

Quantification of the depolarization revealed a moderate reduction (~25 %) after 7 days at 30 °C and a severe reduction (~80 %) after 14 days at 30 °C in lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies, compared with age-matched controls (Figure 2.7A,B). Moreover, shifting the flies to 30 °C also caused a progressive and rapid loss of the ON transients. We found tiny ON transients in lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies after 7 days at 30 °C (~0.7 mV compared to ~3.4 mV in control flies), indicating severely compromised synaptic transmission. Furthermore, we found a lack of ON transients in any lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies after 14 days at 30 °C, indicating a total block of synaptic transmission (Figure 2.7A,C). lGMR; tub-Gal80>2n4r tau^{WT}1 flies showed a dramatic age-related decline in depolarization and ON transients. In lGMR; tub-Gal80>2n4r tau^{WT}1 flies we found depolarization and ON transients were significantly smaller in 7- and 14-day old flies kept at 30 °C, compared to age-matched control flies kept at 18 °C. In contrast, both depolarization and ON transients were well maintained in control flies after being shifted to 30 °C (Figure 2.7). Thus, up to 14 days at 30 °C does not normally have an adverse effect on retinal function.

During the course of this work, Chouhan et al (2016) published a study demonstrating that Rh1-Gal4 driven 0n4r tau^{WT} expression causes an age-related progressive loss of the ERG response. Rh1-Gal4 is expressed in the outer R1-R6 photoreceptors in the late pupa after development has finished (Kumar and Ready, 1995), so tau expression is restricted to adulthood. Thus, this is in agreement with our findings using the TARGET system that adult-onset tau expression compromises retinal function. However, when we tried to replicate these findings we were unable to do so. Instead, we found that ERG responses in Rh1>2n4r tau^{WT}1 eyes were indistinguishable from age-matched controls in flies up until 30-days of age (Figure 2.8).

We next investigated whether adult-onset 2n4r tau^{WT} expression leads to structural degeneration by quantifying the number of photoreceptor rhabdomeres, using the corneal neutralisation technique. Ommatidia in 1-day old lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies raised at 18 °C had a full complement of rhabdomeres (Figure 2.9). In 1-day old lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies raised at 18 °C 97 % of ommatidia contained a full complement of rhabdomeres, similar to 99 % in control flies of the same age (Table 2.2). The spatial organisation and roundness of rhabdomeres was normal in 1-day old lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies raised at 18 °C (Figure 2.9A). After shifting the lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies to 30 °C for up to 14 days, we observed no loss of photoreceptor rhabdomeres. We found no significant differences in the number of rhabdomeres per ommatidium in 7- and 14-day old lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies kept at 30 °C, compared to age-matched control flies kept at 18 °C. Furthermore, we found no significant age-related differences in the number of rhabdomeres per ommatidium within each genotype (Figure 2.9B). In 14-day old flies kept at 30 °C, the fraction of ommatidia with a complete set of rhabdomeres was 94 % in lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies and 97 % in control flies (Table 2.2).


A
IGMR-Gal4; tub-Gal80ts
IGMR; tub-Gal80ts>
2n4r tauWT1
1 d 18 ºC
7 d 30 ºC
14 d 30 ºC
5mV
0.5s
B
Depolarization (mV)
15
12
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6
3
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8 8 8 27 8 11
ns ### ns ###
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1 d 18 °C
7 d 30 °C
14 d 30 °C
IGMR-Gal4; tub-Gal80ts
IGMR; tub-Gal80ts>2n4r tauWT1
C
ON transient (mV)
6
5
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2
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1 d 18 °C
7 d 30 °C
14 d 30 °C

Figure 2.7: Adult-onset 2n4r tau^{WT} expression in the retina induces neuronal dysfunction. (A) ERG recordings in response to a one second light pulse are shown for lGMR-Gal4; tub-Gal80^{ts} and lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies. Flies were raised at 18 °C and after eclosion shifted to 30 °C for up to 14 days to induce 2n4r tau^{WT}1 expression in the eye. 1-day old lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies raised at 18 °C exhibit normal depolarization and ON transients. On the other hand, after shifting lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies to 30 °C, there is a progressive reduction in depolarization and rapid and progressive loss of ON transients. Moderate reduction in depolarization after 7 days at 30 °C and a severe reduction in depolarization after 14 days at 30°C in lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies. Tiny ON transients after 7 days at 30 °C and no ON transients after 14 days at 30 °C in lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies. Whereas, ERGs are well maintained in control flies after shifting to 30 °C. (B) Quantification of ERG depolarization. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on depolarization, with a significant interaction between factors ($p<0.0001$). (C) Quantification of ERG ON transients. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on ON transients, with a significant interaction between factors ($p<0.0001$). Graphs show individual datapoints with mean ± SEM. n = 8-27 flies per genotype/age. ns $p>0.05$, ***$p<0.0001$. Data were normally distributed. Asterisks (in black) show comparisons with the control genotype of the same age and number symbols (in red) show comparisons with 1-day old flies of the same genotype by post-hoc Tukey HSD tests.

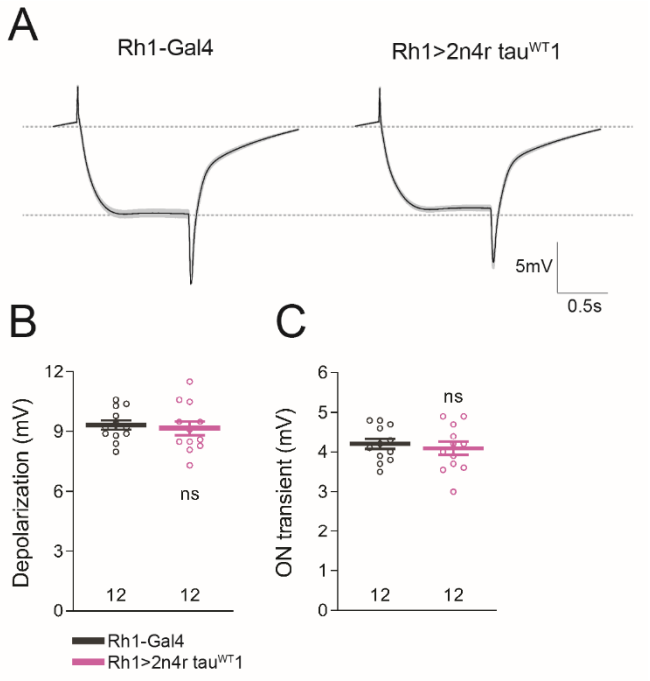


Figure 2.8: Rh1>2n4r tau^{WT} expression fails to induce neuronal dysfunction. (A) ERG recordings in response to a one second light pulse are shown for 30-day old Rh1-Gal4 and Rh1>2n4r tau^{WT}1 flies. Normal depolarization and ON transients in Rh1>2n4r tau^{WT}1 flies. (B) No differences in depolarization are found in Rh1>2n4r tau^{WT}1 flies relative to controls. (C) No differences in ON transients are found in Rh1>2n4r tau^{WT}1 flies relative to controls. Graphs show individual datapoints with mean ± SEM. n = 12 flies per genotype. Data were normally distributed. ns $p>0.05$; two-tailed Student's t-test.

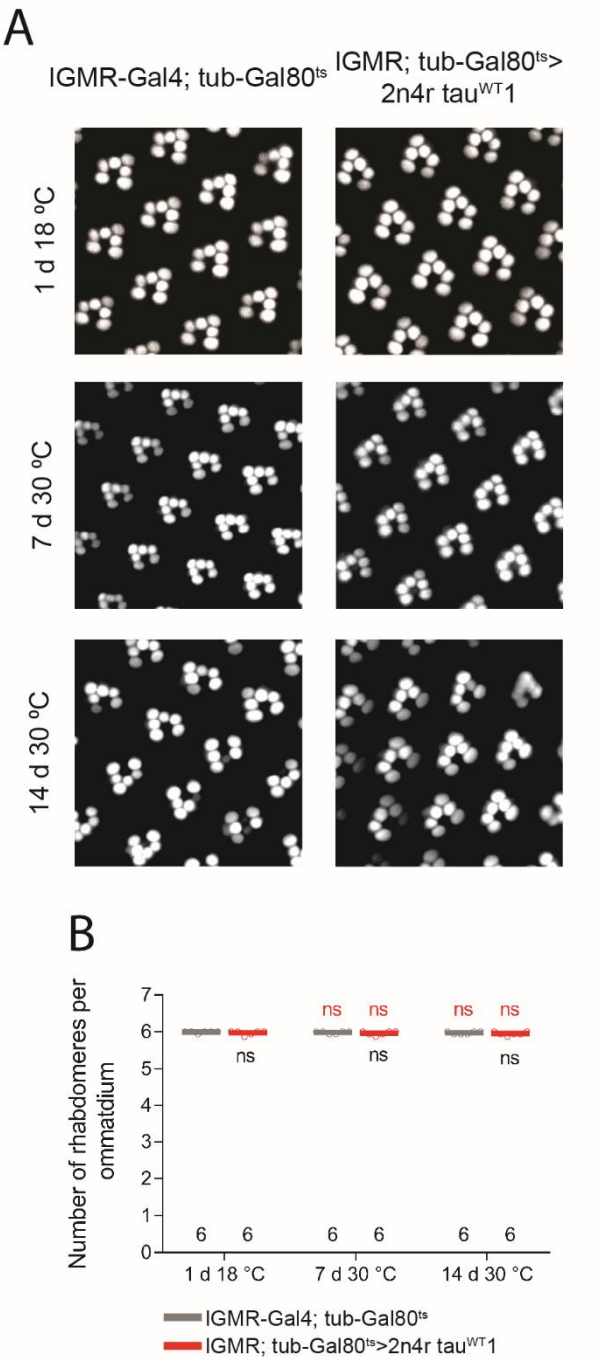


Figure 2.9: Adult-onset 2n4r tau^{WT} expression in the retina does not cause structural degeneration. Water immersion microscopy images of Rh1-eGFP fluorescence in the eyes of lGMR-Gal4; tub-Gal80^{ts} and lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies. Flies were raised at 18 °C and after eclosion shifted to 30 °C for up to 14 days to induce 2n4r tau^{WT} expression in the eye. Outer (R1-R6) photoreceptor rhabdomeres are arranged in an asymmetric trapezoidal pattern. Normal number and arrangement of rhabdomeres in the eyes of lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies. No loss of photoreceptor rhabdomeres is seen in lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies after 14 days at 30 °C. (B) Quantification of the number of photoreceptor rhabdomeres per ommatidium. 2-way ANOVA showed no significant effect of genotype (p=0.1441) and age (p=0.4816) on the number of rhabdomeres per ommatidium, with no significant interaction between factors (p>0.9999). Graphs show individual datapoints with mean ± SEM. n = 6 flies per genotype/age from ~20 ommatidia per fly. ns p>0.05. Data were normally distributed. Asterisks (in black) show comparisons with the control genotype of the same age and number symbols (in red) show comparisons with 1-day old flies of the same genotype by post-hoc Tukey HSD tests.

Taken together, these results show that adult-onset lGMR>2n4r tau^{WT} expression causes progressive neuronal dysfunction, independent of overt neuronal death. Thereby, providing further evidence that tau-evoked functional degeneration is separable from structural degeneration. Additionally, these results suggest that 4r tau-dependent toxicity in adult flies requires a neurodevelopmental component.

2.3.5 Varying the phosphorylation status of tau affects tau-mediated neuronal dysfunction and toxicity

One of the earliest abnormalities detected in tauopathies, such as AD, is an increase in tau phosphorylation. Tau is phosphorylated by several kinases. One of the best studied is the microtubule-affinity regulating kinase (MARK)/PAR1 kinase. The PAR1 kinase has been previously shown to regulate microtubule dynamics via tau phosphorylation (Wang et al., 2007). Tau phosphorylation at the PAR1 kinase sites (Ser262/Ser356) is required for the REP (Nishimura 2004; Chatterjee et al., 2009). Therefore, we sought to investigate whether PAR1 function is also associated with tau-mediated neuronal dysfunction in our model. To this end, we used the UAS-2n4r tau^{S2A} transgene, in which the two PAR1 phosphorylation sites (Ser262/SerS356) have been mutated to phosphoresistant alanine. We tested two well characterised UAS-2n4r tau^{S2A} transgenic lines, which were previously generated by P-element mediated transgenesis (Chatterjee et al., 2009). The depolarization of lGMR>2n4r tau^{S2A}-expressing flies was normal. At both ages analysed, no significant differences in depolarization were seen in lGMR>2n4r tau^{S2A}1 and lGMR>2n4r tau^{S2A}2 flies with respect to age-matched controls. No significant age-related differences in depolarization were seen within each genotype (Figure 2.10A,B). The ON transients were slightly smaller in 1-day old lGMR>2n4r tau^{S2A}1 flies (~2 mV compared to ~3.4 mV in controls), but normal in 30-day old lGMR>2n4r tau^{S2A}1 flies, compared to age-matched controls. Regardless of age, normal ON transients were found in lGMR>2n4r tau^{S2A}2 flies relative to age-matched control flies. In lGMR>2n4r tau^{S2A}1 flies a small, but significant, age-related increase in ON transients was seen. No significant age-related differences in ON transients were found in lGMR>2n4r tau^{S2A} flies. Thus, lGMR>2n4r tau^{S2A} expression failed to produce an age-related decline in ON transients (Figure 2.10A,C). These results show that lGMR>2n4r tau^{S2A} expression is insufficient to cause neuronal dysfunction and suggest tau phosphorylation by the PAR-1 kinase plays an essential role in tau-mediated neuronal dysfunction. An alternative explanation is a conformational change in 2n4r tau^{S2A} as a result of the S262A and S356A mutations, modifies its toxicity independently of PAR1 function. Another possible explanation for the different levels of toxicity mediated by the UAS-2n4r tau^{WT} and UAS-2n4r tau^{S2A} transgenes are position effects altering transgene expression levels.

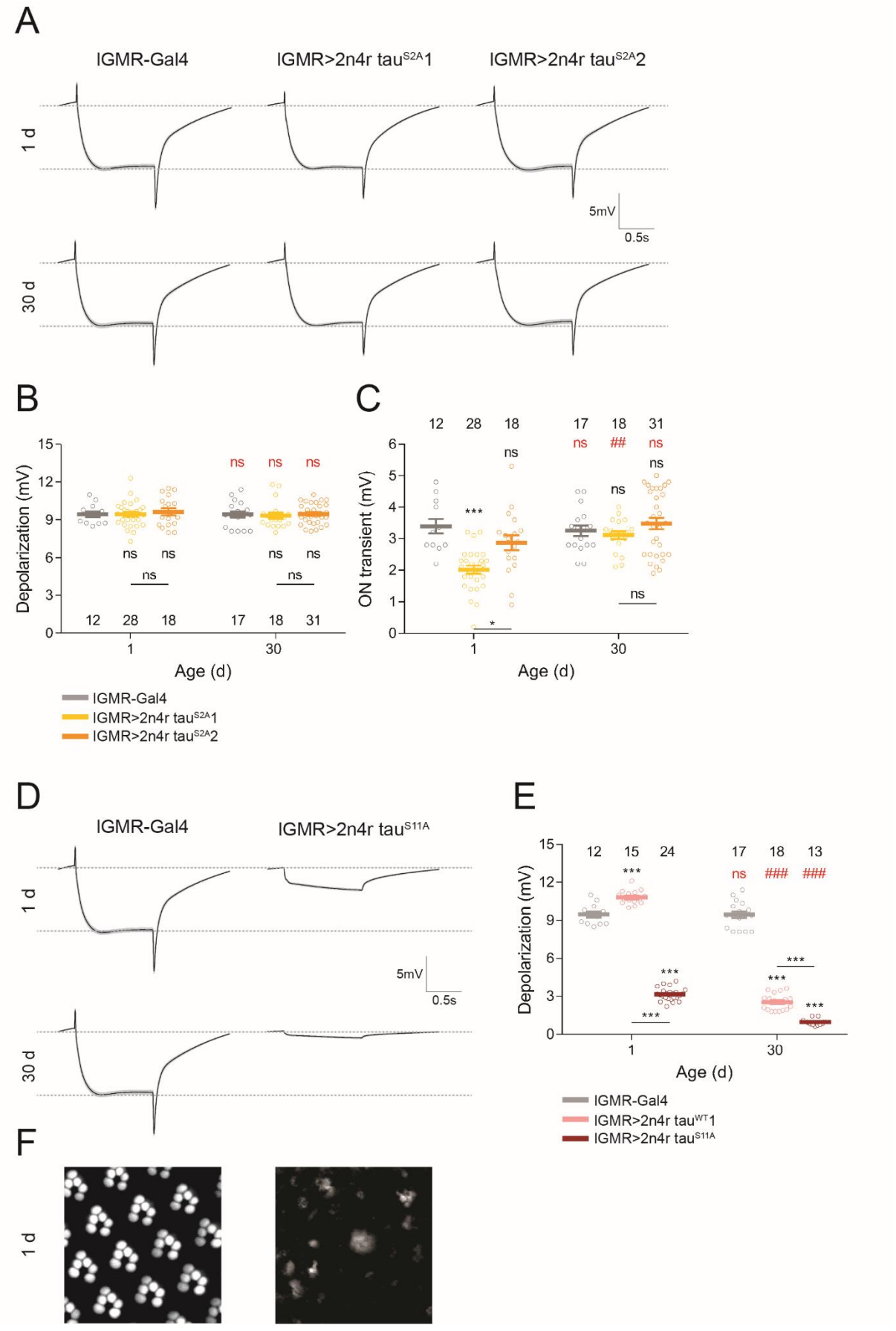

A
IGMR-Gal4
IGMR>2n4r tauS2A1
IGMR>2n4r tauS2A2
1 d
30 d
5mV
0.5s
B
Depolarization (mV)
Age (d)
C
ON transient (mV)
Age (d)
IGMR-Gal4
IGMR>2n4r tauS2A1
IGMR>2n4r tauS2A2
D
IGMR-Gal4
IGMR>2n4r tauS11A
1 d
30 d
5mV
0.5s
E
Depolarization (mV)
Age (d)
IGMR-Gal4
IGMR>2n4r tauWT1
IGMR>2n4r tauS11A
F
1 d

Figure 2.10: Varying tau phosphorylation status affects tau-mediated functional and structural degeneration. (A) ERG recordings from 1- and 30-day old lGMR-Gal4, lGMR>2n4r tau^{S2A}1 and lGMR>2n4r tau^{S2A}2 flies. Normal depolarization in 1- and 30-day old 2n4r tau^{S2A}-expressing flies. No age-related reduction in depolarization in 2n4r tau^{S2A}-expressing flies. Normal ON transients in 2n4r tau^{S2A}-expressing flies, except for a small reduction in 1-day old lGMR>2n4r tau^{S2A}1 flies. (B) Quantification of ERG depolarization. 2-way ANOVA showed no significant effect of genotype ($p=0.7178$) and age ($p=0.6102$) on depolarization, with no significant interaction between factors ($p=0.9478$). (C) Quantification of ERG ON transients. 2-way ANOVA showed a significant effect of genotype ($p=0.0098$) and age ($p=0.0012$) on ON transients, with a significant interaction between factors ($p=0.0098$). n = 12-31 flies per genotype/age. (D) ERG recordings from 1- and 30-day old lGMR-Gal4 and lGMR>2n4r tau^{S11A} flies. Severe reduction in depolarization in lGMR>2n4r tau^{S11A} flies at both ages analysed. Dramatic age-related decline in depolarization in lGMR>2n4r tau^{S11A} flies. Lack of ON transients in lGMR>2n4r tau^{S11A} flies at both ages analysed. (E) Quantification of ERG depolarization. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on depolarization, with a significant interaction between factors ($p<0.0001$). Graphs show individual datapoints with mean ± SEM. n = 12-24 flies per genotype/age. ns $p>0.05$, *$p<0.05$, **$p<0.001$, ***$p<0.0001$. (B,C,E) Data were normally distributed. Asterisks (in black) show comparisons with the control genotype of the same age, asterisks (in black) above horizontal lines show comparisons between experimental genotypes of the same age and number symbols (in red) show comparisons with 1-day old flies of the same genotype by post-hoc Tukey HSD tests. (F) Water immersion microscopy images of Rh1-eGFP florescence in the eyes of 1-day old lGMR-Gal4 and lGMR>2n4r tau^{S11A} flies. Very few discernible photoreceptor rhabdomeres in lGMR>2n4r tau^{S11A} flies.

We next investigated whether the 2n4r tau^{S11A} construct (in which 11 glycogen synthase kinase (GSK)/ shaggy (sgg) (serine/threonine) phosphorylation sites have been mutated to phosphoresistant alanine) with increased microtubule binding affinity precipitates a more severe phenotype than 2n4r tau^{WT} in the fly eye. Previous studies have demonstrated that tau^{S11A} is more toxic than wild-type tau when overexpressed in the developing *Drosophila* visual system (Chatterjee et al., 2009). At both ages analysed, depolarization in lGMR>2n4r tau^{S11A} flies was substantially reduced compared to both age-matched lGMR>2n4r tau^{WT}1 and control flies. Quantification of depolarization revealed a ~70 % reduction in 1-day old lGMR>2n4r tau^{S11A} flies and a ~90 % reduction in 30-day old lGMR>2n4r tau^{S11A} flies, compared with control flies of the same age. Also, quantification of depolarization revealed a ~70 % reduction in 1-day old lGMR>2n4r tau^{S11A} flies and a ~60 % reduction in 30-day old lGMR>2n4r tau^{S11A} flies, compared with age-matched lGMR>2n4r tau^{WT}1 flies. Thus, there was accelerated loss of the ERG response in lGMR>2n4r tau^{S11A} flies compared to

lGMR>2n4r tau^{WT}1 flies (Figure 2.10D,E). We found a lack of ON transients in any lGMR>2n4r tau^{S11A} flies, indicating a total block of synaptic transmission (Figure 2.10D). We observed severe structural defects in 1-day old lGMR>2n4r tau^{S11A} flies as evidenced by a complete loss of photoreceptor rhabdomeres in the vast majority of ommatidia (Figure 2.10F). It is likely the functional and structural defects in 1-day old lGMR>2n4r tau^{S11A} flies are due to developmental toxic mechanisms. These results support the idea that the microtubule binding properties of tau directly affect its toxicity.

2.3.6 Aβ42 exacerbates tau-mediated neuronal dysfunction

Some tauopathies like AD, are characterised by additional filamentous lesions. The pathological hallmarks of AD are the aggregation of hyperphosphorylated tau protein into intraneuronal NFTs, and the aggregation of amyloid beta (Aβ) peptides into extracellular neuritic plagues. We therefore sought to investigate whether Aβ expression enhances tau-evoked neuronal dysfunction. But first we investigated whether Aβ expression alone is sufficient to cause functional degeneration. Many studies have shown Aβ expression in the *Drosophila* brain results in amyloid deposition, premature death, cognitive deficits and age-dependent neurodegeneration, recapitulating several key features of AD (Iijima et al., 2004; Finelli et al., 2004; Crowther et al., 2005; Iijima et al., 2008; Jahn et al., 2011).

We expressed the previously published and well characterised UAS-Aβ42 line (Iijima et al., 2004) using the lGMR-Gal4 driver. ERG recordings from lGMR>Aβ42 animals did not show any evidence of neuronal dysfunction. At both ages analysed, no significant differences in depolarization were seen in lGMR>Aβ42 flies compared with age-matched UAS control flies. Between the 1- and 30-day old age groups, no significant differences in depolarization were found within genotypes (Figure 2.11A,B). The ON transients were a little smaller in 1- and 30-day old lGMR>Aβ42 flies compared with age-matched controls. We found no age-related differences in ON transients in lGMR>Aβ42 flies (Figure 2.11A,C). Thus, in flies up to 30-days of age, lGMR>Aβ42 expression fails to inhibit synaptic transmission. Collectively, these results show that unlike lGMR>4r tau expression, lGMR>Aβ42 expression is insufficient to cause significant neuronal dysfunction in our model.

Finally, we analysed whether coexpression of Aβ42 with 2n4r tau^{WT}1 exacerbates tau-mediated neuronal dysfunction. We coexpressed a single copy of UAS-2n4r tau^{WT} 1 and UAS-Aβ42 using a single copy of the lGMR-Gal4 driver. At both ages analysed, we found a highly significant reduction in depolarization in lGMR>2n4r tau^{WT}1; Aβ42 flies compared with age-matched control flies. Regardless of age, a significant reduction in depolarization was seen in lGMR>2n4r tau^{WT}1; Aβ42 flies with respect to age-matched lGMR>2n4r tau^{WT}1 flies. A more substantial age-related reduction in depolarization was seen in lGMR>2n4r tau^{WT}1; Aβ42 flies compared to lGMR>2n4r tau^{WT}1 flies. Quantification of the depolarization revealed a ~15 % reduction in 1-day old lGMR>2n4r tau^{WT}1; Aβ42 flies and a ~90 % reduction in 30-day old lGMR>2n4r tau^{WT}1; Aβ42 flies, compared with age-matched control flies (Figure 2.11D,E). We found minute ON transients in 1-day old lGMR>2n4r tau^{WT}1; Aβ42 flies (only four out of 15 flies tested had an ON transient. No ON transients were found in 30-day old lGMR>2n4r tau^{WT}1; Aβ42 flies, indicating a total block of synaptic transmission (Figure 2.11F). These results demonstrate that coexpression of Aβ42 with 2n4r tau^{WT}1 aggravates tau-mediated functional degeneration in our model. Different tau expression levels as a result of UAS copy number imbalance may account for the stronger phenotype in lGMR>2n4r tau^{WT}1; Aβ42 flies compared to lGMR>2n4r tau^{WT}1 flies.

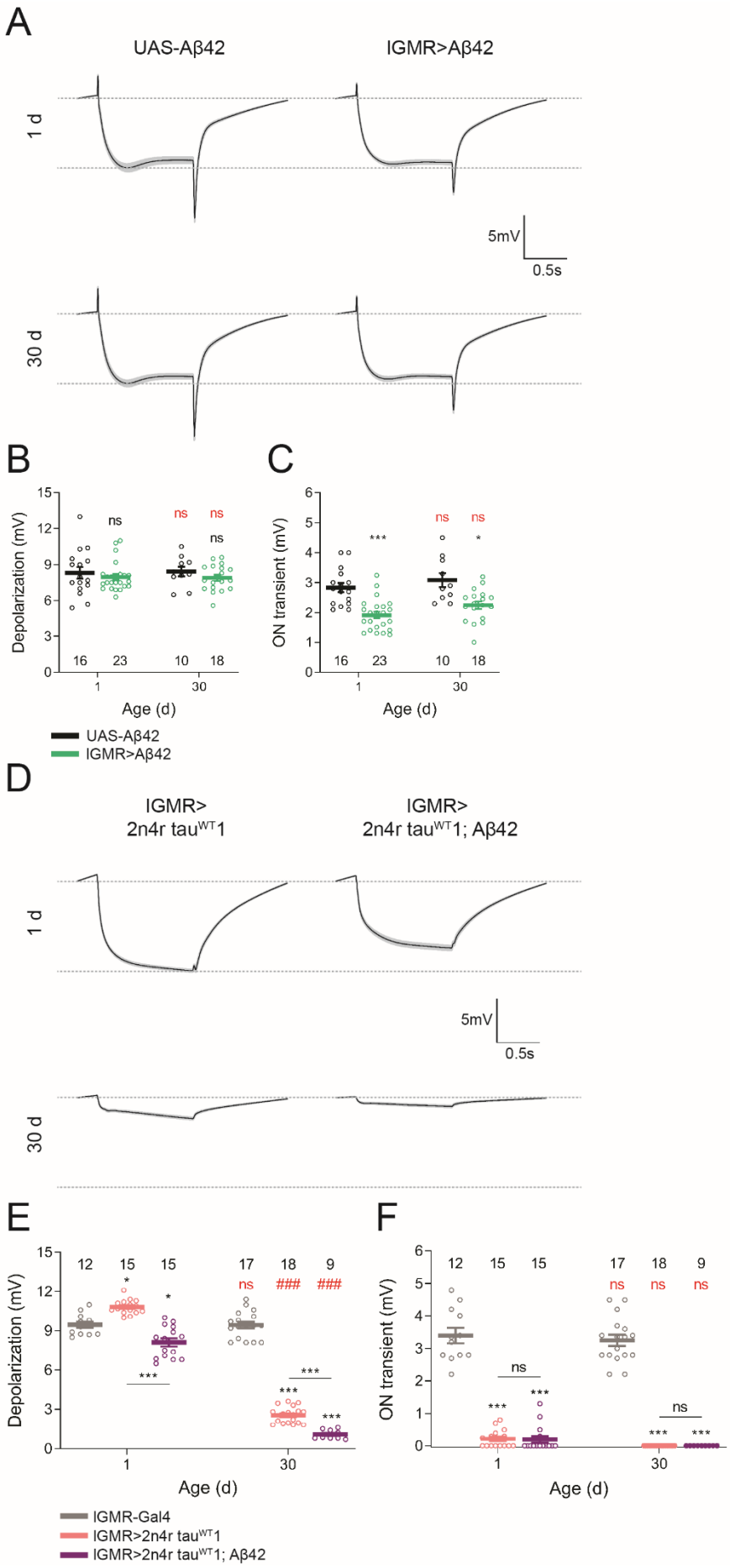

A
UAS-Aβ42
IGMR>Aβ42
1 d
30 d
5mV
0.5s
B
C
Depolarization (mV)
ON transient (mV)
Age (d)
UAS-Aβ42
IGMR>Aβ42
D
IGMR>
2n4r tau^WT1
IGMR>
2n4r tau^WT1; Aβ42
E
F
IGMR-Gal4
IGMR>2n4r tau^WT1
IGMR>2n4r tau^WT1; Aβ42

Figure 2.11: Aβ42 expression potently exacerbates tau-mediated neuronal dysfunction. (A) ERG recordings in response to a one second light pulse of 1- and 30-day old UAS-Aβ42 and lGMR>Aβ42 flies. Normal depolarization in Aβ42-expressing flies. Moderate reduction in ON transients in Aβ-expressing flies. No age-related differences in depolarization and ON transients in lGMR>Aβ42 flies. (B) Quantification of ERG depolarization. 2-way ANOVA showed no significant effect of genotype ($p=0.2228$) and age ($p=0.9609$) on depolarization, with no significant interaction between factors ($p=0.8090$). (C) Quantification of ERG ON transients. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) but not age ($p=0.0596$) on ON transients, with no significant interaction between factors ($p=0.7944$). n = 10-23 flies per genotype/age. (D) ERG recordings in response to a one second light pulse of 1- and 30-day old lGMR>2n4r $tau^{WT}1$ and lGMR>2n4r $tau^{WT}1$; Aβ42 flies. Lack of ON transients in lGMR>2n4r $tau^{WT}1$; Aβ42 flies. At both ages analysed, reduction in depolarization in lGMR>2n4r $tau^{WT}1$; Aβ42 flies compared with age-matched control and lGMR>2n4r $tau^{WT}1$ flies. Substantial age-related reduction in depolarization in both lGMR>2n4r $tau^{WT}1$ and lGMR>2n4r $tau^{WT}1$; Aβ42 flies. (E) Quantification of ERG depolarization. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) and age ($p<0.0001$) on depolarization, with a significant interaction between factors ($p<0.0001$). (F) Quantification of ERG ON transients. 2-way ANOVA showed a significant effect of genotype ($p<0.0001$) but not age ($p=0.0826$) on ON transients, with no significant interaction between factors ($p=0.9535$). n = 9-18 flies per genotype/age. Graphs show individual datapoints with mean ± SEM. ns $p>0.05$, *$p<0.05$, **$p<0.001$, ***$p<0.0001$. Data were normally distributed. Asterisks (in black) show comparisons with the control genotype of the same age, asterisks (in black) above horizontal lines show comparisons between experimental genotypes of the same age and number symbols (in red) show comparisons with 1-day old flies of the same genotype by post-hoc Tukey HSD tests.

Genotype	Age	n	ERG ON transient (mV ± SEM)	ERG depolarization (mV ± SEM)	n	Number of photoreceptor rhabdomeres per ommatidium	Percentage of ommatidia with a complete set of photoreceptor rhabdomeres	Colour
lGMR-Gal4	1	12	3.4 ± 0.25	9.5 ± 0.23	6	6.0 ± 0.01	97	
lGMR-Gal4	5	10	3.4 ± 0.25	9.4 ± 0.20				
lGMR-Gal4	10	13	3.4 ± 0.21	9.2 ± 0.24	6	6.0 ± 0.01	96	
lGMR-Gal4	30	17	3.3 ± 0.17	9.4 ± 0.25	6	6.0 ± 0.01	95	
lGMR>0n4r tau^{WT}	1	10	3.4 ± 0.23	9.2 ± 0.26	6	6.0 ± 0.01	95	
lGMR>0n4r tau^{WT}	5	8	3.8 ± 0.34	9.0 ± 0.41				
lGMR>0n4r tau^{WT}	10	32	1.1 ± 0.25	7.1 ± 0.23	6	6.0 ± 0.01	95	
lGMR>0n4r tau^{WT}	30	19	0 ± 0	4.1 ± 0.17	6	5.9 ± 0.01	92	
lGMR>0n4r tau^{R406W}	1	21	3.3 ± 0.15	10.1 ± 0.22	6	5.9 ± 0.02	94	
lGMR>0n4r tau^{R406W}	5	17	2.3 ± 0.26	9.6 ± 0.24				
lGMR>0n4r tau^{R406W}	10	20	0.1 ± 0.07	5.6 ± 0.21	6	5.9 ± 0.04	93	
lGMR>0n4r tau^{R406W}	30	14	0 ± 0	2.2 ± 0.10	6	5.9 ± 0.03	92	
lGMR>2n4r tau^{WT}1	1	15	0.2 ± 0.07	10.8 ± 0.14	8	5.9 ± 0.01	84	
lGMR>2n4r tau^{WT}1	5	17	0 ± 0	9.4 ± 0.19				
lGMR>2n4r tau^{WT}1	10	14	0 ± 0	5.7 ± 0.26	6	4.7 ± 0.09	25	
lGMR>2n4r tau^{WT}1	30	18	0 ± 0	2.5 ± 0.15	11	2.9 ± 0.18	<1	
lGMR>2n4r tau^{WT}2	1	18	1.3 ± 0.19	10.4 ± 0.20	13	6.0 ± 0.04	93	
lGMR>2n4r tau^{WT}2	5	22	0 ± 0	10.9 ± 0.22				
lGMR>2n4r tau^{WT}2	10	16	0 ± 0	8.1 ± 0.18	6	5.8 ± 0.06	81	
lGMR>2n4r tau^{WT}2	30	12	0 ± 0	3.8 ± 0.19	6	2.9 ± 0.11	<1	
Elav-Gal4	30	12	3.4 ± 0.19	8.4 ± 0.36				
Elav>2n4r tau^{WT}1	30	10	3.3 ± 0.13	8.8 ± 0.41				
lGMR-Gal4; tub-Gal80^{ts} (18 °C)	1	8	3.4 ± 0.14	8.4 ± 0.20	6	6.0 ± 0.01	99	
lGMR-Gal4; tub-Gal80^{ts} (30 °C)	7	8	3.5 ± 0.26	8.3 ± 0.10	6	6.0 ± 0.01	98	

lGMR-Gal4; tub-Gal80^{ts} (30 °C)	14	8	3.4 ± 0.27	8.5 ± 0.09	6	6.0 ± 0.02	97	
lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 (18 °C)	1	8	3.1 ± 0.22	8.3 ± 0.41	6	6.0 ± 0.02	97	
lGMR; tub-Gal80ts>2n4r tau^{WT}1 (30 °C)	7	27	0.7 ± 0.20	6.5 ± 0.23	6	6.0 ± 0.02	96	
lGMR; tub-Gal80ts>2n4r tau^{WT}1 (30 °C)	14	11	0 ± 0	1.6 ± 0.16	6	5.9 ± 0.02	94	
Rh1-Gal4	30	12	4.2 ± 0.13	9.3 ± 0.23				
Rh1>2n4r tau^{WT}1	30	12	4.1 ± 0.17	9.2 ± 0.35				
lGMR>2n4r tau^{S2A}1	1	28	2 ± 0.13	9.4 ± 0.19				
lGMR>2n4r tau^{S2A}1	30	18	3.1 ± 0.13	9.3 ± 0.26				
lGMR>2n4r tau^{S2A}2	1	18	2.9 ± 0.24	9.7 ± 0.28				
lGMR>2n4r tau^{S2A}2	30	31	3.5 ± 0.18	9.5 ± 0.15				
lGMR>2n4r tau^{S11A}	1	24	0 ± 0	3.1 ± 0.10				
lGMR>2n4r tau^{S11A}	30	13	0 ± 0	1.0 ± 0.07				
UAS-Aβ42	1	16	2.8 ± 0.15	8.3 ± 0.49				
UAS-Aβ42	30	10	3.1 ± 0.24	8.4 ± 0.40				
lGMR>Aβ42	1	23	2.0 ± 0.11	8.0 ± 0.26				
lGMR>Aβ42	30	18	2.2 ± 0.13	7.9 ± 0.26				
lGMR>2n4r tau^{WT}1; Aβ42	1	15	0.2 ± 0.10	8.1 ± 0.31				
lGMR>2n4r tau^{WT}1; Aβ42	30	9	0 ± 0	1.1 ± 0.12				

Table 2.2: Quantification of ERG ON transients and ERG depolarization by genotype and age. Quantification of the number of photoreceptor rhabdomeres per ommatidium and the percentage of ommatidia with a full complement of photoreceptor rhabdomeres by genotype and age. n = number of flies tested per genotype/age. Colour corresponds to the colour used in the graphs.

2.4 Discussion

In this study we expressed different isoforms of human tau in the *Drosophila* retina and performed a systematic assessment of the resulting functional and structural changes. First, we show that 0n4r tau expression causes adult-onset progressive functional degeneration without structural degeneration. Whereas, 2n4r tau expression causes progressive functional degeneration, which precedes progressive structural degeneration. Thus, we show 4r tau isoform-specific differences. Next, we show adult-onset 2n4r tau expression causes functional degeneration in the absence of structural degeneration. Our results show that one of the earliest manifestations of human tau expression in the *Drosophila* eye is neuronal dysfunction without neuronal death. Thus, tau-mediated neuronal dysfunction is separate from neuronal death. These results suggest that tau-mediated neuronal dysfunction may underlie early symptoms in tauopathies, such as AD. Further understanding of the contribution of tau-evoked neuronal dysfunction, in the early stages of the disease process, may provide novel therapeutic options to slow down or even halt disease progression. Next, we show that tau phosphorylation via the PAR1 kinase plays a role in tau-mediated neuronal dysfunction in our model. Finally, we show that coexpression of Aβ42 with 2n4r $tau^{WT}1$ exacerbates tau-evoked neuronal dysfunction.

2.4.1 Neuronal dysfunction in tau-expressing eyes

We expressed several different isoforms of human tau in the *Drosophila* eye and monitored age-related changes in function using retinal ERGs. We selected a Gal4 driver that expressed tau at low enough levels so as not to produce a rough eye, which allowed us to study age-dependent changes. This is the first study to compare the ability of different 4r tau isoforms to produce neuronal dysfunction. We demonstrate that the overaccumulation of either 0n4r or 2n4r tau in the fly eye causes neuronal and synaptic dysfunction. However, they produce distinct pathological phenotypes. lGMR>0n4r tau expression causes adult-onset progressive neuronal dysfunction. We found intact retinal function in young flies (<5-day old) as indicated by the normal ERG responses (Figure 2.1) and visual behaviour in the flight simulator system (Figure 2.5). Whereas, retinal function was compromised in aged flies (>5-

day old) as indicated by the progressive reduction in ERG depolarization, which coincides with the rapid and progressive loss of ERG ON transients (Figure 2.1). These results clearly show that human tau is capable of causing adult-onset neuronal dysfunction. lGMR>2n4r tau expression also causes progressive neuronal dysfunction, however, it is not adult-onset. A clear phenotype was observed in the eyes of young (<5-day old) lGMR>2n4r tau^{WT} flies. We found severe defects in synaptic transmission in young flies (<5-day old) as shown both electrophysiologically (lack of ERG ON transients) (Figure 2.3) and behaviourally (abnormal optomotor responses in the flight simulator system) (Figure 2.5). We found that in lGMR>2n4r tau^{WT} flies a progressive reduction in ERG depolarization occurs as a function of age (Figure 2.3). The synaptic transmission defects in newly eclosed adult flies are likely related to tau-dependent toxicity during development. These results show that 2n4r tau is more toxic during development than 0n4r tau in the fly eye. And, synaptic transmission is more sensitive to lGMR>2n4r tau expression than lGMR>0n4r tau expression in newborn adult flies. lGMR>2n4r tau^{WT} expression has a more marked effect on synaptic transmission than the phototransduction cascade in young flies. Zhou et al. (2017) showed in *Drosophila* larval NMJs, mutant 0n4r tau binds to synaptic vesicles via its N-terminal domain thereby crosslinking them and restricting their mobilisation via presynaptic actin polymerization, which reduces their release rate and interferes with synaptic transmission. A possible explanation for the more dramatic effect of 2n4r tau on synaptic transmission compared to 0n4r tau, is 2n4r tau binds synaptic vesicles more strongly due to the presence of the two N-terminal inserts and therefore has a more of an effect on presynaptic functions. The authors did not compare the abilities of different 4r tau proteins to bind synaptic vesicles and interfere with synaptic transmission. Wild-type and R406W tau are associated with different tauopathies, which have distinct pathology, symptomology and molecular aetiology (Williams, 2006; Kovacs, 2015), however, they both cause a similar pathological phenotype in our model. We found a stronger phenotype in lGMR>0n4r tau^{R406W} flies compared to lGMR>0n4r tau^{WT} flies, as indicated by the accelerated and more dramatic reduction in ERG depolarization and the accelerated loss of ERG ON transients (Figure 2.1).

The ability of tau to cause neuronal dysfunction has been investigated in the *Drosophila* NMJ. Expression of human tau in larval motor neurons causes neuronal dysfunction,

characterised by axonal transport and locomotor function defects (Mudher et al., 2004; Chee et al., 2005; Ubhi et al., 2007; Talmat-Amar et al., 2011). There is further evidence from other experimental animals that tau is capable of causing neuronal dysfunction. Firstly, injection of human tau blocks synaptic transmission in squid axons (Moreno et al., 2011). Secondly, tau expression in *Aplysia* motor neurons disrupts axonal transport and synaptic transmission (Erez et al., 2014). Finally, many murine studies have indicated expression of human wild-type or mutant tau leads to synaptic and neuronal dysfunction (Polydoro et al., 2009; Hoover et al., 2010; Crimins et al., 2012). However, this is the first study to show 4r tau isoform-specific differences in mediating neuronal dysfunction. A major goal is to delineate the mechanisms by which tau causes neuronal dysfunction in tauopathies. Our model provides such an opportunity because it allows the combination of sophisticated genetic manipulation with rapid and reproducible measures of age-dependent changes in function and structure; something, which is not possible in other animal models of tauopathy. Additionally, our model is suitable for medium to high-throughput screening of potential therapeutic agents.

All the UAS-tau transgenic lines used in this study were created by P-element mediated transgenesis, in which cDNA inserts at random into the Drosophila genome. We attempted to standardise expression by using the same driver line and crossing all transgenic lines into a common background. Despite this, it is still possible that positional effects on levels of transgene activity and expression contribute to the different pathological phenotypes, as tau-induced neurotoxicity has been shown to be highly dose-dependent. For example, the tau H1 haplotype which drives slightly higher expression of tau is associated with increased risk for AD, PSD and PD (Houlden et al., 2001; Kwok et al., 2004; Refenes et al., 2009; Allen et al. 2014). The use of site specific integration of tau transgenes (using the c31 system) would eliminate position effects, and therefore allow for better control of the level of transgene expression and permit direct comparisons of the neurotoxicity mediated by different tau proteins (Groth et al., 2004). However, because we obtained similar results from two independently derived UAS-0n4r tau and UAS-2n4r tau transgenic lines, it makes it more likely the distinct pathological phenotypes produced by different 4r tau isoforms are not a consequence of differences in transgene expression levels due to position effects.

However, the milder phenotype in lGMR>2n4r tau^{WT}2 flies compared to lGMR>2n4r tau^{WT}1 flies is most likely related to differences in transgene expression levels (Figure 2.3 and Figure 2.5). We are unable to determine whether the stronger phenotype in lGMR>0n4r tau^{R406W} flies compared to lGMR>0n4r tau^{WT} flies, is due to R406W tau being more toxic than wild-type tau or differences in transgene expression levels (Figure 2.1). Previous studies have reported 0n4r tau^{R406W} is more than toxic than 0n4r tau^{WT} when ectopically expressed in the developing *Drosophila* brain or visual system (Wittmann et al., 2001; Jackson et al., 2002; Khurana et al., 2006). However, all of these studies used the same UAS-0n4r^{WT} and UAS-0n4r tau^{R406W} lines used in this study and as such did not control for position effects. It has been a long held belief that the aggregates exert toxicity leading to neurodegeneration. However, it is becoming clear that the aggregates are in fact relatively inert and instead soluble tau monomers and oligomers are the toxic species. Therefore, higher tau expression levels might not necessarily coincide with increased neurotoxicity, as they might promote aggregate formation and thereby reduce the amount of toxic soluble tau species. However, tau aggregates have not been observed in *Drosophila* models of tauopathy (reviewed in Cowan et al., 2011 and Gistelinck et al., 2012).

2.4.2 Neuronal death in tau-expressing eyes

Tau expression throughout development (using either sGMR-gal4 or Elav-Gal4) produces a REP (Wittmann et al., 2001; Jackson et al., 2002; Grammenoudi et al., 2008). The *Drosophila* eye emerges during late larval and pupal stages from a monolayer epithelium, called the eye-antennal imaginal disc (Moses, 1991; Ellis et al., 1993). Therefore, a rough eye in newborn adult flies is probably as a result of tau-dependent toxicity *per se* rather than degeneration. Bona fide degeneration would be when normal ommatidia in newborn adult flies subsequently die. We next investigated whether we would find bona fide degeneration in our model. To this end, we quantified the number of photoreceptor rhabdomeres at different time points *in vivo*, using the corneal neutralisation technique (Franceschini and Kirschfeld, 1971; Mollereau et al., 2000; Pichard and Desplan, 2001; Gambis et al., 2011). We found no structural degeneration in the eyes of 0n4r tau-expressing flies up to 30-days of age. Ommatidia in the eyes of 1-day old 0n4r tau-expressing flies had a normal number

and arrangement of rhabdomeres. With aging, there was no loss of photoreceptor rhabdomeres in the eyes of 0n4r tau-expressing flies (Figure 2.2). It is possible ultrastructural defects are present in the photoreceptors of 1-day old 0n4r tau-expressing flies, however this was beyond the scope of this investigation. In contrast, we found age-related progressive structural degeneration in 2n4r tau-expressing flies. Ommatidia in the eyes of newborn 2n4r-tau expressing flies contained a full complement of rhabdomeres, but there were minor abnormalities in the spatial organisation of rhabdomeres and rhabdomere roundness. A progressive loss of photoreceptor rhabdomeres occurs in the eyes of 2n4r tau-expressing flies as a consequence of aging. In 30-day old 2n4r-tau expressing flies, ommatidia contained on average half their normal number of rhabdomeres. Furthermore, less than 1 % of ommatidia had a complete set of rhabdomeres in 30-day old 2n4r-tau expressing flies. Whereas, 95 % of ommatidia contained a complete set of rhabdomeres in 30-day old control flies (Figure 2.4 and Table 2.2). These results suggest defects in photoreceptor development due to 2n4r tau-dependent toxicity. Therefore, it is not possible to determine whether the subsequent age-related structural degeneration is due to the delayed contribution of developmental toxic mechanisms.

Our results show isoform-specific effects in tau-mediated neuronal dysfunction and neuronal death. Many *in vitro* studies have shown that different 4r tau isoforms display different phosphorylation susceptibilities (Goedert and Jakes, 1990; Goedert et al., 1992; Buee et al., 2000), microtubule binding abilities (Kanaan et al., 2011; Matsumoto et al., 2015), subcellular distribution (Liu and Gotz, 2013; Paholikova et al., 2015) and interaction partners (Liu et al., 2016). These differences most likely contribute to our observed isoform-specific effects. Our results suggest that different 4r tau isoforms should not be used interchangeably when modelling tauopathies in animal models. These data show that human 4r tau overaccumulation in the *Drosophila* eye fails to cause bona fide structural degeneration, but not functional degeneration. Thus, our results strongly suggest that tau-mediated neuronal dysfunction is independent from neuronal death. This opens up the possibility that, at least partially, non-overlapping mechanisms mediate tau-evoked functional and structural degeneration. Tau-induced neuronal dysfunction before irreversible neuronal death offers a promising avenue for therapeutic intervention. Several

previous studies in *Drosophila* have also reported tau-mediated neuronal dysfunction in the absence of neuronal death. For example, tau expression in adult MB neurons impairs associative learning without overt neuronal loss (Mershin et al., 2004; Kosmidis et al., 2010; Papanikolopoulou et al., 2010). Similarly, tau expression in larval motor neurons disrupts NMJ morphology, axonal transport and synaptic transmission without apparent cell death (Mudher et al., 2004; Chee et al., 2005; Ubhi et al., 2007; Talmat-Amar et al., 2011). Collectively, these results show in *Drosophila*, irrespective of neuronal type or life stage, human tau is able to cause functional degeneration separable from structural degeneration.

Elav driven tau expression caused a rough eye phenotype (Figure 2.6A). The REP correlates well with loss of retinal cells (Feany et al., 1996; Wittmann et al., 2001; Jackson et al., 2002; Chatterjee et al., 2009). Elav>2nr $tau^{WT}1$ photoreceptors retained functionality, as measured using retinal ERGs, despite the REP (Figure 2.6). Thus, despite tau-dependent toxicity during development, function is preserved in adult photoreceptors. It is unclear why Elav>2n4r tau^{WT} expression, unlike lGMR>2n4r tau^{WT} expression, fails to cause neuronal dysfunction; but this could, for example, be due to different expression levels at specific stages of development. These results provide further evidence that tau-induced neuronal dysfunction is separate from neuronal death.

2.4.3 Adult-onset tau expression causes functional degeneration without structural degeneration

Most *Drosophila* models of tauopathy have used Gal4 drivers that express during development (for example, Elav and sGMR). Therefore, we were interested to know whether human 2n4r tau^{WT} tau expression throughout the adult retina is sufficient to cause neuronal dysfunction and death. We found dramatic effects on retinal function that were specifically attributable to adult-onset 2n4r tau^{WT} expression, as shown using retinal ERGs (Figure 2.7). However, adult-onset 2n4r tau^{WT} expression failed to recapitulate the loss of ERG ON transients and increased ERG depolarization found in 1-day old lGMR>2n4r tau^{WT} flies (Figure 2.3). This suggests that tau-dependent toxicity during development contributes to the synaptic transmission defects seen in newborn lGMR>2n4r tau^{WT} flies.

During the course of this work, a study was published that expressed human 0n4r tau^{WT} exclusively in adult outer R1-R6 photoreceptors (using Rh1-Gal4) and monitored age-dependent changes in function using retinal ERGs. The authors showed that Rh1>0n4r tau^{WT} expression causes age-related progressive neuronal dysfunction (Chouhan et al., 2016). However, we were unable to replicate these results. We found normal ERG responses in Rh1>2n4r tau^{WT} flies up to 30-days of age (Figure 2.8). Our experimental conditions were the same as Chouhan et al. (2016): we both recorded ERGs from flies with a single copy of the Gal4 driver and UAS transgene which were raised at 25 °C. Both studies used different tau isoforms; we used 2n4r, whereas Chouhan et al (2016) used 0n4r. However, it is unlikely the different results can be attributed to this, as we have shown that both lGMR>0n4r tau and lGMR>2n4r tau expression produce similar pathological phenotypes. Because Rh1-Gal4 is a very weak driver (compared to lGMR-Gal4) and the effects of tau are highly dose dependent, it is possible that tau expression levels were insufficient to cause neuronal dysfunction. However, this does not explain Chouhan et al. (2016) findings. The other major difference between Rh1 and lGMR is that whereas Rh1 is expressed only in the outer R1-R6 photoreceptors, lGMR is expressed in all retinal cell types (including glia). The overaccumulation of human tau in glial cells causes apoptotic cell death in both glia and neurons and premature death (Colodner and Feany, 2010). However, whether glial expression of tau causes neuronal dysfunction is not known. Although, it is possible that tau expression in glia contributes to tau-mediated neuronal dysfunction in lGMR>4r tau flies.

We found 2n4r tau^{WT} expression throughout the adult retina does not cause neuronal death. We found lGMR; tub-Gal80^{ts}>2n4r tau^{WT}1 flies retained a normal number of rhabdomeres per ommatidia after up to 14-days at 30 °C (Figure 2.9). Similarily, Malmanche et al. (2017) showed no loss of photoreceptor rhabdomeres upon eye-specific late pupal- or adult-onset induction of 4r tau expression, using sGMR-Gal4; tub-Gal80^{ts} or Rh1-Gal4, respectively. Likewise, Chouhan et al. (2016) reported no loss of photoreceptor rhabdomeres in Rh1>0n4r tau^{WT} flies up to 30-days old. However, conflicting results were reported by Gorsky et al. (2016). The authors showed pan-neuronal adult-onset 2n4r tau^{WT} expression, using the elav GeneSwitch driver, causes mild but progressive photoreceptor rhabdomere loss. These discrepancies could be due to differences in transgene expression

levels. These results suggest a note of caution when interpreting results from models, which use different expression systems. Our results show 4r tau expression during development is required for adult-onset progressive retinal structural degeneration.

A few studies have shown that human tau expression in adult MB neurons precipitates associative learning and memory deficits in absence of neurodegeneration (Mershin et al., 2004; Papanikolopoulou et al., 2010). However, MB neuron confined neurodegeneration detected in a few animals over 45-days old (Mershin et al., 2004). Therefore, it is possible that in our model, if we age our flies for longer, photoreceptor rhabdomere loss might become detectable. These studies in combination with ours demonstrate that adult-onset tau expression, irrespective of the neuronal population, is capable of causing neuronal dysfunction in the absence of neuronal death. Similarly, Kimura et al. (2007) showed mice overexpressing human tau exhibit age-related place learning and memory impairments without neuronal loss.

2.4.4 Varying the phosphorylation status of tau influences tau-mediated neuronal dysfunction and toxicity

The next question is to understand what regulates tau-mediated neuronal dysfunction. We show a possible role for the PAR1 kinase in tau-induced neuronal dysfunction. Expression of a 2n4r tau^{S2A} construct, which cannot be phosphorylated at the two PAR1 phosphorylation sites (S262 and S356), in the *Drosophila* eye fails to cause neuronal dysfunction, as shown using retinal ERGs (Figure 2.10). Similar findings reported by Papanikolopoulou et al. (2010), who showed expression of 2n4r tau^{S2A} in adult MB neurons does not impair associative learning and memory. Results from mice models of tauopathy also suggest that tau hyperphosphorylation is required for tau-mediated neuronal dysfunction (Kimura et al., 2007). Another possible explanation for the lack of phenotype in lGMR>2n4r tau^{S2A} flies compared to lGMR>2n4r tau^{WT} flies are differences in transgene expression levels. However, because we obtained consistent results from two independently derived UAS-2n4r tau^{S2A} and UAS-2n4r tau^{WT} transgenic lines this is less likely.

Mechanistically how hyperphosphorylated tau disrupts neuronal function is not well understood. It has been proposed that hyperphosphorylated tau directly or indirectly disrupts axonal transport and synaptic transmission. For example, overexpressing tau in *Drosophila* larval motor neurons disrupts axonal transport, leading to vesicle aggregation in the axon and a reduction in mitochondria in the presynaptic terminal (Mudher et al., 2004; Chee et al., 2005). Similarly, expression of human tau in *Aplysia* glutamatergic sensory-motor neuron synapses leads to reduced releasable presynaptic vesicles pools, as a result of impaired axoplasmic transport (Erez et al., 2014). Additionally, human tau injected directly into the presynaptic axon terminal of the squid giant synapse blocks synaptic transmission and causes synaptic vesicles to aggregate in the active zone (Moreno et al., 2011). The next step is to study the ultrastructure of the photoreceptor terminals to see if any of these pathological features exist in our model.

Other studies have shown PAR1 phosphorylation is required for tau-dependent toxicity in the developing eye (Nishimura et al., 2004; Chatterjee et al., 2009) and MB neuroblasts (Kosmidis et al., 2010). Taken together, these studies suggest PAR1 phosphorylation is a common mechanism underlying tau-mediated neuronal dysfunction and toxicity. Our model provides the opportunity to examine the role of other tau kinases; for example, GSK3β, cdk5 and PKA, which have been previously implicated in tauopathies (Avila, 2008; Guo et al., 2017), in tau-mediated functional and structural degeneration. We found that lGMR>2n4r tau^{S11A} expression (with a higher microtubule binding affinity than 2n4r tau^{WT}) precipitated a stronger phenotype than lGMR>2n4r tau^{WT} expression. The degenerative phenotype in lGMR>2n4r tau^{S11A} flies is likely related to tau-dependent toxicity during development, as it is already established upon adult emergence (Figure 2.10D,E,F). Similarily, 0n4r tau^{AP} (with a higher microtubule-binding affinity than 0n4r tau^{WT}) has a more dramatic effect on axonal transport in the *Drosophila* larval NMJ than 0n4r tau^{WT} (Talmat-Amar et al., 2011). These results show the microtubule binding properties of tau directly influence tau-mediated neurotoxicity.

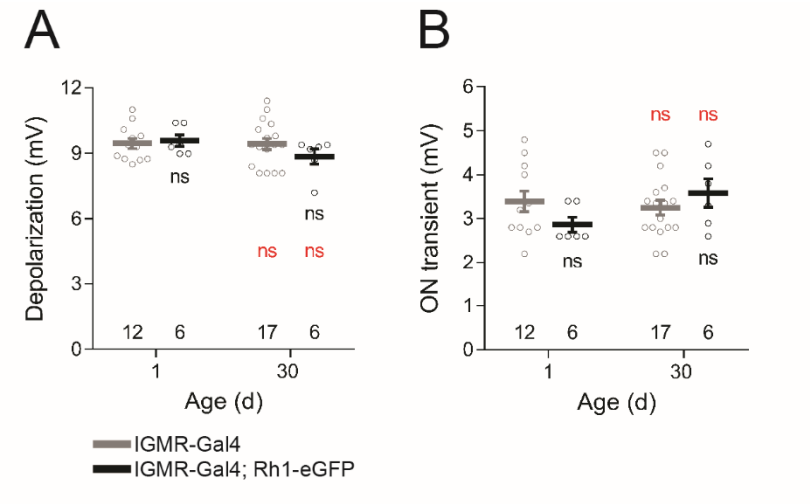
2.4.5 Aβ42 enhances tau-mediated neuronal dysfunction

Next, we investigated whether human Aβ expression produces neuronal dysfunction under similar experimental conditions. We found lGMR>Aβ42 expression had little effect on the ERG response (Figure 2.11A,B,C). Similar findings reported by Chouhan et al. (2016), who showed that Rh1>Aβ42 expression also has little effect on the ERG response. However, this is in contrast to previous studies, which have shown Aβ expression induces significant neuronal dysfunction. For example, Zhao et al. (2010) showed Aβ42 expression in the *Drosophila* giant fibre system causes age-related synaptic transmission defects and depletion of mitochondria from the presynaptic terminal (Huang et al., 2013). In addition, patch clamp analysis revealed a depression of *Drosophila* cholinergic synapses upon Aβ42 expression (Fang et al., 2012). It is unknown why Aβ42 expression in the *Drosophila* eye fails to recapitulate the neuronal dysfunction observed in the *Drosophila* giant fibre system and cholinergic synapses upon Aβ42 expression. However, these results suggest that specific neuronal populations are differentially susceptible to Aβ42-evoked neuronal dysfunction. It is possible that increasing the level of Aβ expression, by introducing additional copies of the Gal4 driver or UAS transgene, or aging the flies for longer might produce a phenotype.

We found that coexpression of Aβ42 with human 2n4r tau^{WT} potently exacerbates tau-mediated neuronal dysfunction in the *Drosophila* eye (Figure 2.11D,E,F). Expression levels of tau are likely to differ in lGMR>2n4r $tau^{WT}1$ and lGMR>2n4r $tau^{WT}1$; Aβ42 flies due to UAS-copy number imbalance. These differences in the level of UAS-2n4r tau^{WT} expression may contribute to the different phenotype strengths in lGMR>2n4r $tau^{WT}1$ and lGMR>2n4r $tau^{WT}1$; Aβ42 flies. These findings suggest that tau is an important downstream mediator of Aβ-mediated neuronal dysfunction. In support of this, Folwell et al. (2010) showed coexpression of human tau^{WT} and Aβ42 enhances tau-mediated neuronal dysfunction in *Drosophila* larval motor neurons. Similar findings have been reported in AD mice models. For example, reduction of endogenous tau in an AD mice model mitigates both Aβ-dependent excitotoxicity and cognitive deficits (Roberson et al., 2011). Thus, in both mouse and *Drosophila* models, tau abnormalities are required for Aβ-mediated neuronal

dysfunction. The next step is to investigate the mechanisms by which human Aβ and tau interact to cause neuronal dysfunction.

Much of the research in tauopathies, such as AD, has focused on aggregate formation and neurodegeneration. However, recent evidence has shown clinical symptoms appear in the absence of aggregate formation and prior to neuronal death (reviewed in Guo et al., 2017), indicating a period of cellular dysfunction may precede cell death in the early stages of tauopathies. There are a range of tauopathies with different clinical profiles, which are characterised by abnormalities in specific tau proteins, suggesting potential-isoform specific differences. Currently, little is known about the ability of different tau proteins to precipitate neuronal dysfunction in the same neuronal population. We show in a *Drosophila* eye model, 0n4r tau causes age-related progressive neuronal dysfunction (Figure 2.1 and Figure 2.5) in the absence of neuronal death (Figure 2.2). Whereas 2n4r tau causes progressive neuronal dysfunction (Figure 2.3 and Figure 2.5), which precedes neuronal death (Figure 2.4). Thus, our *Drosophila* model recapitulates the tau-mediated functional degeneration without structural degeneration found in the early stages of human tauopathies. We further show 2n4r tau expression in the adult *Drosophila* retina is capable of causing neuronal dysfunction (Figure 2.7), but not cell death (Figure 2.9). Thus, adult tau-induced structural degeneration, but not functional degeneration, requires a neurodevelopmental component. Collectively, these results demonstrate tau-mediated neuronal dysfunction is uncoupled from neuronal death. We also suggest tau phosphorylation by the PAR1 kinase is involved in tau-mediated neuronal dysfunction, because mutant tau that cannot be phosphorylated at the PAR1 phosphorylation sites fails to cause neuronal dysfunction in our model (Figure 2.10). Finally, we demonstrate that Aβ42 expression alone is insufficient to cause dysfunction, but coexpression of Aβ42 with 2n4r tau^{WT} potently exacerbates tau-mediated neuronal dysfunction (Figure 2.11). These results suggest tau is required by Aβ42 to mediate its pathogenic effects.



Supplementary Figure 2.1: Presence of Rh1-eGFP does not interfere with retinal function. (A) No differences in ERG depolarization are found in lGMR-Gal4; Rh1-eGFP flies relative to lGMR-Gal4 flies. (B) No differences in ERG ON transients are found in lGMR-Gal4; Rh1-eGFP flies relative to lGMR-Gal4 flies. Graphs show individual datapoints with mean ± SEM. n =6-17 flies per genotype/age. ns p>0.05; two-tailed Student's t-test. Asterisks (in black) show comparisons between different genotypes of the same age and number symbols (in red) show comparisons between different ages of the same genotype.

Chapter 3: Characterisation of sleep-wake and circadian rhythm disturbances in *Drosophila* models of tauopathy

3.1 Introduction

Alzheimer's disease (AD) is the most common form of dementia among the elderly. It is estimated to affect 46 million people worldwide (see the UsAgainstAlzheimer's website) and increases in prevalence with age. Therefore, because of the aging human population it is becoming more common. By 2050, it is expected to affect over 130 million people worldwide. Currently, there is no effective treatment to prevent, cure or inhibit AD (Coughlin and Irwin, 2017). AD is histologically characterised by the dual pathologies of β-amyloidosis, the formation of extracellular amyloid plagues due to the accumulation of Aβ peptides, and tauopathy, the formation of intraneuronal neurofibrillary tangles (NFTs) due to the accumulation of hyperphosphorylated tau protein (Braak et al., 1994; Braak and Braak, 1994).

As well as the memory deficits that typify AD, the vast majority of AD patients also exhibit disturbances in their sleep-wake cycle and circadian rhythms. In early AD, the sleep disturbances are characterised by increased daytime naps and reduced and fragmented night-time sleep (Satlin et al., 1995; Volicer et al., 2001; Bliwise, 2004; Bliwise et al., 2011). Furthermore, a large number of AD patients display sundowning, which is a significant increase in behavioural symptoms in the hours before bedtime (Volicer et al., 2001). Additionally, a large proportion of individuals with AD also show reduced amplitude daily rhythms of activity and body temperature with marked phase shifts (Satlin et al., 1995; Harper et al., 2001; Volicer et al., 2001; Tranah et al., 2011). These sleep and circadian disturbances are a massive burden for AD patients and their caregivers (Gallagher-Thompson, 1992; Gaugler et al., 2006).

At a cellular level, circadian rhythms are based upon autoregulatory interlocking transcription/translation feedback loops of 'clock genes'. In mammals, the bHLH/PAS

transcription factors BMAL1 and CLOCK, heterodimerise and activate transcription of a large number of genes, including *Period* (*Per*) and *Cryptochrome* (*Cry*). PERIOD (PER) and CRYPTOCHROME (CRY) after a time delay inhibit BMAL1 and CLOCK activity, resulting in ~24 hour oscillations in gene expression (Mohawk et al., 2012; Hardin and Panda, 2013). In mammals, the vast majority of cells in the body exhibit circadian oscillations, however the ~20,000 neurons in the suprachiasmatic nucleus (SCN) of the hypothalamus are the master pacemakers. SCN neurons are divided into a dorsal shell (arginine vasopressin (AVP)-positive) and ventral core (vasoactive intestinal polypeptide (VIP)-positive). Circadian oscillators in the SCN receive direct photic inputs (from the retinohypothalamic tract) and entrain to light-dark (LD) cycles. The SCN then synchronises through a variety of electrical, endocrine and metabolic signalling pathways all the other circadian oscillators operating throughout the brain and body, so that they all oscillate in phase with each other and the environment (Reppert and Weaver, 2002; Welsh et al., 2010; Herzog et al., 2017). Free-running circadian rhythms persist in the absence of external cues (i.e. constant darkness) because the circadian oscillators in the SCN are self-sustaining.

Studies of circadian locomotor activity, using animal models of β-amyloidosis, have yielded a complex and confusing picture. For example, in LD conditions, mice overexpressing mutant human APP (amyloid β precursor protein; which is proteolytically cleaved to produce Aβ peptides) display normal activity rhythms (Wisor et al., 2005; Ambree et al., 2006; Gorman and Yellon, 2010). Whereas, mice overexpressing mutant APP and PS1 (presenilin 1; secretase that proteolytically cleaves APP to produce Aβ peptides) show a two hour phase delay in wakefulness (Duncan et al., 2012). Surprisingly, these APP and APP/PS1 mice have relatively normal activity rhythms in constant darkness (Wisor et al., 2005; Gorman and Yellon, 2010; Bano Otalora et al., 2012). However, two studies have shown that *Drosophila* models of β-amyloidosis exhibit age-related progressive behavioural arrhythmia in both LD and DD conditions (Chen et al., 2014; Long et al., 2014).

In comparison to Aβ pathology, very few studies have assessed the contribution of tau pathology to sleep-wake cycle and circadian rhythm disturbances in AD. Sterniczuk et al. (2010) showed 3xTg-AD mice, which overexpress a combination of mutant human APP, PS1

and tau, exhibit increased activity during the day (inactive phase) in LD conditions, but only minor deficits in activity rhythms in DD conditions. Additionally, aged 3xTg-AD mice exhibit core body temperature rhythm disturbances (Knight et al., 2013). Although, because Aβ pathology is also present, it is not possible to identify how tau specifically contributes to the observed circadian behaviour abnormalities. More recently, it has been shown that Tg4510 mice, which overexpress P301L tau in the forebrain (under the control of the forebrain-specific CaMKII promoter), exhibit increased activity during the day (inactive phase) in LD conditions and mild abnormalities in free-running behaviour (Stevanovic et al., 2017). However, $PLB2_{Tau}$ mice, which express P301L and R406W tau in the forebrain (under the control of the CaMKII promotor), show normal activity rhythms but abnormalities in sleep electroencephalograms (EEGs) (Koss et al., 2016). For these reasons, the role of tau in AD-linked sleep-wake cycle and circadian rhythm disturbances remains elusive. In *Drosophila,* disruption of the circadian Doubletime (Dbt) kinase causes cleavage of endogenous tau, which leads to circadian behavioural deficits and a shortened lifespan. In addition, disruption of Dbt also leads to enhanced neurodegeneration, produced by overexpression of human tau in the *Drosophila* visual system (Means et al., 2015). These results establish a link between the circadian clock and tau pathology.

Studies on post-mortem brains of human AD patients show a dramatic loss of AVP- and VIP-expressing neurons in the SCN (Swaab et al., 1985; Wu and Swaab, 2007), suggesting that damage to the SCN might cause circadian behavioural deficits. In support of this, 3xTg-AD mice exhibit substantial reductions in AVP- and VIP-containing neurons and APP/PS1 mice have disrupted endogenous *Per2* mRNA oscillations in the SCN (Sterniczuk et al., 2010; Duncan et al., 2012). Additionally, Tg4510 mice have disrupted PER2 protein oscillations in the hypothalamus and hippocampus (Stevanovic et al., 2017). In *Drosophila*, boosting the cleavage of APPL (the Drosophila homolog of APP) by overexpressing β-secretase disrupts activity rhythms and PER expression in the central clock neurons (Blake et al., 2015). However, results from *Drosophila* overexpressing human Aβ suggest that defects in the output pathways, rather than disrupted oscillations in the central clock, are the cause of the circadian behavioural abnormalities. For example, *Drosophila* overexpressing human Aβ,

show age-induced progressive behavioural arrhythmia in free-running conditions, despite normal oscillations in their master pacemaker neurons (Chen et al., 2014; Long et al., 2014).

The remarkable conservation of circadian mechanisms between humans and *Drosophila* justifies the use of *Drosophila* as a model organism (Bell-Pedersen et al., 2005). Circadian locomotor activity in *Drosophila* is driven by ~150 neurons in the fly brain that express a molecular clock. The ~150 neurons can be divided into several groups: small ventrolateral neurons (sLNvs), large ventrolateral neurons (lLNvs), dorsolateral neurons (LNds), lateral posterior neurons (LPNs), and three groups of dorsal neurons (DN1s, DN2s and DN3s), based on their anatomical position (Peschel and Helfrich-Forster, 2011). The neuropeptide pigment dispersing factor (PDF) is released from all the ventrolateral neurons, except the 5th sLNv (~16 neurons). PDF paracrinely synchronises the oscillations of the clock neurons so that they oscillate in phase with one another (Renn et al., 1999; Hyun et al., 2005; Stoleru et al., 2005; Cusumano et al., 2009). The sLNvs are the master pacemakers in *Drosophila*, because in free-running conditions they control the speed of behavioural and molecular rhythms in a PDF-dependent manner (Stoleru et al., 2005; Yao and Shafter, 2014). Clock neurons make synaptic contacts with specific non-clock neurons in the pars intercerebralis (PI) and lateral horn (LH), which show circadian production of the neuropeptides diuretic hormone 44 (DH44) and leucokinin (LK), respectively. Correct signalling at these synapses is needed for normal free-running circadian behaviour (Cavanaugh et al., 2014; Cavey et al., 2016).

Numerous *Drosophila* models of tauopathy have been produced by overexpressing human wild-type (WT) tau or FTD-linked mutant (R406W/V337M) tau in the developing fly brain (Wittmann et al., 2001). These fly models of tauopathy replicate many of the key features of human AD, including progressive age-related neurodegeneration (Wittmann et al., 2001; Nishimura et al., 2004), a reduced lifespan (Wittmann et al., 2001) and associative learning and memory deficits (Mershin et al., 2004). To date, no studies have described in detail the circadian dysfunction that can occur in *Drosophila* overexpressing human tau.

First, we show pan-neuronal tau expression (either wild-type or R406W) in the fly brain results in elevated night-time activity and daytime and night-time sleep loss in LD conditions, and progressive behavioural arrhythmia in DD conditions. Next, we show tau expression exclusively in PDF neurons causes elevated night-time activity, daytime sleep gains and night-time sleep loss in LD conditions, but is insufficient to induce progressive behavioural arrhythmia in DD conditions. In contrast, tau expression in all clock neurons causes progressive behavioural arrhythmia in DD. We found no detectable loss of PDF neurons in either Pdf>tau or tim>tau flies. These results suggest that the tau-evoked elevated night-time activity, daytime sleep gains and night-time sleep loss in LD conditions are as a result of dysfunction in the central pacemaker neurons. These results also indicate that the non-PDF clock neurons are the primary target for mediating tau-linked progressive behavioural arrhythmia in DD. In support of these ideas, suppression of tau expression in PDF neurons is sufficient to rescue the elevated night-time activity and night-time sleep loss in LD conditions, but not progressive behavioural arrhythmia in DD conditions in tau-expressing flies. Tau expression in the PDF neurons also prolongs the free-running period and makes the flies hyperactive in DD. Collectively, these experiments show that distinct groups of neurons mediate different tau-evoked circadian behavioural abnormalities. Recent evidence has shown in tauopathies dysfunction in the circadian system contributes to the disease process (Musiek et al., 2015; Hood and Amir, 2017). Thus, treating the circadian misalignment and sleep disruption early in the disease process might not just improve patient wellbeing but also clinical outcome. The development of a fly model that faithfully recapitulates AD-linked sleep-wake cycle and circadian rhythm disturbances provides a platform to screen for potential therapeutic agents. Given the projected increase in prevalence in AD and other tauopathies, effective treatment options are urgently needed.

3.2 Materials and Methods

3.2.1 Fly strains

All flies were raised on standard food (containing 0.8 % agar, 1.0 % soy flour, 8.0 % medium cornmeal, 8.0 % malt extract, 1.8 % yeast, 4.0 % molasses, 2.5 % 10% Nipagin in absolute

ethanol and 0.4 % propionic acid). Flies were raised under a 12 h light: 12 h dark (LD) cycle at 25 °C, except for flies carrying tub-Gal80^{ts} which were raised at 18 °C. Mated male flies of 5- or 25-days of age were used in all experiments. The following lines were used in the study: Canton-S, Elav^{C155}-Gal4, ElavGS-Gal4, tim-Gal4, Pdf-Gal4, Pdf-Gal80, repo-Gal80, tub-Gal80^{ts}, UAS-2n4r tau^{WT}, UAS-0n4r tau^{R406W}, UAS-0n4r tau^{WT} and UAS-2n4r tau^{S11A}, for details see Table 2.1. To reduce the variation from genetic background all lines were backcrossed against Canton-S for five generations. All lines have been previously described (for references see Table 2.1). The various UAS-tau lines used were previously generated by P-element mediated transgenesis. The pan-neuronal expression of tau is driven by Elav-Gal4. The pan-clock expression of tau is driven by tim-Gal4. Expression of tau exclusively in PDF clock neurons is driven by Pdf-Gal4. The tim-Gal4, repo-Gal80 line which labels all *timeless* clock neurons was generated by combining two transgenes: tim-Gal4 and repo-Gal80. The Elav-Gal4, Pdf-Gal80 line which labels all neurons apart from the PDF clock neurons was generated by combining transgenes: Elav-Gal4 and Pdf-Gal80.

Adult-onset pan-neuronal tau expression was achieved by using the Elav GeneSwitch (ElavGS)-UAS system (Osterwalder et al., 2001). Adult-onset pan-neuronal tau expression by ElavGS was induced by treatment with 500 µM mifepristone (RU486) added to standard food. Tau expression in the adult clock neurons was achieved by using the TARGET system: temperature sensitive tub-Gal80^{ts} combined with tim-Gal4. At 18 °C (permissive temperature), Gal80^{ts} is active and represses Gal4 activity. After shifting adult flies to 30 °C, Gal80^{ts} becomes inactive and no longer represses Gal4 activity (McGuire et al., 2003). The same Elav-Gal4, tim-Gal4 and Pdf-Gal4 driver lines were used in all experiments.

3.2.2 Lifespan experiments

Elav-Gal4 females were crossed to males carrying either UAS-2n4r tau^{WT} or UAS-0n4r tau^{R406W}. UAS-tau flies served as controls. Crosses were set up in bottles. Adult males were collected 0-48 h after eclosion and transferred under short CO_2 anaesthesia to vials containing standard food. All flies were kept in continuous LD cycles (12 h: 12 h) at 25 °C and 70 % humidity. Flies were kept at an initial density of 20 individuals per vial and transferred

without anaesthesia to new vials containing fresh food three times a week. Deaths were scored every day, except for weekends. 20 vials per genotype were used. Log-rank analysis of survivorship curves was performed using Graph Pad Prism 7.

3.2.3 Locomotor behaviour assay

Adult males were collected within a few hours of eclosion and no more than 20 were aged on standard food and were tipped on to new food every two-three days. Flies were housed individually in 65 mm x 5 mm glass tubes containing 5 % sucrose with 2 % agarose at one end of the tube (approximately 1/3 of the tube). Cotton wool was used to plug the open end of the tube. Locomotor activity was collected with a *Drosophila* activity monitor (DAM2) system (TriKinetics, Waltham, US) in one minute bins. The glass tubes containing the flies are placed in a DAM2 where an infrared beam bisects each tube. When the fly is active it breaks this beam and activity is recorded (Chiu et al., 2010; Pfeiffenberger et al., 2010). Monitors were placed in an incubator at 25 °C and 50-70 % humidity (Panasonic Mir155). Flies were entrained in continuous LD cycles (12 h: 12 h) for three-four full days, after which the lights were switched off and activity was monitored in constant darkness (DD) for seven-nine full days. Average daily activity histograms were produced from activity data from three consecutive days of LD or seven-nine consecutive days of DD. Daytime and night-time activity is the total number of beam counts during the light and dark phase of LD, averaged across three consecutive days. A fly was characterised as being asleep when it was inactive for at least five minutes (Hendricks et al., 2000; Shaw et al., 2000). Sleep analysis was conducted using a custom-written Excel macro (Donlea et al., 2014). Average daily sleep profiles were produced from activity data from three consecutive days of LD. Daytime and night-time sleep is the total amount of sleep during the light and dark phase of LD, averaged across three consecutive days. GraphPad prism was used for plotting.

Circadian rhythm strength (power, PN) was assessed by Lomb-Scargle periodogram analysis of the DD data using an Image J plugin, Actogram J (Schmid et al., 2011). Flies were characterised as rhythmic or arrhythmic based upon the presence or absence of any peak above the 0.05 significance line (which is usually at 12.5 PN), respectively (Van Dongen et

al., 1999). Average power and period length were only calculated from flies with rhythmic activity profiles. In flies with complex rhythms (more than a single peak above the 0.05 significance line), the largest peak was used for analysis. Activity in DD is the average number of activity counts/24 h averaged across seven-nine days. For temperature shift experiments, power and period length were calculated from the initial 6 days in DD at 20 °C and the subsequent six days at 30 °C (flies were first entrained to LD cycles for three days). Graph Pad Prism 7 was used for plotting.

3.2.4 Immunohistochemistry

To visualise the clock neurons, we used tim-Gal4 and Pdf-Gal4 to drive expression of a UAS-mCD8::GFP transgene. Fly brains were dissected in phosphate buffered saline (PBS). After dissection, brains were fixed in freshly prepared 4 % paraformaldehyde in PBS for 30 minutes. Brains were rinsed three times in PBS with 0.5 % Triton X100 (PBS-T 0.5 %) and mounted on microscope slides in Vectashield mounting media (Vector Laboratories, Burlingame, US). Slides were stored at 4 °C and imaged within a week. Images were taken with an Olympus FV1000 confocal microscope, using Fluoview acquisition software. Images were analysed using ImageJ and Adobe Photoshop.

3.2.5 Statistical analysis

Graph Pad Prism 7 was used for statistical analysis. Sample sizes are reported in figures. Box plots show median with interquartile range and the 10 % and 90 % percentiles as whiskers. Data from flies which died before the end of the experiment was excluded from analysis. All data were tested for normal or lognormal distribution by a Kolmogorov-Smirnov test. Activity (LD and DD) and power datasets were lognormally distributed. Log-transformed activity and power datasets were analysed by a two-tailed Student's t-test if there were two groups, 1-way ANOVA if there were more than two groups with a single factor (genotype) and 2-way ANOVA if there were more than two groups with two factors (genotype and age) followed by post-hoc tests. Multiple comparisons after ANOVA were performed by a Tukey HSD test. Sleep and period datasets were neither normally nor lognormally distributed,

therefore we chose to use non-parametric tests rather than parametric tests (i.e. t-test and ANOVA), which assume normal distribution. Sleep and period datasets were analysed by a Mann-Whitney U-test if there were two groups and Kruskal-Wallis ANOVA if there were more than two groups with a single factor (genotype) followed by post-hoc tests. Sleep and period datasets with more than two groups with two factors (genotype and age) were analysed in two ways. First, Kruskal-Wallis ANOVA followed by post-hoc tests were used to check for differences between different genotypes of the same age (either 5- or 25-days old). Multiple comparisons after ANOVA were performed by a Dunn's test. Second, a Mann-Whitney U-test was used to check for differences between different ages (5- and 25-day old) of the same genotype. No statistical tests were done to predetermine sample size. However, sample size are consistent with previous publications in the field (Chen et al., 2014; Long et al., 2014; Julienne et al., 2017). P levels are indicated as ns $p>0.05$, * $p<0.05$, ** $p<0.001$ or *** $p<0.0001$.

3.3 Results

3.3.1 Pan-neuronal human wild-type tau (2n4r isoform) expression affects activity and sleep in LD conditions

First, we expressed human full-length wild-type tau (2n4r isoform) (Jackson et al., 2002) in all neurons using the Gal4/UAS system (driven by the pan-neuronal Elav-Gal4 driver) and investigated whether the flies showed normal circadian behaviour by monitoring their locomotor activity in LD and DD conditions. Several studies have shown human wild-type tau overexpression in the developing Drosophila brain or visual system results in neurodegeneration, which resembles several key features of tauopathies, including age-dependency, cell-type specificity, and accumulation and mislocalisation of abnormally phosphorylated tau (Jackson et al., 2002; Mershin et al., 2004; Nishimura et al., 2004; Chen et al., 2007; Kosmidis et al., 2010).

In LD conditions, *Drosophila* normally exhibit a morning activity peak (centred around the dark-to-light transition) and evening activity peak (centred around the light-to-dark

transition). Flies anticipate the light transitions by gradually increasing their activity in advance of them. The timing of the activity peaks is controlled by the clock. Independent of the clock, flies also exhibit startle responses, which are transient bursts of activity in response to the light transitions. *Drosophila* show a midday siesta between the two activity peaks and a period of consolidated sleep at night (reviewed in Dubowy and Sehgal, 2017). Both Gal4 control and Elav>2n4r tau^{WT} flies showed a normal bimodal activity profile (Figure 3.1A, left column and middle column). However, Elav>2n4r tau^{WT} flies seemed to show elevated basal activity. Elav>2n4r tau^{WT} flies appeared to show normal morning and evening anticipation, but largely abolished morning and evening startle responses (Figure 3.1A). We found that levels of daytime activity were unaffected, whereas levels of night-time activity were substantially increased, in both 5- and 25-day old Elav>2n4r tau^{WT} flies compared to age-matched controls. Between the 5- and 25-day old age groups, we found no significant differences in levels of daytime and night-time activity in either Elav>2n4r tau^{WT} or control flies (Figure 3.1B,C).

Next, we investigated whether the elevated night-time activity in Elav>2n4r tau^{WT} flies coincides with sleep loss. We found a small loss of daytime sleep in Elav>2n4r tau^{WT} flies compared to age-matched controls. The daytime sleep loss just failed to reach significance in 5-day old Elav>2n4r tau^{WT} flies. No significant age-related differences in daytime sleep were seen in Elav>2n4r tau^{WT} flies. On the other hand, we found a significant age-related increase in daytime sleep in Gal4 control flies. As a result, the daytime sleep loss reached significance in 25-day old Elav>2n4r tau^{WT} flies (Figure 3.2A,C). Regardless of age, we found a large loss of night-time sleep in both 5- and 25-day old Elav>2n4r tau^{WT} flies, compared to age-matched controls. Elav>2n4r tau^{WT} flies showed a significant age-related reduction in night-time sleep (Figure 3.2A,D). These results demonstrate that pan-neuronal human wild-type tau (2n4r isoform) expression promotes night-time activity and suppresses daytime and night-time sleep in LD conditions.

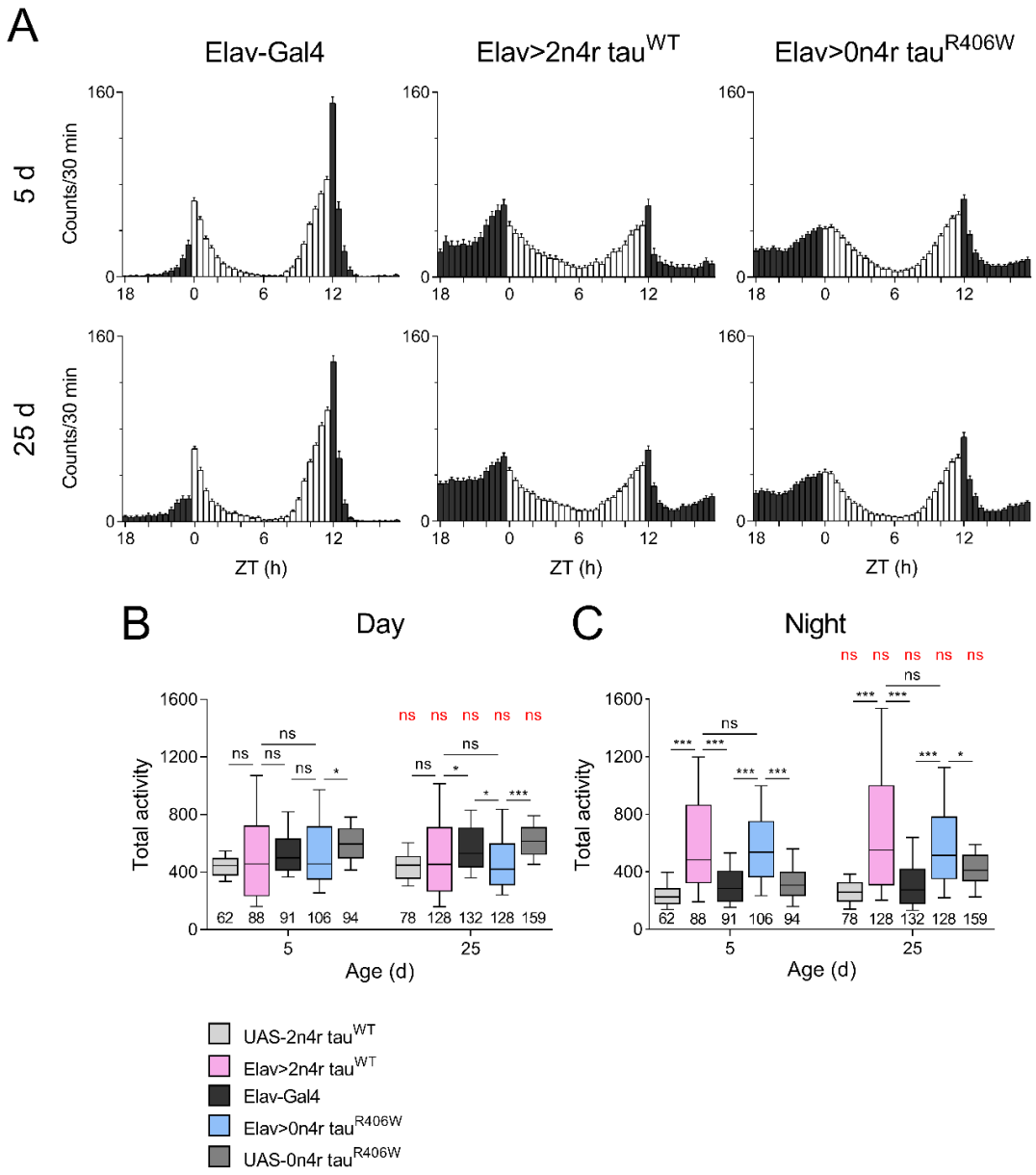


Figure 3.1: Locomotor activity of tau-expressing flies in LD conditions. (A) Average daily activity histograms for three days of LD conditions are shown for 5- and 25-day old Gal4 control (left column), Elav>2n4r tau^{WT} (middle column) and Elav>0n4r tau^{R406W} (right column) flies. All genotypes exhibit normal bimodal activity rhythms. Elevated baseline activity in tau-expressing flies. Vertical bars represent activity counts displayed in 30-min bins (mean ± SEM). Lights-on (white bars). Lights-off (grey bars). (B-C) Quantification of daytime and night-time activity. Elav>2n4r tau^{WT} expression has no effect on daytime activity, but increases night-time activity, relative to controls. Elav>0n4r tau^{R406W} expression decreases daytime activity (in 25-day old, but not in 5-day old flies), but increases night-time activity, relative to controls. Graphs show median with second and third quartiles and 10th and 90th percentiles. n= 62-159 flies per genotype/age from 4-8 independent experiments. ns p>0.05; *p<0.05; ***p<0.0001. Data were lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data.

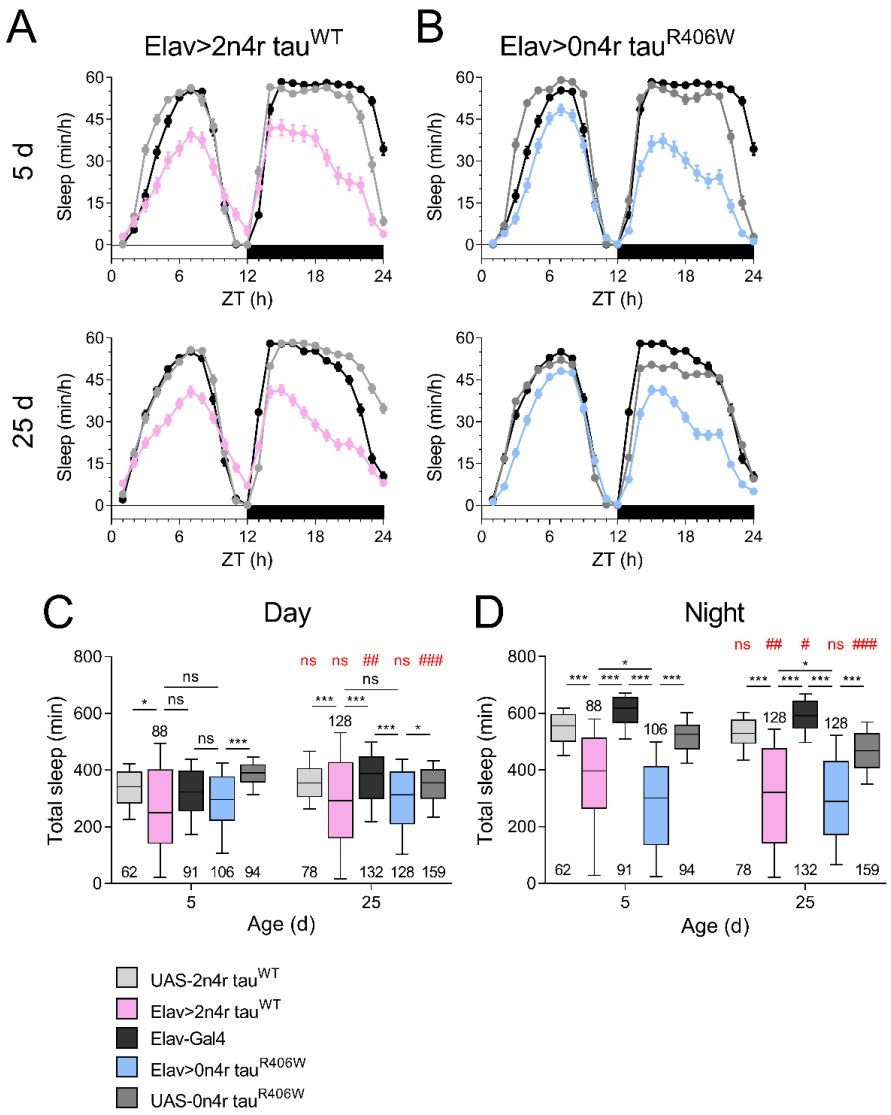


Figure 3.2: Pan-neuronal tau expression reduces sleep. (A) Average daily sleep profile for three days of LD conditions are shown for 5- and 25-day old Elav>2n4r tau^{WT} and control flies. (B) Average daily sleep profile for three days of LD conditions are shown for 5- and 25-day old Elav>0n4r tau^{R406W} and control flies. All genotypes display a bimodal profile, with peaks at midday and midnight and troughs at dawn and dusk. The horizontal bars show when the lights were on (white) or off (black). Graphs show mean ± SEM. (C-D) Quantification of daytime and night-time sleep. Elav>2n4r tau^{WT} expression reduces daytime sleep (difference does not quite reach the level of significance in 5-day old flies) and night-time sleep relative to controls. Elav>0n4r tau^{R406W} expression reduces daytime sleep (difference does not reach significance in 5-day old flies) and night-time sleep relative to controls. Graphs show median with second and third quartiles and 10th and 90th percentiles. n = 62-159 flies per genotype/age from 4-8 independent experiments. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. Data were neither normally nor lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.

3.3.2 Pan-neuronal human wild-type tau (2n4r isoform) expression affects free-running circadian rhythms

In DD conditions, Gal4 control flies maintained a rhythm of daytime activity and night-time activity, with a period of nearly 24 hours. Similarly, Elav>2n4r tau^{WT} flies were more active during the day than at night. However, in Elav>2n4r tau^{WT} flies relative night-time activity appeared to be elevated, so the distinction between daytime activity and night-time inactivity was less clear. Average daily activity histograms and representative double-plotted actograms for Gal4 control and Elav>2n4r tau^{WT} flies can be seen in Figure 3.3 (left column and middle column). To quantify rhythm strength (power), we used Lomb-Scargle periodogram analysis (Van Dongen et al., 1999). The power of Elav>2n4r tau^{WT} flies was substantially reduced compared with age-matched controls at both ages analysed (Figure 3.3B). By 25-days of age, only 85 % of Elav>2n4r tau^{WT} flies remained rhythmic, compared to 100 % of control flies (Table 3.1). A representative actogram for a rhythmic and arrhythmic 25-day old Elav>2n4r tau^{WT} fly is shown in Figure 3.3 (middle column, bottom row). Between the 5- and 25-day old age groups, all genotypes exhibited a significant reduction in overall rhythmicity (Figure 3.3B).

Pan-neuronal tau expression had no effect on the period length of rhythms (Figure 3.3C). It is possible the dysrhythmia in Elav>2n4r tau^{WT} flies could be due to reduced activity levels. In support of this, Ali et al. (2011) showed pan-neuronal tau^{WT} expression resulted in locomotor activity deficits. However, we found pan-neuronal 2n4r tau^{WT} expression resulted in age-induced hyperactivity in DD. We found normal activity levels in 5-day old Elav>2n4r tau^{WT} flies, but increased levels of activity in 25-day old Elav>2n4r tau^{WT} flies, compared to age-matched controls. A small, but significant, age-related decline in activity levels was seen in control flies. Whereas, we found in Elav>2n4r tau^{WT} flies activity levels were well maintained with aging (Figure 3.3D). Thus, the reduced rhythmicity in Elav>2n4r tau^{WT} flies was not an artifact of reduced activity levels. These results show that the overaccumulation of human wild-type tau (2n4r isoform) in all neurons causes progressive behavioural arrhythmia and age-induced hyperactivity in DD conditions.

3.3.3 Pan-neuronal human R406W tau (0n4r isoform) expression affects circadian locomotor behaviour in LD and DD conditions

Different tau proteins are associated with different diseases with distinct pathology, molecular aetiology and symptomology (reviewed in Guo et al., 2017). Therefore, it remains unclear whether different tau proteins would precipitate equivalent results when studied in insolation. To explore this we monitored the locomotor activity (in LD and DD conditions) of flies pan-neuronally expressing human wild-type or R406W tau (0n4r isoform). Both human 0n4r tau^{WT} and 0n4r tau^{R406W} have been previously shown to cause premature death, adult-onset progressive neurodegeneration and associative memory deficits when expressed ectopically in the *Drosophila* brain (Wittmann et al. 2001; Kosmidis et al., 2010; Sealey et al., 2017). We found Elav>2n4r tau^{WT} expression reduced lifespan by 16 % and Elav>0n4r tau^{R406W} expression reduced lifespan by 20 % (Supplementary Figure 3.1). Previous studies have shown that Elav>tau expression results in a more dramatic lifespan reduction (Wittmann et al., 2001; Gorsky et al., 2016). This discrepancy could be due to differences in the genetic background or experimental protocol, we used male flies kept on standard food at a low population density (≤20 flies per vial), tipped three times a week on to fresh food. As the median lifespan of Elav>2n4r tau^{WT} flies was 57 days and Elav>0n4r tau^{R406W} flies was 63 days and we used flies up 35-days old in our experiments, pan-neuronal tau expression does not shorten lifespan sufficiently to influence our observations of circadian behaviour (Supplementary Figure 3.1). As such, a similar number of Elav>2n4r tau^{WT}, Elav>0n4r tau^{R406W} and control flies survived to the end of the ten day experiment (Table 3.1).

Pan-neuronal 0n4r tau^{R406W} expression had little effect on the shape of the bimodal activity profile in LD conditions. However, basal activity appeared to be elevated in Elav>0n4r tau^{R406W} flies. The timing of morning and evening anticipation seemed to be normal in Elav>0n4r tau^{R406W} flies. However, morning and evening startle responses were largely abolished in Elav>0n4r tau^{R406W} flies (Figure 3.1A, right column). We found levels of daytime activity were unaffected in 5-day old Elav>0n4r tau^{R406W} flies, but slightly reduced in 25-day old Elav>0n4r tau^{R406W}, compared to age-matched controls. On the other hand, levels of night-time activity were substantially increased in Elav>0n4r tau^{R406W} flies compared to age-

matched controls at both ages analysed. No significant age-related differences in daytime or night-time activity were seen in Elav>0n4r tau^{R406W} flies (Figure 3.1B,C).

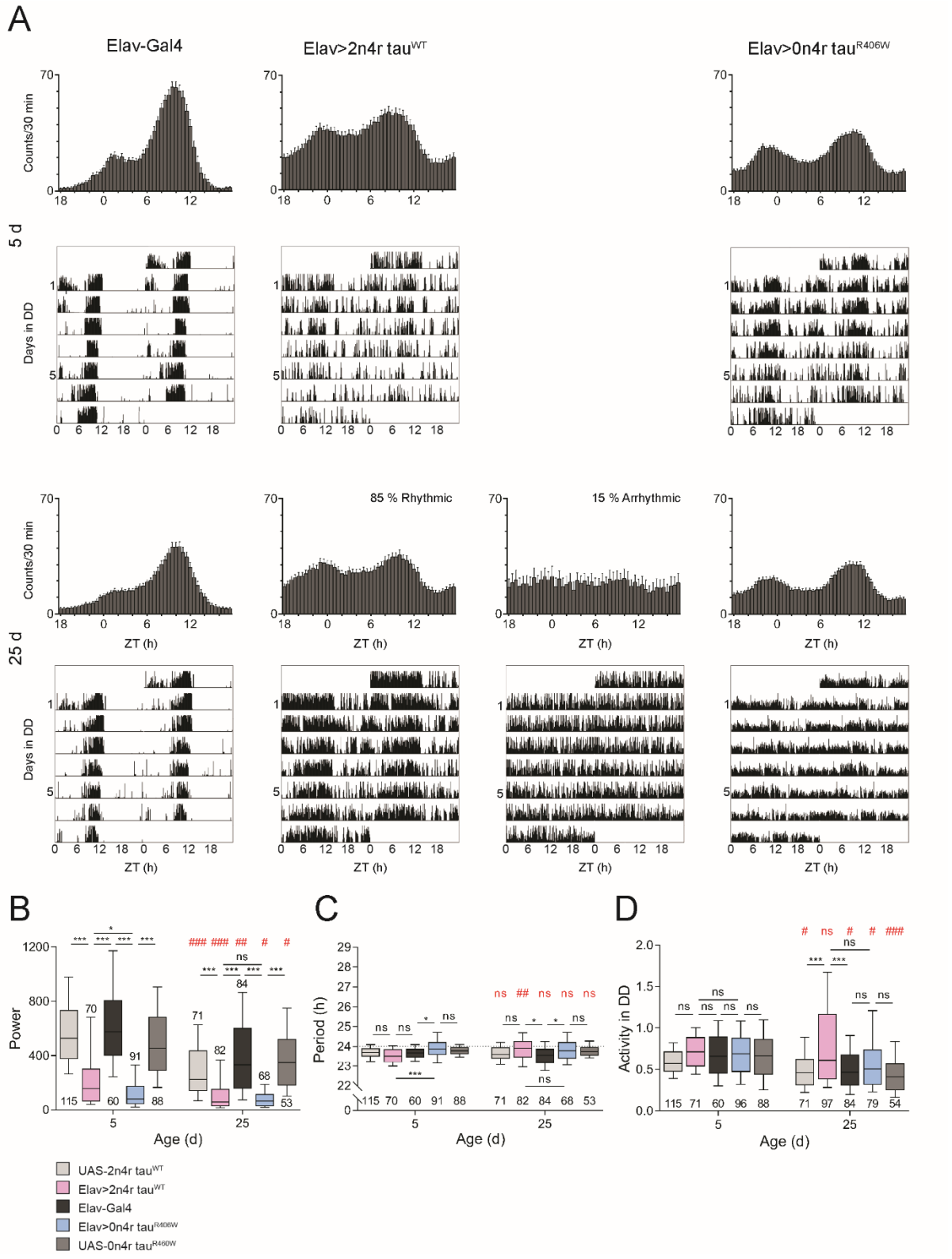


Figure 3.3: Pan-neuronal tau expression causes behavioural arrhythmia. (A) Average daily activity histograms and representative double-plotted actograms for seven days of DD conditions are shown for 5- and 25-day old Gal4 control (left column), Elav>2n4r tau^{WT} (middle column) and Elav>0n4r tau^{R406W} (right column) flies. Flies were entrained in standard LD cycles for three days and then monitored in DD for seven days. Control flies exhibit robust rhythms of daytime activity and night-time inactivity, with a period of just under 24 h. Tau-expressing flies exhibit either weak rhythms with a normal period or are arrhythmic. Vertical bars represent activity counts displayed in 30-min bins (mean ± SEM). (B) Pan-neuronal tau expression severely reduces power, determined by Lomb-Scarle analysis. See Table 3.1 for the percentage of rhythmic flies. (C) Pan-neuronal tau expression has no effect on the period length of rhythms. (D) Pan-neuronal 2n4r tau^{WT} expression causes age-induced hyperactivity in DD. Pan-neuronal 0n4r tau^{R460W} expression has no effect on activity levels in DD. Graphs show median with second and third quartiles and 10th and 90th percentiles. n = 53-115 flies per genotype/age from 4-6 independent experiments. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. (B,D) Data were lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C) Data were neither normally nor lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.

Elav>0n4r tau^{R406W} expression resulted in a small loss of daytime sleep. Daytime sleep loss approached but did not quite achieve significance in 5-day old Elav>0n4r tau^{R406W} flies. Between the 5- and 25-day old age groups, no significant differences in daytime sleep were seen in Elav>0n4r tau^{R460W} flies. Therefore, as a result of the age-related increase in daytime sleep in Gal4 control flies, the daytime sleep loss reached significance in 25-day old Elav>0n4r tau^{R406W} flies (Figure 3.2B,C). Elav>2n4r tau^{WT} and Elav>0n4r tau^{R406W} expression produced a similar daytime sleep loss phenotype at both ages analysed (Figure 3.2C). We found Elav>0n4r tau^{R406W} expression resulted in the loss of a large proportion of night-time sleep. The night-time sleep loss phenotype in Elav>0n4r tau^{R406W} flies was not progressive (Figure 3.2B,D). Elav>2n4r tau^{WT} and Elav>0n4r tau^{R406W} expression produced similar night-time activity phenotypes (Figure 3.1C). In 5-day old flies, we found a stronger night-time sleep loss phenotype in Elav>0n4r tau^{R406W} flies compared to Elav>2n4r tau^{WT} flies. However, we found a similar night-time sleep loss phenotype in 25-day old Elav>2n4r tau^{WT} and Elav>0n4r tau^{R406W} flies, as a result of the age-related reduction in night-time sleep in Elav>2n4r tau^{WT} flies (Figure 3.2D). Similar to Elav>2n4rtau^{WT} expression, Elav>0n4r tau^{R406W}

expression promotes night-time activity and suppresses daytime and night-time sleep in LD conditions.

Compared to Gal4 control flies, Elav>0n4r tau^{R406W} flies appeared to show less distinction between daytime activity and night-time inactivity and relative night-time activity seemed to be elevated in free-running conditions. Average daily activity histograms and representative actograms for Elav>0n4r tau^{R406W} flies can be seen in Figure 3.3 (right column). Both 5- and 25-day old Elav>0n4r tau^{R406W} flies showed a substantial reduction in power compared with age-matched controls (Figure 3.3B). Additionally, 5 % of 5-day old Elav>0n4r tau^{R406W} flies and 15 % of 25-day old Elav>0n4r tau^{R406W} flies were arrhythmic (active around the clock), compared to 0 % of control flies (Table 3.1). Elav>0n4r tau^{R406W} and control flies showed a similar age-related decline in rhythmicity. In 5-day old flies, Elav>0n4r tau^{R406W} expression produced a more severe reduction in overall rhythmicity compared to Elav>2n4r tau^{WT} expression. We found a more substantial age-related reduction in rhythmicity in Elav>2n4r tau^{WT} flies compared to Elav>0n4r tau^{R406W} flies. As a result, in 25-day old flies, Elav>0n4r tau^{R406W} and Elav>2n4r tau^{WT} expression produced a similar reduction in rhythm strength (Figure 3.3B). The period length of rhythms was normal in Elav>0n4r tau^{R406W} flies (Figure 3.3C). No significant differences in levels of activity in DD were seen in Elav>0n4r tau^{R406W} flies compared with age-matched controls at both ages analysed. Between the 5- and 25-day old age groups, both Elav>0n4r tau^{R406W} and control flies showed a small, but significant, reduction in activity levels (Figure 3.3D). These results show that Elav>0n4r tau^{R406W} expression reduces overall rhythmicity, but has no effect on the period length of rhythms or activity levels in DD conditions.

3.3.4 Pan-neuronal 0n4r tau^{WT} expression fails to induce behavioural arrhythmia

Surprisingly, we found that Elav>0n4r tau^{WT} expression exhibited robust behaviour, in contrast to the behavioural arrhythmia produced by Elav>2n4r tau^{WT} and Elav>0n4r tau^{R406W} expression (Supplementary Figure 3.2A,B,C). Regardless of age, power and period length did not significantly differ between Elav>0n4r tau^{WT} flies and age-matched controls (Supplementary Figure 3.2B,C). The only remarkable difference between Elav>0n4r tau^{WT}

and control flies was activity levels in DD. In free-running conditions, we found a substantial increase in levels of activity in Elav>0n4r tau^{WT} flies compared to controls. As the Elav>0n4r tau^{WT} flies aged the elevated activity levels were stable (Supplementary Figure 3.2D). Thus, Elav>0n4r tau^{R406W} expression reduces overall rhythmicity without affecting activity levels, in comparison Elav>0n4r tau^{WT} expression fails to affect general rhythmicity but increases activity levels.

3.3.5 Adult-onset pan-neuronal human tau expression does not affect circadian locomotor behaviour

To determine whether adult-onset pan-neuronal tau expression can affect rhythmicity, we employed the GeneSwitch (GS) inducible transgene expression system (Osterwalder et al., 2001). To ensure the effectiveness of ElavGS, we induced tetanus toxin (which inactivates the synaptic release machinery; Sweeney et al., 1995) using ElavGS. Flies, in which tetanus toxin expression was induced by treatment with RU486, died within two days (data not shown).

ElavGS>2n4r tau^{WT} flies were fed on RU486- or vehicle-containing standard food from one-two days post eclosion for ten days. Flies were subsequently loaded into the DAMs and their locomotor activity was monitored in both LD and DD conditions. In LD conditions, RU486-fed flies exhibited bimodal activity rhythms that were essentially identical to those found in vehicle-fed flies. (Figure 3.4A). No significant differences in activity and sleep were seen between RU486- and vehicle-fed flies (Supplementary Figure 3.3A,B,C). In DD conditions, both RU486- and vehicle-fed flies maintained robust rhythms of daytime activity and night-time inactivity, with a period of just under 24 hours (Figure 3.4B). We found power and period length did not significantly differ between RU486- and vehicle-fed flies (Figure 4.4C,D). Levels of activity in DD in RU486-fed flies were not significantly different from in vehicle-fed flies (Figure 3.4E), but were dramatically reduced compared to other genotypes (Table 3.1). This cannot be attributed to the effects of ethanol exposure as Wolf et al., (2002) demonstrated that low doses of ethanol increase locomotor activity. These results

indicate that adult-onset pan-neuronal 2n4r tau^{WT} expression (via ElavGS) is insufficient to cause circadian behavioural deficits.

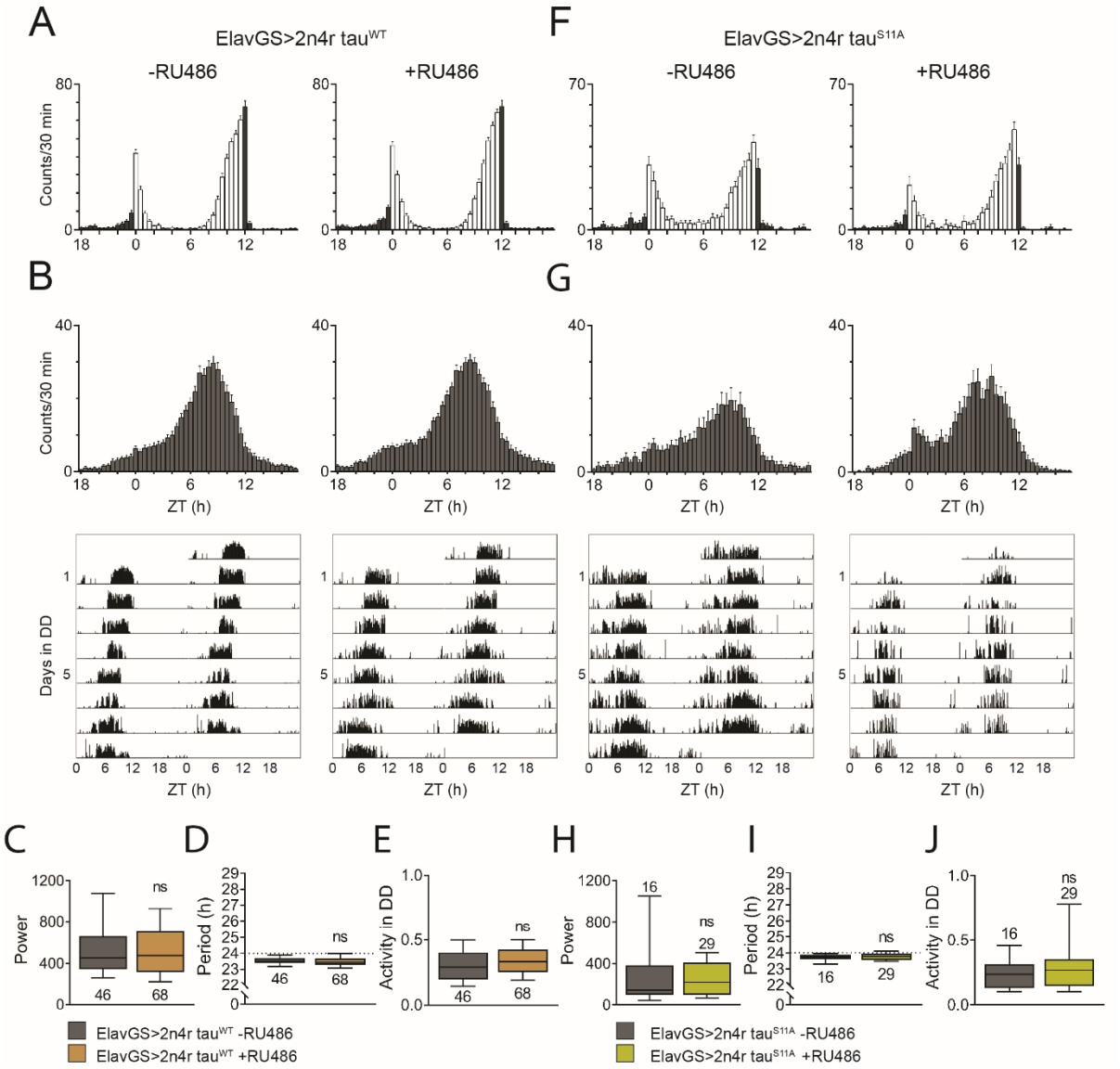


Figure 3.4: Adult-onset pan-neuronal tau expression (via Elav-GS) is insufficient to disrupt normal circadian locomotor rhythms. ElavGS>2n4r tau^{WT} (A-E) and ElavGS>2n4r tau^{S11A} (F-J) flies were raised on standard food during development and were transferred within two days of eclosion onto RU486- or vehicle-containing standard food for ten days. Flies were subsequently loaded into the DAMs and their locomotor activity was recorded during 3LD cycles followed by 8DD cycles. (A) Average daily activity histograms for three days of LD conditions are shown for RU486- and vehicle-fed ElavGS>2n4r tau^{WT} flies. Activity rhythms are essentially identical in RU486- and vehicle-fed flies. (B) Average daily activity histograms and representative double-plotted actograms for eight days of DD conditions are shown for RU486- and vehicle-fed ElavGS>2n4r tau^{WT} flies. Both RU486- and vehicle-fed flies show robust rhythms of daytime activity and night-time inactivity, with a period of just under 24 h. (C) Power. (D) Period length. (E) Activity in DD. n = 46-68 flies per group from 3-4 independent experiments. (F) Average daily activity histograms for three days of LD conditions are shown for RU486- and vehicle-fed ElavGS>2n4r tau^{S11A} flies. Activity rhythms are virtually identical in RU486- and vehicle-fed flies. (G) Average daily activity histograms and representative double-plotted actograms for eight days of DD conditions are shown for RU486- and vehicle-fed ElavGS>2n4r tau^{S11A} flies. Both RU486- and vehicle-fed flies show robust rhythms of daytime activity and night-time inactivity, with a period of nearly 24 h. (H) Power. (I) Period length. (J) Activity in DD. n = 16-29 flies per group from a single experiment. Graphs show median with second and third quartiles and 10th and 90th percentiles. ns p>0.05; two-tailed Student's t-test with log-transformed data for (C,E,H,J); Man-Whitney U-test for (D,I).

Next, we tested whether adult-onset pan-neuronal expression of the highly toxic 2n4r tau^{S11A} construct could induce behavioural arrhythmia under similar conditions. Previous studies have shown that 2n4r tau^{S11A} is more toxic than either 0n4r tau^{WT} or 2n4r tau^{WT} when expressed in the *Drosophila* larval neuromuscular junction (NMJ) or adult visual system (Chatterjee et al., 2009; Talmat-Amar et al., 2011). Indeed, Elav-driven 2n4r tau^{S11A} expression at both 18 °C and 25 °C, resulted in lethality at the pupal stage (data not shown). Both RU486- and vehicle-fed ElavGS>2n4r tau^{S11A} flies showed bimodal activity rhythms in LD conditions (Figure 3.4F). We found no significant differences in levels of activity and sleep between RU486- and vehicle-fed flies (Supplementary Figure 3.3D,E). In DD conditions, RU486-fed flies retained significant rhythmic behaviour (Figure 3.4G). No significant differences in power, period length and activity in DD were seen between RU486- and vehicle-fed flies (Figure 3.4H,I,J). Taken together, these results show adult-onset pan-neuronal expression of either 2n4r tau^{WT} or highly toxic 2n4r tau^{S11A} (via ElavGS) fails to induce circadian behavioural abnormalities. However, the lack of a phenotype is likely due to insufficient tau expression levels, it is possible increasing the concentration of RU486,

longer exposure to RU486, or introducing additional copies of Gal4/UAS might produce a phenotype. In comparison, TNT is effective in the nanomolar concentration range (Sweeney et al., 1995; Thum et al., 2006).

3.3.6 Human wild-type tau (2n4r isoform) expression in the clock neurons affects activity and sleep in LD conditions

Studies on post mortem brains from AD patients have shown substantial cell loss in the SCN, suggesting that damage to the master pacemaker might cause circadian behavioural abnormalities (Swaab et al., 1985; Wu and Swaab, 2007). Because of this, we wanted to explore the effects of restricting tau expression to the clock system. To achieve this, we used the Pdf-Gal4 and tim-Gal4 driver lines to express tau either in the PDF-positive clock neurons (~16 neurons) or in all clock neurons (~150 neurons), respectively and recorded locomotor activity in LD and DD conditions.

In LD conditions, both tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} flies showed normal bimodal activity rhythms with morning and evening peaks. Except for a loss of morning anticipation in 25-day old tim>2n4r tau^{WT} flies, morning and evening anticipation appeared to be normal in tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} flies. Morning and evening startle responses seemed to be normal in tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} flies. Baseline activity levels appeared to be slightly elevated in tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} (Figure 3.5A). We found levels of daytime activity were unaffected in tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} flies, whereas levels of night-time activity were substantially increased, compared to age-matched controls at both ages analysed (Figure 3.5B,C). No significant age-related differences in daytime activity were observed in tim>2n4r tau^{WT}, Pdf>2n4r tau^{WT} and control flies (Figure 3.5B). In 5-day old flies, tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} expression produced a similar night-time activity phenotype. With aging, levels of night-time activity were well maintained in Pdf>2n4r tau^{WT} flies, but not in tim>2n4r tau^{WT} flies. A severe age-related reduction in night-time activity was seen in tim>2n4r tau^{WT} flies. Therefore, in 25-day old flies the night-time activity phenotype was milder in tim>2n4r tau^{WT} flies than in Pdf>2n4r tau^{WT} flies. Control

flies showed a small, but significant, age-related reduction in night-time activity (Figure 3.5C).

Regardless of age, tim>2n4r tau^{WT} expression had no effect on daytime sleep (Figure 3.7A,C). On the other hand, Pdf>2n4r tau^{WT} expression resulted in a small gain in daytime sleep in 5-day old flies, but not in 25-day old flies. We found a moderate reduction in daytime sleep in Pdf>2n4r tau^{WT} flies as a function of age (Figure 3.7B,C). Whereas, no significant age-related differences in daytime sleep were seen in either tim>2n4r tau^{WT} or control flies (Figure 3.7A,C). At both ages analysed, tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} expression resulted in the loss of a large proportion of night-time sleep. In 5-day old flies, the night-time sleep loss phenotype was stronger in tim>2n4r tau^{WT} flies than in Pdf>2n4r tau^{WT} flies. We found levels of night-time sleep were stable in Pdf>2n4r tau^{WT} flies, but not in tim>2n4r tau^{WT} flies, with aging. As a result, in 25-day old flies, the night-time sleep loss phenotype was similar in tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} flies. No significant age-related differences in night-time sleep were seen in control flies (Figure 3.7A,B,D). These results show that 2n4r tau^{WT} expression in the clock system promotes daytime sleep in an age-dependent manner and promotes night-time activity and suppresses night-time sleep in an age-independent manner.

3.3.7 Human wild-type tau (2n4r isoform) expression in all clock neurons is sufficient to trigger behavioural arrhythmia in DD conditions

In DD conditions, UAS control flies were active during the day and inactive at night (Figure 3.8A, left column). Whereas, tim>2n4r tau^{WT} flies failed to show a clear distinction between their daytime activity and night-time inactivity because relative night-time activity seemed to be elevated (Figure 3.8A, middle column). Both 5-and 25-day old tim>2n4r tau^{WT} flies showed a substantial reduction in power compared with -age-matched controls. All genotypes exhibited an age-related decline in overall rhythmicity (Figure 3.8B). By 25-days of age, the fraction of tim>2n4r tau^{WT} flies that remained rhythmic was only 70 %, compared to 100 % of controls (Table 3.1). An example actogram for a rhythmic and arrhythmic 25-day old tim>2n4r tau^{WT} fly is shown in Figure 3.8 (middle column, bottom row). Surprisingly,

tim>2n4r tau^{WT} flies were less rhythmic than Elav>2n4r tau^{WT} flies at the same age – as shown by reduced overall rhythmicity and an increased arrhythmic sub-population (Supplementary Figure 3.7A and Table 3.1).

Furthermore, tim>2n4r tau^{WT} expression prolonged the free-running period. The average period length of 5-day old tim>2n4r tau^{WT} flies was 0.3-0.4 h longer than in control flies. Similarly, the average period length of 25-day old tim>2n4r tau^{WT} flies was 1.1 h longer than in control flies. Period lengths in tim>2n4r tau^{WT} flies were much more variable than in control flies. For example, in 25-day old tim>2n4r tau^{WT} flies period lengths ranged from between 19.1-31.5 h, compared to 22.7-24.7 h in the Gal4 control and 22.6-25.1 h in the UAS control. Between the 5- and 25-day old age groups, no significant differences in the period length of rhythms were seen in either tim>2n4r tau^{WT} or control flies (Figure 3.8C and Table 3.1). We found a substantial increase in activity levels in DD in 5-day old tim>2n4r tau^{WT} flies with respect to controls. Tim>2n4r tau^{WT} flies showed a dramatic age-related decline in levels of activity. In 25-day old tim>2n4r tau^{WT} flies, activity levels were reduced to the point where they no longer significantly differed from age-matched controls. On the other hand, control flies showed a moderate age-related reduction in activity levels (Figure 3.8D). These results show that 2n4r tau^{WT} overexpression in all clock neurons causes progressive behavioural arrhythmia and hyperactivity, and prolongs the period length of rhythms in DD conditions.

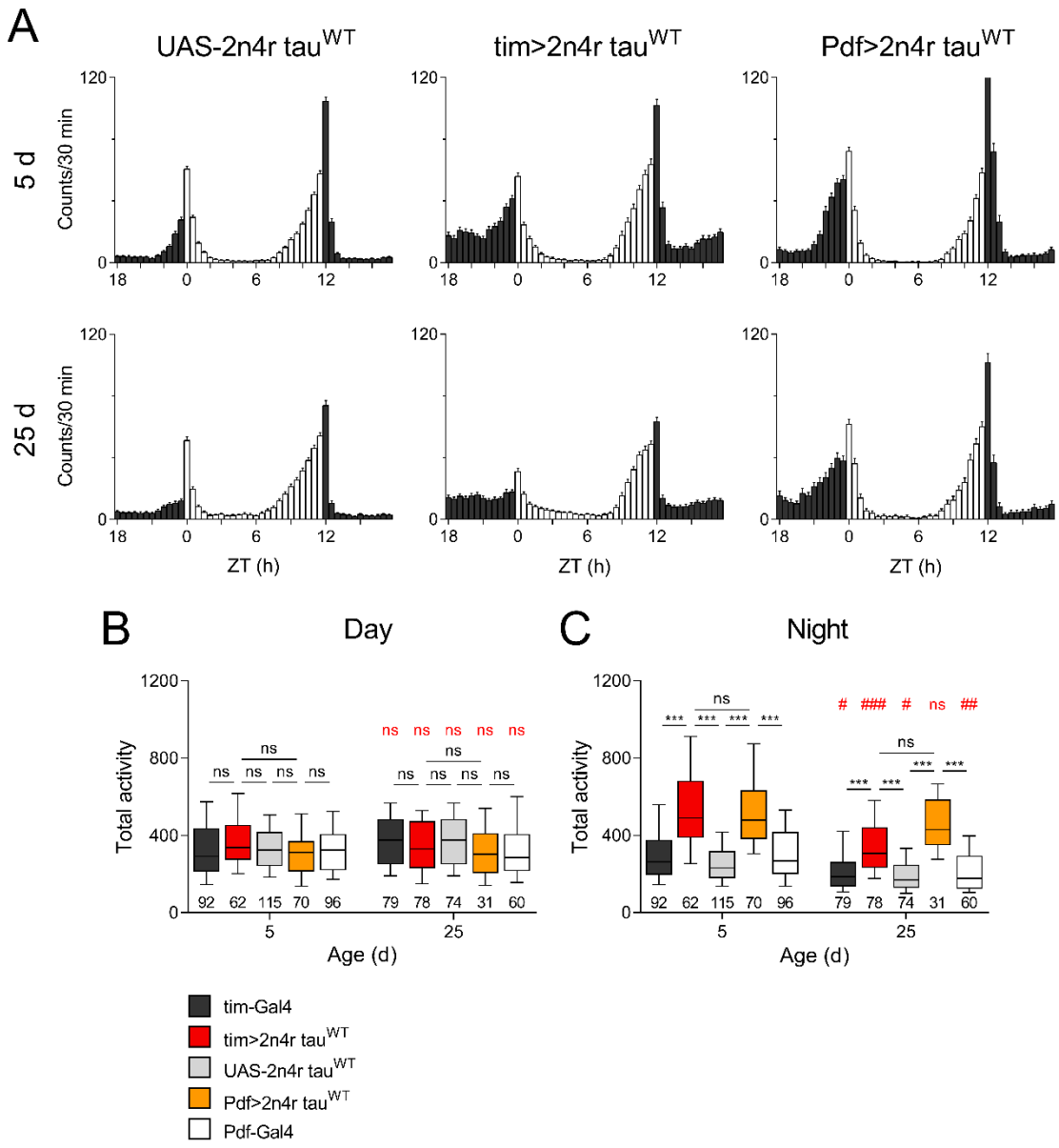


Figure 3.5: Locomotor activity of flies expressing 2n4r tau^{WT} in the clock system in LD conditions. (A) Average daily activity histograms for three days of LD conditions are shown for 5- and 25-day old UAS control (left column), tim>2n4r tau^{WT} (middle column) and Pdf>2n4r tau^{WT} (right column) flies. All genotypes exhibit bimodal activity rhythms. Elevated baseline activity in tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} flies. Loss of morning anticipation in 25-day old tim>2n4r tau^{WT} flies. Vertical bars represent activity counts displayed in 30-min bins (mean ± SEM). Lights-on (white bars). Lights-off (grey bars). (B-C) Quantification of daytime and night-time activity. Tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} expression has no effect on daytime activity, but increases night-time activity, relative to controls. Graphs show median with second and third quartiles and 10th and 90th percentiles. n = 31-115 flies per genotype from 2-6 independent experiments. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. Data were lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data.

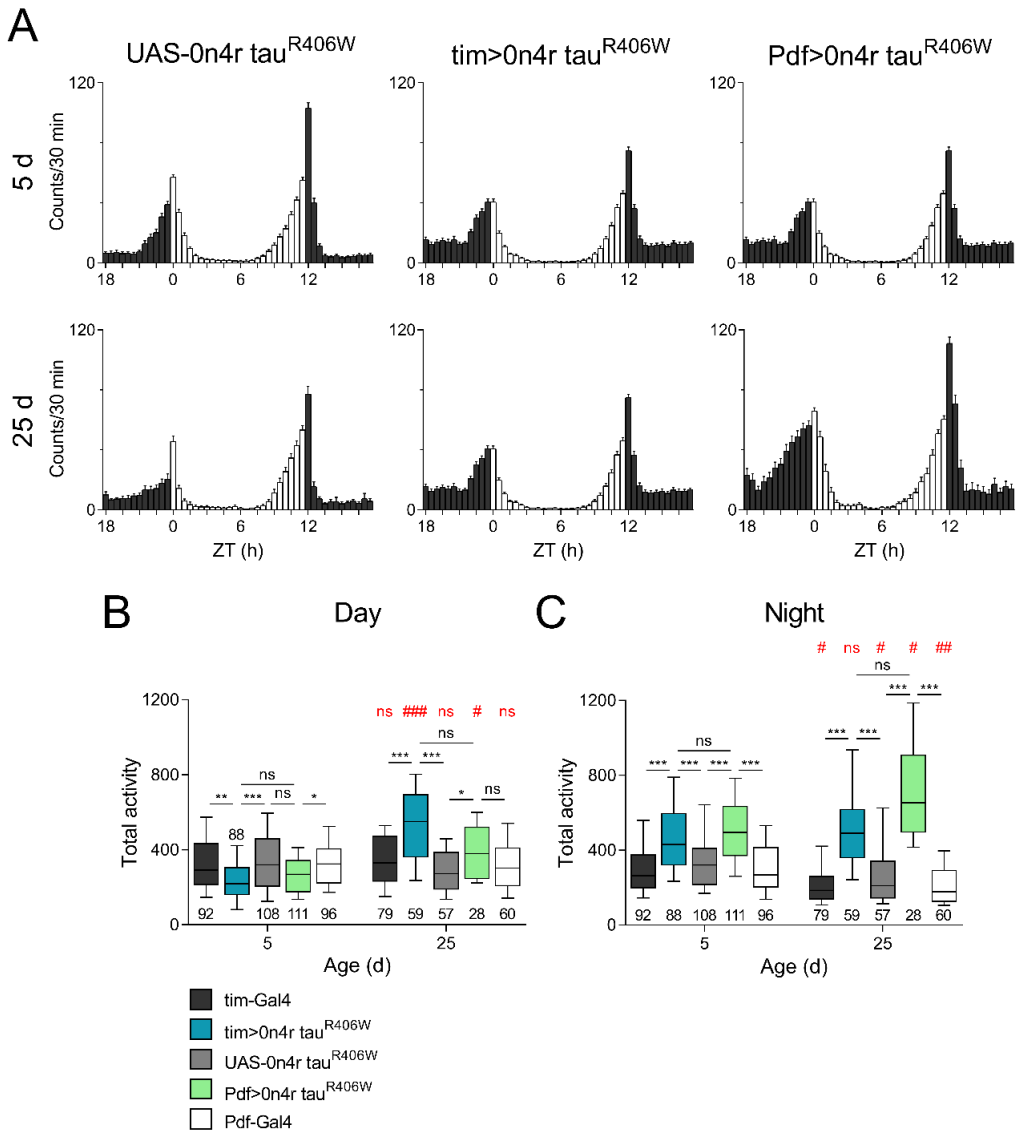


Figure 3.6: Locomotor activity of flies expressing 0n4r tau^{R406W} in the clock system in LD conditions. (A) Average daily activity histograms for three days of LD conditions are shown for 5- and 25-day old UAS control (left column), tim>0n4r tau^{R406W} (middle column) and Pdf>0n4r tau^{R406W} (right column) flies. All genotypes exhibit bimodal activity rhythms. Elevated basal activity in tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} flies. Vertical bars represent activity counts displayed in 30-min bins (mean ± SEM). Lights-on (white bars). Lights-off (grey bars). (B-C) Quantification of daytime and night-time activity. Tim>0n4r tau^{R460W} and Pdf>0n4r tau^{R460W} expression decreases daytime activity in 5-day old flies, but increases daytime activity in 25-day old flies, relative to controls (albeit differences approached but fell just short of significance in Pdf>0n4r tau^{R460W} flies). Tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R460W} expression increases night-time activity relative to controls. Graphs show median with second and third quartiles and 10th and 90th percentiles. n = 28-111 flies per genotype from 2-6 independent experiments. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. Data were lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data.

Surprisingly, Pdf>2n4r tau^{WT} expression flies robust circadian behaviour, which was in contrast to the behavioural arrhythmia produced when 2n4r tau^{WT} was expressed pan-neuronally or pan-clock. Rhythm strength in Pdf>2n4r tau^{WT} flies did not significantly differ from age-matched controls at both ages analysed (Figure 3.8A,B). We found a highly significant increase in overall rhythmicity in Pdf>2n4r tau^{WT} flies, compared to tim>2n4r tau^{WT} and Elav>2n4r tau^{WT} flies at both ages analysed (Figure 3.8B and Supplementary Figure 3.7A). It is possible that the lack of arrhythmicity is due to the weaker expression of Pdf, as compared to tim and Elav lines. However, this is unlikely as Chen et al. (2014) showed using a GFP reporter that expression levels specifically in the PDF neurons are very similar for the Elav, Pdf and tim drivers. Furthermore, Pdf>2n4r tau^{WT} expression prolonged the free-running period by 0.7-0.8 h in 5-day old flies and 0.7-0.9 h in 25-day old flies compared to controls. Period lengths in Pdf>2n4r tau^{WT} flies were less variable than in tim>2n4r tau^{WT} flies. The period-lengthening effects were similar in tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} flies (Figure 3.8C). Regardless of age, we found a substantial increase in activity levels in DD in Pdf>2n4r tau^{WT} flies compared to controls. The elevated activity levels were well maintained in Pdf>2n4r tau^{WT} flies with aging. Thus, tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} expression produced a similar increase in activity levels in young flies, but not in aged flies (Figure 3.8D). These data show that 2n4r tau^{WT} expression in PDF-positive clock neurons lengthens the free-running period and causes hyperactivity, but is insufficient to trigger behavioural arrhythmicity in DD conditions.

3.3.8 0n4r tau^{R406W} and 2n4r tau^{WT} expression in the clock system produce similar effects on circadian locomotor behaviour

Next, we tested whether we would obtain similar results if we expressed 0n4r tau^{R406W} in the clock system. Both tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} flies displayed bimodal activity rhythms with elevated basal activity in LD conditions. Morning and evening anticipation and startle responses appeared to be normal in tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} flies (Figure 3.6A). We found levels of daytime activity were reduced in 5-day old tim>0n4r tau^{R406W} flies, but increased in 25-day old tim>0n4r tau^{R406W} flies, relative to age-matched controls. We found a similar daytime activity pattern in Pdf>0n4r tau^{R406W} flies,

although the differences fell just short of significance. Both tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} flies showed an age-related increase in daytime activity (larger in tim>0n4r tau^{R406W} flies). Whereas, levels of daytime activity were stable in control flies as they aged (Figure 3.6B).

Regardless of age, levels of night-time activity were substantially increased in both tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} flies with respect to age-matched controls. We found no significant age-related differences in night-time activity in tim>0n4r tau^{R4046W} flies. Whereas, we found a significant age-related increase in night-time activity in Pdf>0n4r tau^{R460W} flies. Control flies showed a small, but significant, age-related decrease in night-time activity. Therefore, tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} expression produced a similar increase in night-time activity in 5-day old flies. Whereas, in 25-day old flies, Pdf>0n4r tau^{R460W} expression produced a stronger night-time activity phenotype than tim>0n4r tau^{R460W} expression (Figure 3.6C).

We found a mild gain in daytime sleep in 5-day old tim>0n4r tau^{R406W} flies, but a large loss of daytime sleep in 25-day old tim>0n4r tau^{R406W} flies, compared to age-matched controls (Figure 3.7E,G). Whereas, Pdf>0n4r tau^{R406W} expression had on effect on daytime sleep at both ages analysed (Figure 3.7F,G). Regardless of age, we found strong night-time sleep loss in both tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R460W} flies compared to age-matched controls. The night-time sleep loss phenotype was progressive in both tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} flies. In control flies levels of night-time sleep were stable with aging. Tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} expression resulted in similar night-time sleep loss phenotypes at both ages analysed (Figure 3.7E,F,H).

Tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} expression had differential effects on daytime activity and sleep. Tim>2n4r tau^{WT} expression had no effect on daytime activity and sleep, whereas tim>0n4r tau^{R460W} expression produced age-dependent daytime activity and sleep phenotypes (Supplementary Figure 3.5A,C). Tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} expression resulted in similar night-time activity and sleep phenotypes in 5-day old flies. As a result of aging, the night-time activity and sleep phenotypes weakened in tim>2n4r tau^{WT}

flies, whereas the night-time sleep phenotypes strengthened in tim>0n4r tau^{R406W} flies. As a result, in 25-day old flies, we found milder night-time activity and sleep phenotypes in tim>2n4r tau^{WT} flies compared to tim>0n4r tau^{R406W} flies (Supplementary Figure 3.5B,D). Regardless of age, no significant differences in daytime activity and sleep were seen between Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} flies (Supplementary Figure 3.6A,C). At both ages analysed, Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} expression predominately produced similar elevated night-time activity and night-time sleep loss phenotypes (Supplementary Figure 3.6B,D).

These results show that 0n4r tau^{R406W} expression in the clock system affects daytime activity and sleep in an age-dependent manner – it suppresses daytime activity and promotes daytime sleep in young flies, but promotes daytime activity and suppresses daytime sleep in aged flies. Whereas, 0n4r tau^{R406W} expression in the clock neurons affects night-time activity and sleep in an age-independent manner – it promotes night-time activity and suppresses night-time sleep in both young and aged flies.

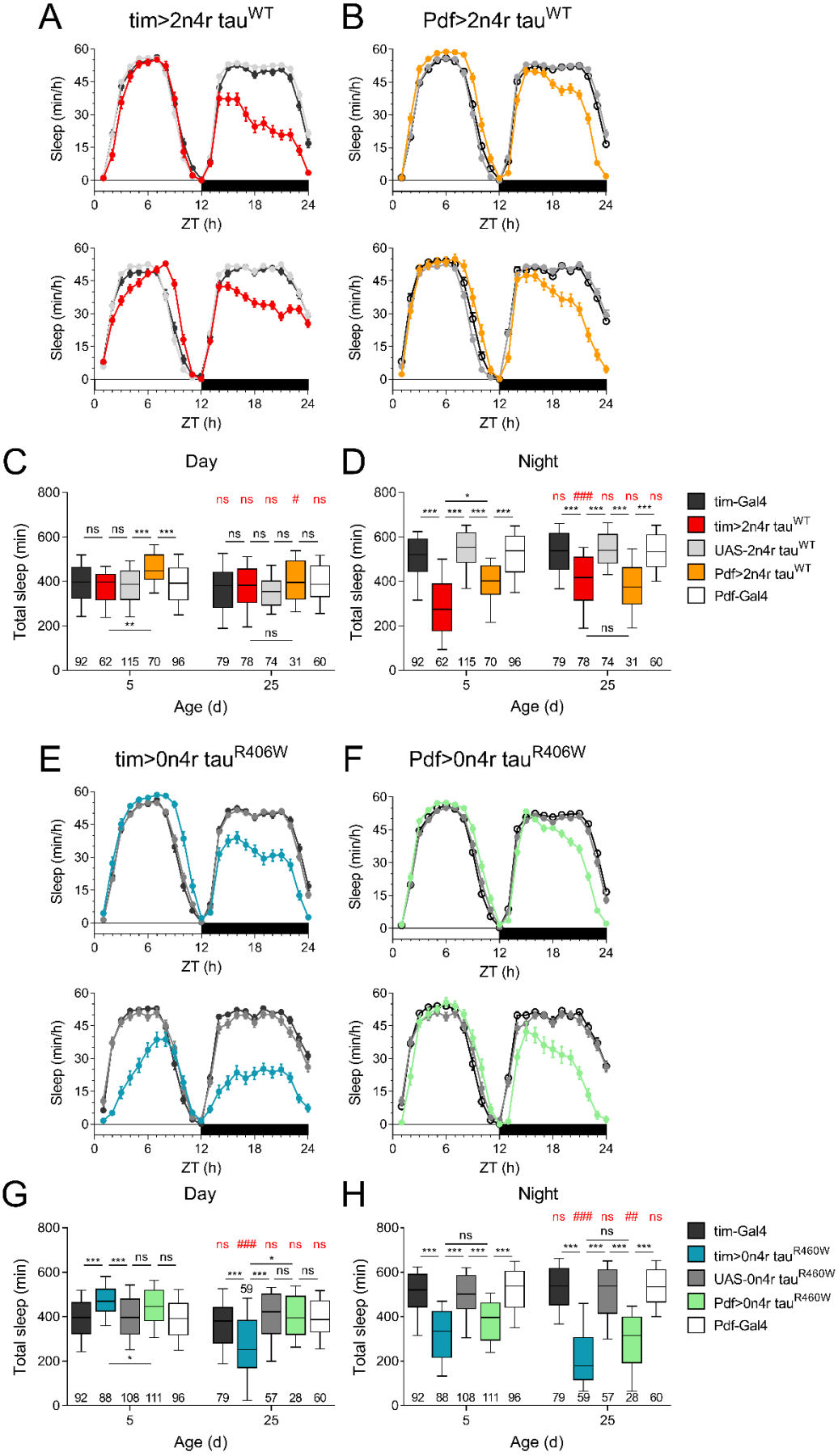

A
tim>2n4r tau^WT
B
Pdf>2n4r tau^WT
Sleep (min/h)
ZT (h)
C
Day
D
Night
Total sleep (min)
Age (d)
tim-Gal4
tim>2n4r tau^WT
UAS-2n4r tau^WT
Pdf>2n4r tau^WT
Pdf-Gal4
E
tim>0n4r tau^R406W
F
Pdf>0n4r tau^R406W
G
Day
H
Night
tim-Gal4
tim>0n4r tau^R460W
UAS-0n4r tau^R460W
Pdf>0n4r tau^R460W
Pdf-Gal4

Figure 3.7: Tau expression in the clock system promotes daytime sleep and suppresses night-time sleep. (A) Average daily sleep profile for three days of LD conditions are shown for 5- and 25-day old tim>2n4r tau^{WT} and control flies. (B) Average daily sleep profile for three days of LD conditions are shown for 5- and 25-day old Pdf>2n4r tau^{WT} and control flies. (C-D) Quantification of daytime and night-time sleep. Tim>2n4r tau^{WT} expression has no effect on daytime sleep, but decreases night-time sleep, relative to controls. Pdf>2n4r tau^{WT} expression increases daytime sleep (in 5-day old, but not in 25-day old flies), but decreases night-time sleep, relative to controls. (D) Average daily sleep profile for three days of LD conditions are shown for 5- and 25-day old tim>0n4r tau^{R406W} and control flies. (F) Average daily sleep profile for three days of LD conditions are shown for 5- and 25-day old control and Pdf>0n4r tau^{R406W} and control flies. All genotypes display a bimodal pattern, with peaks at midday and midnight and troughs at dawn and dusk. (G-H) Quantification of daytime and night-time sleep. Tim>0n4r tau^{R406W} expression increases daytime sleep in 5-day old flies, but decreases daytime sleep in 25-day old flies, relative to controls. Tim>0n4r tau^{R406W} expression decreases night-time sleep relative to controls. Pdf>0n4r tau^{R406W} expression has no effect on daytime sleep, but decreases night-time sleep, relative to controls. The horizontal bars show when the lights were on (white) or off (black). (A-D) n = 31-115 flies per genotype/age from 2-6 independent experiments. (E-H) n = 28-111 flies per genotype/age from 2-6 independent experiments. (A,B,E,F) Graphs show mean ± SEM. (C,D,G,H) Graphs show median with second and third quartiles and 10th and 90th percentiles. ns $p>0.05$; *$p<0.05$; **$p<0.001$; ***$p<0.0001$. Data were neither normally nor lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's test. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.

UAS control flies were active during the day and inactive during the night in DD conditions (Fig 3.9A, left column). Tim>0n4r tau^{R406W} flies maintained some observable rhythmicity in DD. However, relative night-time activity appeared to be elevated, so the distinction between daytime activity and night-time inactivity was less marked (Figure 3.9A, middle column). The power of both 5- and 25-day old tim>0n4r tau^{R406W} flies was substantially reduced compared with age-matched controls (Figure 3.9B). <5 % of 5- and 25-day old tim>0n4r tau^{R406W} flies were arrhythmic (Table 3.1). Between the 5- and 25-day age groups, a decline in behavioural rhythmicity was seen in all genotypes (Figure 3.9B). At both ages analysed, tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} expression produced a similar reduction in overall rhythmicity (Supplementary Figure 3.5E). However, tim>2n4r tau^{WT} flies showed an increased arrhythmic sub-population compared to tim>0n4r tau^{R4606W} flies (Table 3.1). Elav>0n4r tau^{R406W} and tim>0n4r tau^{R406W} expression resulted in a similar decline in overall rhythmicity (Supplementary Figure 3.7B).

Additionally, tim>0n4r tau^{R406W} expression prolonged the free-running period. The average period length of 5-day old tim>0n4r tau^{R406W} flies was 0.4 h longer than in control flies. Furthermore, the average period length of 25-day old tim>0n4r tau^{R406W} was 0.9-1.1 h longer than in control flies (Figure 3.9C). There was a significant age-related increase in the period length of rhythms in tim>0n4r tau^{R406W} flies, which was evident in the histograms and actograms (Figure 3.9A, middle column; rightward shift in the evening activity peak). Periods lengths in tim>0n4r tau^{R406W} flies were much more widely variable than in control flies. For example, in 25-day old tim>0n4r tau^{R406W} flies period lengths ranged from between 18.6-30.9 h, compared to 22.7-24.7 h in the Gal4 control and 23.2-24.7 h in the UAS control (Figure 3.9C and Table 3.1). We found similar period-lengthening effects in tim>2n4 tau^{WT} and tim> 0n4r tau^{R406W} flies (Supplementary Figure 3.5F). Tim>0n4r tau^{R406W} expression caused a slight increase in levels of activity in DD in 5-day old flies, and a considerable increase in levels of activity in DD in 25-day old flies, compared to age-matched controls. We found no significant age-related differences in activity levels in DD in tim>0n4r tau^{R406W} flies. Regardless of age, tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} expression produced a similar increase in activity levels in constant darkness (Fig 3.9D). In 5-day old flies, tim>0n4r tau^{R406W} expression produced a more substantial increase in activity levels in DD with respect to tim>2n4r tau^{WT} expression. Whereas, in 25-day old flies we saw the opposite (Supplementary Figure 3.5G). These data show similar to pan-clock 2n4r tau^{WT} expression, pan-clock 0n4r tau^{R406W} expression produces behavioural arrhythmicity and hyperactivity, and lengthens the free-running period in DD conditions.

We found a small reduction in power in 5-day old, but not in 25-day old, Pdf>0n4r tau^{R046W} flies compared to age-matched controls (Fig 3.9B). However, the actograms of Pdf>0n4r tau^{R406W} flies showed that the vast majority of animals maintained a pattern of daytime activity and night-time inactivity similar to control flies. So, despite the reduced power, Pdf>0n4r tau^{R406W} flies clearly maintained a high degree of rhythmicity (Fig 3.9A, right column). Furthermore, we found no age-related decline in rhythm robustness in Pdf>0n4r tau^{R406W} flies and no Pdf>0n4r tau^{R406W} flies were arrhythmic (Figure 3.9B and Table 3.1). At both ages analysed, Pdf>0n4r tau^{R406W} flies were more rhythmic than tim>0n4r tau^{R406W} and Elav>0n4r tau^{R406W} flies as evidenced by increased overall rhythmicity and a reduced

arrhythmic sub-population (Figure 3.9B, Supplementary Figure 3.7B and Table 3.1). Rhythm strength was significantly reduced in 5-day old, but not in 25-day old, Pdf>0n4r tau^{R406W} flies compared to Pdf>2n4r tau^{WT} flies of the same age (Supplementary Figure 6E).

Pdf>0n4r tau^{R460W} expression prolonged the free-running period by 0.5 h in both 5- and 25-day old flies, compared with age-matched controls. No significant age-related differences in the period length of rhythms was seen in either Pdf>0n4r tau^{R406W} or control flies. We found similar period-lengthening effects in tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R406W} flies, despite the significant age-related increase in period length in tim>0n4r tau^{R460W} flies. Compared to tim>0n4r tau^{R460W} flies, period lengths in Pdf>0n4r tau^{R460W} flies were not as widely variable (Figure 3.9C). We found similar period-lengthening effects in Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} flies (Supplementary Figure 3.6F). Regardless of age, we found a dramatic increase in activity levels in DD in Pdf>0n4r tau^{R406W} flies compared to age-matched controls. The elevated activity levels were well maintained in Pdf>0n4r tau^{R406W} flies with aging. At both ages analysed, tim>0n4r tau^{R406W} and Pdf>0n4r tau^{R460W} expression produced a similar increase in activity levels (Figure 3.9D). Regardless of age, Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} expression triggered a similar increase in levels of activity (Supplementary Figure 3.6G). Similar to Pdf>2n4r tau^{WT} expression, Pdf>0n4r tau^{R406W} expression lengthens the free-running period and causes hyperactivity, but is insufficient to produce progressive behavioural arrhythmia in DD conditions.

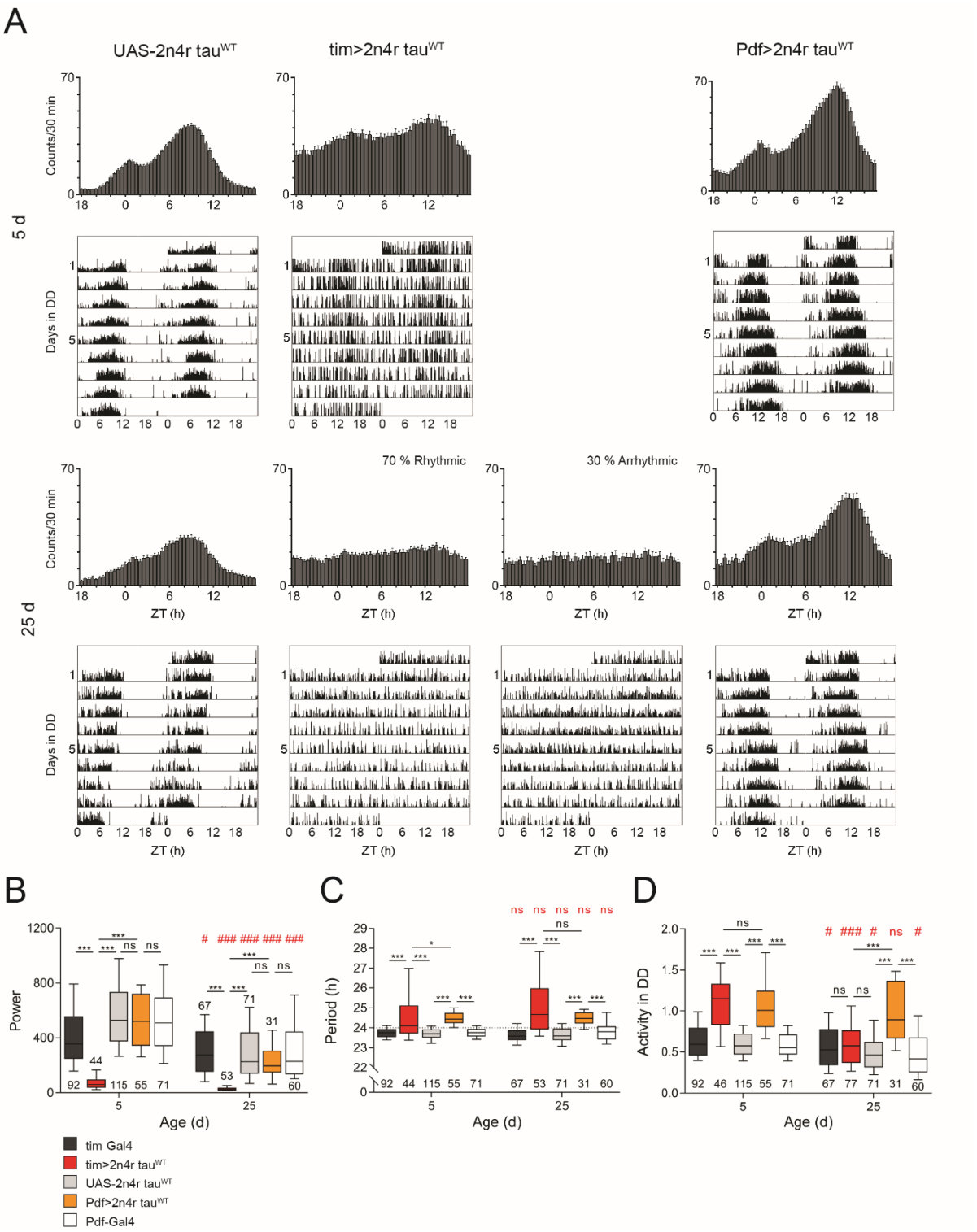

A
UAS-2n4r tau^WT
tim>2n4r tau^WT
Pdf>2n4r tau^WT
5 d
25 d
Counts/30 min
Days in DD
ZT (h)
70 % Rhythmic
30 % Arrhythmic
B
C
D
Power
Period (h)
Activity in DD
Age (d)
tim-Gal4
tim>2n4r tau^WT
UAS-2n4r tau^WT
Pdf>2n4r tau^WT
Pdf-Gal4

Figure 3.8: 2n4r tau^{WT} expression in the clock system disrupts normal circadian locomotor rhythms. (A) Average daily activity histograms and representative double-plotted actograms for nine days of DD conditions are shown for 5- and 25-day old UAS control (left column), tim>2n4r tau^{WT} (middle column) and Pdf>2n4r tau^{WT} (right column) flies. Flies were entrained in standard LD cycles for three days and then monitored in DD for nine days. UAS control flies exhibit robust rhythms of daytime activity and night-time inactivity, with a period of just under 24 h. Tim>2n4r tau^{WT} flies exhibit either very weak rhythms with a long-period (>24 h) or are arrhythmic. Pdf>2n4r tau^{WT} flies exhibit robust rhythms with a long-period (>24 h). Vertical bars represent activity counts displayed in 30-min bins (mean ± SEM). (B) Tim>2n4r tau^{WT} expression severely reduces power relative to controls. Pdf>2n4r tau^{WT} expression has no effect on power relative to controls. See Table 3.1 for the percentage of rhythmic flies. (C) Tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} expression prolongs the free-running period. (D) Tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT} expression increases activity levels in DD relative to controls. Graphs show second and third quartiles and 10th and 90th percentiles. n = 31-115 flies per genotype/age from 2-6 independent experiments. ns $p>0.05$; *$p<0.05$; ***$p<0.0001$. (B,D) Data were lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C) Data were neither normally nor lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.

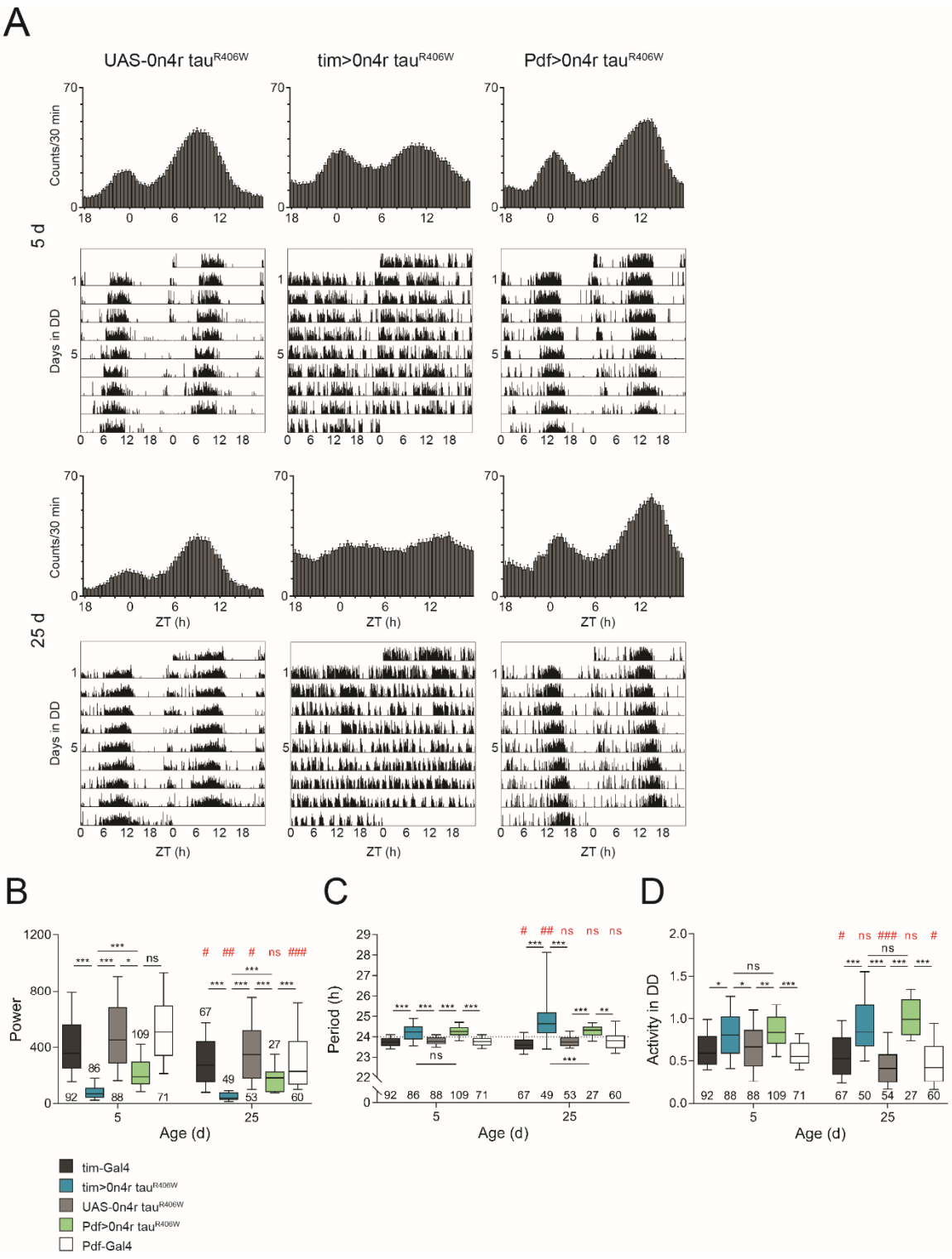

A
UAS-0n4r tauR406W
tim>0n4r tauR406W
Pdf>0n4r tauR406W
5 d
25 d
Counts/30 min
Days in DD
ZT (h)
B
C
D
Power
Period (h)
Activity in DD
Age (d)
tim-Gal4
tim>0n4r tauR406W
UAS-0n4r tauR406W
Pdf>0n4r tauR406W
Pdf-Gal4

Figure 3.9: 0n4r tau^{R406W} expression in the clock system disrupts normal circadian locomotor rhythms. (A) Average daily activity histograms and representative double-plotted actograms for nine days of DD conditions are shown for UAS control (left column), tim>0n4r tau^{R406W} (middle column) and Pdf>0n4r tau^{R406W} (right column) flies. Flies were entrained in standard LD cycles for three days and then released in DD for nine days. UAS control flies show robust rhythms of daytime activity and night-time inactivity, with a period of nearly 24 h. tim>0n4r tau^{R406W} flies exhibit either very weak rhythms with a long period (>24 h) or are arrhythmic. Pdf>0n4r tau^{R406W} flies exhibit robust rhythms with a long period (>24 h). Vertical bars represent activity counts displayed in 30-min bins (mean ± SEM). (B) Severe reduction in power is found in 5 and 25-day old tim>0n4r tau^{R406W} flies relative to controls. Moderate reduction in power is found in 5-day old, but in not 25-day old, Pdf>0n4r tau^{R406W} flies relative to controls. See Table 3.1 for the percentage of rhythmic flies. (C) Prolonged free-running period is found in tim>0n4r tau^{R460W} and Pdf>0n4r tau^{R406W} flies relative to controls. (D) Increased activity levels in DD are found in tim>0n4r tau^{R460W} and Pdf>0n4rtau^{R460W} flies relative to controls. Graphs show second and third quartiles and 10th and 90th percentiles. n = 27-109 flies per genotype/age from 2-6 independent experiments. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. (B,D) Data were lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C) Data were neither normally nor lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.

3.3.9 Highly toxic 2n4r tau^{S11A} expression in the clock system induces behavioural arrhythmia in DD conditions

Next, we tested whether pan-clock expression of the highly toxic phosphorylation-resistant 2n4r tau^{S11A} construct is associated with a more severe phenotype than pan-clock 2n4r tau^{WT} expression. We found a highly significant reduction in power in tim>2n4r tau^{S11A} flies compared to age-matched controls at both ages analysed. At both ages analysed, overall rhythmicity did not significantly differ between tim>2n4r tau^{S11A} flies and tim>2n4r tau^{WT} flies (Supplementary Figure 3.4A,B). However, an increased arrhythmic sub-population was found in tim>2n4r tau^{S11A} flies compared to tim>2n4r tau^{WT} flies. At 5-days of age, 80 % of tim>2n4r tau^{S11A} flies were rhythmic, compared to 95 % of tim>2n4r tau^{WT} flies. By 25-days of age, only 60 % of tim>2n4r tau^{S11A} flies remained rhythmic, compared to 70 % of tim>2n4r tau^{WT} flies (Supplementary Figure 3.4A and Table 3.1). Tim> 2n4r tau^{S11A}

expression prolonged the free-running period in an age-dependent manner. The period length of rhythms was normal in 5-day old tim>2n4r tau^{S11A} flies. Whereas, the average period length of 25-day old tim>2n4r tau^{S11A} flies was 1.0 h longer than in age-matched controls. Period lengths in 25-day old tim>2n4r tau^{S11A} flies varied greatly, they ranged from between 18.6-32.6 h, compared to 22.7-24.7 h in the Gal4 control and 23.2-24.2 h in the UAS control. By 25-days of age, the period-lengthening effects were similar in tim>2n4r tau^{S11A} and tim>2n4r tau^{WT} flies (Supplementary Figure 3.4C). Tim>2n4r tau^{S11A} expression had no significant effect on activity levels in DD irrespective of age (Supplementary Figure 3.4D)

3.3.10 Tau expression does not cause structural degeneration of the central pacemaker neurons

Next, we wished to investigate whether structural damage to the central pacemaker coincides with the behavioural abnormalities. We used a mCD8::GFP transgene to directly visualise the morphology of the PDF-positive clock neurons (sLNvs and lLNvs), which are the master pacemakers. We observed no obvious loss of sLNvs and lLNvs neurons in either Pdf>tau or tim>tau flies up to 25-days of age (Figure 3.10B,C). The PDF neurons have dense arborisations in the optic lobes (Helfrich-Forster and Homberg, 1993). The density of the arborisation pattern appeared to be similar in Pdf>2n4r tau^{WT} and Gal4 control flies (Figure 3.10B).

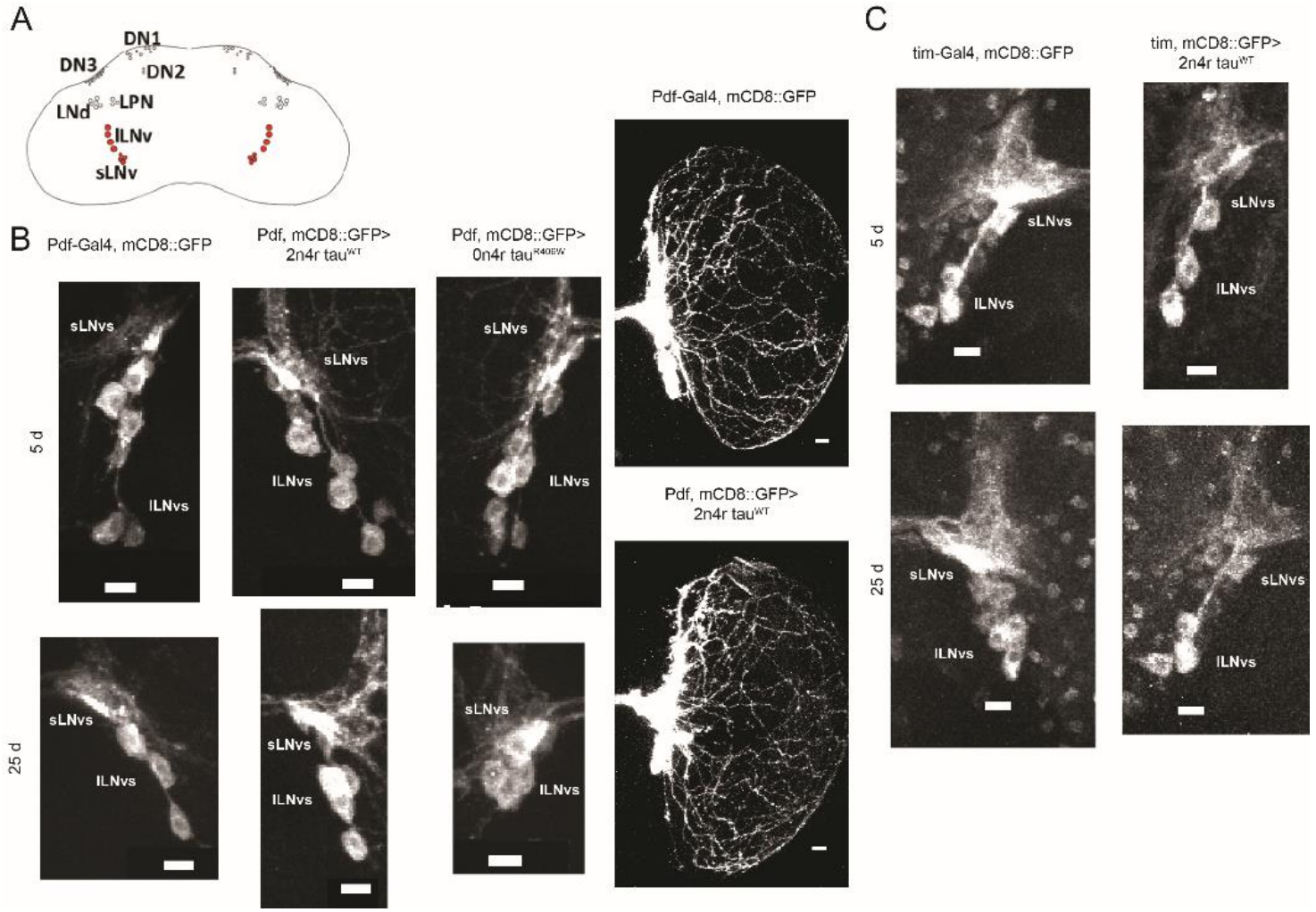


Figure 3.10: Tau expression in the clock system results in no loss of PDF neurons. (A) Graphic showing the ~150 clock neurons in the *Drosophila* brain subdivided into seven groups. Red circles indicate the PDF-positive clock neurons (sLNvs and lLNvs), which are the master pacemakers. (B) Image stacks of sLNvs and lLNvs neurons for 5- and 25-day old control, Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} flies. sLNvs and lLNvs neurons appear morphologically normal in Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} flies compared to controls. (C) Image stacks of sLNvs and lLNvs neurons for 5- and 25-day old control and tim>2n4r tau^{WT} flies. sLNvs and lLNvs neurons appear morphologically normal in tim>2n4r tau^{WT} flies compared to controls. Scale bars: 10µ

3.3.11 Elevated night-time activity and night-time sleep loss in LD in tau-expressing flies is dependent upon tau expression in the master pacemaker neurons

We have shown that tau expression in all neurons, and exclusively in PDF neurons, causes a similar increase in night-time activity and loss of night-time sleep (Supplementary Figure 3.8B,D). Therefore, we sought to investigate whether the night-time activity and sleep phenotypes in tau-expressing flies are related to the effects of tau expression in the PDF neurons. To achieve this, we used the pan-neuronal Elav-Gal4 driver combined with Pdf-Gal80 to block Gal4 activity exclusively in the PDF neurons (Stoleru et al., 2004). We found levels of both daytime and night-time activity in 5-day old Elav, Pdf-Gal80>0n4r tau^{R406W} flies did not significantly differ from age-matched controls. Levels of night-time activity were significantly lower in Elav, Pdf-Gal80>0n4r tau^{R460W} compared to Elav>0n4r tau^{R460W} flies (Figure 3.11B). No significant differences in levels of daytime and night-time sleep were seen in 5-day old Elav, Pdf-Gal80>tau^{R406W} flies relative to age-matched controls. Levels of night-time sleep were significantly higher in Elav, Pdf-Gal80>0n4r tau^{R460W} flies compared to Elav>0n4r tau^{R460W} flies (Figure 3.11C). These results show blocking tau expression exclusively in the PDF neurons rescues the night-time activity and sleep phenotypes in tau-expressing flies. These results suggest tau acts in the master pacemaker neurons to cause elevated night-time activity and night-time sleep loss in LD conditions.

3.3 12 Behavioural arrhythmia in DD in tau-expressing flies is not dependent upon tau expression in the master pacemaker neurons

We have previously shown that tau expression exclusively in the PDF neurons fails to cause progressive behavioural arrhythmia (Figure 3.8 and Figure 3.9). Therefore, we were interested to know whether tau expression in all neurons apart from the PDF neurons was sufficient to produce progressive behavioural arrhythmia in DD conditions. In 5-day old Elav, Pdf-Gal80>tau^{R406W} flies, relative night-time activity seemed to be elevated, so there was less distinction between daytime activity and night-time inactivity (Figure 3.11D). We found that 5-day old Elav, Pdf-Gal80>0n4r tau^{R406W} flies showed a substantial reduction in power compared with controls of the same age (Figure 3.11E). The period length of rhythms was normal in 5-day old Elav, Pdf-Gal80>0n4r tau^{R406W} flies (Figure 3.11F). We found no

significant differences in levels of activity in DD in 5-day old Elav, Pdf-Gal80>0n4r tau^{R406W} flies with respect to age-matched controls (Figure 3.11G). In DD conditions, Elav, Pdf-Gal80>0n4r tau^{R406W} flies were essentially identical to Elav>0n4r tau^{R406W} flies of the same age – we found no significant differences in overall rhythmicity, period length and activity levels (Figure 3.11E,F,G). These results show that suppression of tau expression exclusively in the PDF neurons is insufficient to rescue tau-evoked behavioural arrhythmia in DD conditions. These data further highlight the contribution of neurons downstream of the master pacemaker in tau-mediated circadian behavioural arrhythmicity.

3.3.13 Tau expression in tim-positive glial cells plays a role in tau-evoked circadian behavioural arrhythmia in DD conditions

We have already established that tim>2n4r tau^{WT} flies are less rhythmic than Elav>2n4r tau^{WT} flies as shown by reduced overall rhythmicity and an increased arrhythmic sub-population (Supplementary Figure 7A and Table 3.1). Elav-Gal4 labels all neurons but not glia, whereas tim-Gal4 labels PDF neurons, other nearby clock neurons and glia. Therefore, we were interested to know whether tau expression in glia contributes to the more severe phenotype in tim>2n4r tau^{WT} flies. Colodner and Feany (2010) showed that wild-type tau expression in glia causes aggregate formation and cell death of neurons and glia. We used a repo-Gal80 transgene to block tau expression in the glial subset of tim-positive cells (Awasaki et al., 2008). At both ages analysed, tim, repo-Gal80>2n4r tau^{WT} flies showed a substantial reduction in power compared with age-matched controls. We found a similar reduction in overall rhythmicity in 5-day old tim, repo-Gal80>2n4r tau^{WT} and tim>2n4r tau^{WT} flies. However, tim, repo-Gal80>2n4r tau^{WT} flies exhibited a less dramatic age-related decline in rhythmicity compared to tim>2n4r tau^{WT} flies. Therefore, we found a significantly smaller reduction in overall rhythmicity in 25-day old tim, repo-Gal80>2n4r tau^{WT} flies compared to age-matched tim>2n4r tau^{WT} flies (Figure 3.12A,B). Additionally, in 25-day old flies, we found a reduced arrhythmic sub-population in tim, repo-Gal80>2n4r tau^{WT} flies compared to tim>2n4r tau^{WT} flies (Table 3.1). These data suggest that 2n4r tau^{WT} expression in the glial subset of tim-positive cells may contribute to the progressive behavioural arrhythmicity in tim>2n4r tau^{WT} flies.

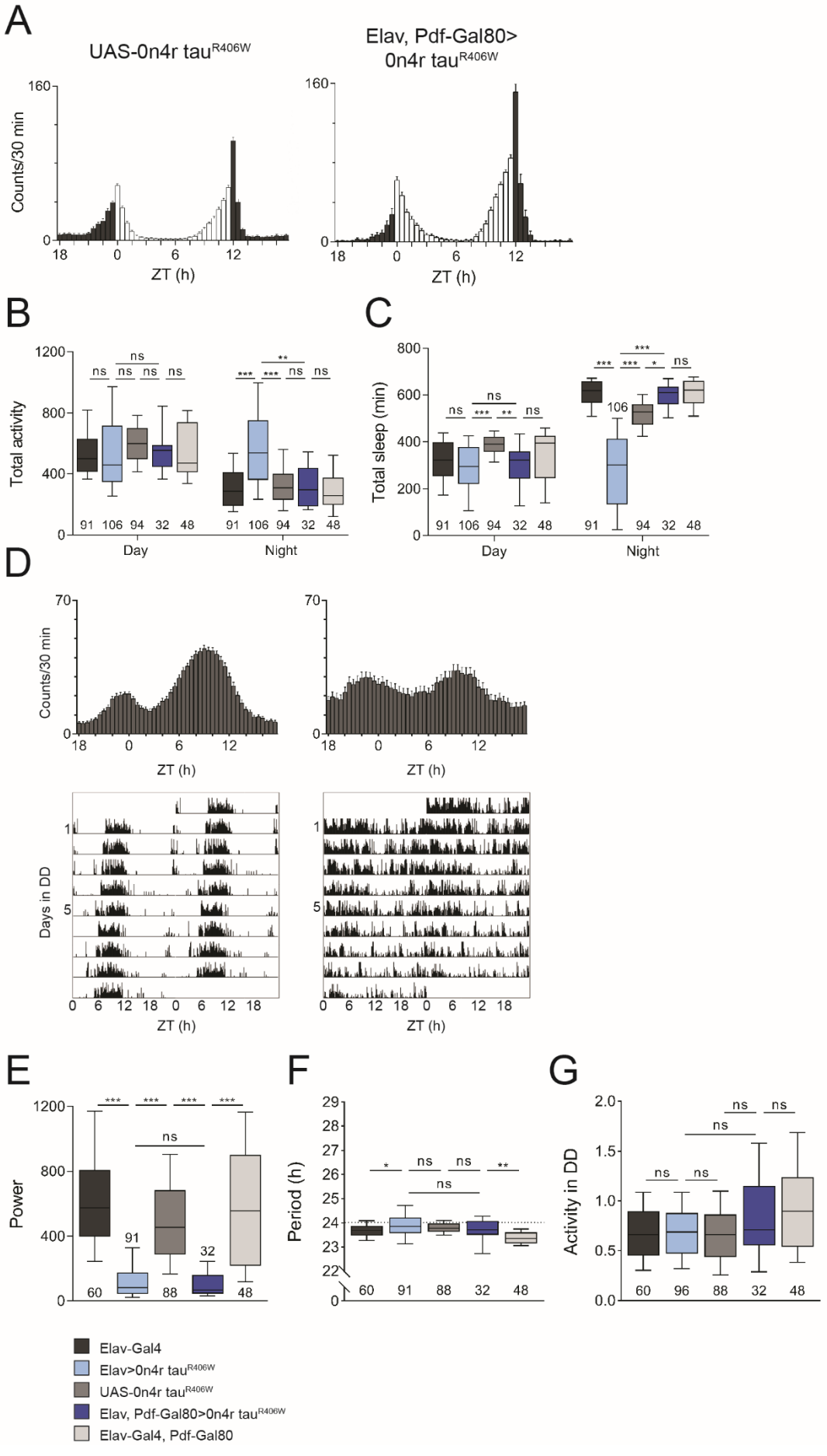

A
UAS-0n4r tauR406W
Elav, Pdf-Gal80>
0n4r tauR406W
Counts/30 min
ZT (h)
B
Total activity
Day
Night
C
Total sleep (min)
D
Days in DD
E
Power
F
Period (h)
G
Activity in DD
Elav-Gal4
Elav>0n4r tauR406W
UAS-0n4r tauR406W
Elav, Pdf-Gal80>0n4r tauR406W
Elav-Gal4, Pdf-Gal80

Figure 3.11: Circadian locomotor behaviour in Elav, Pdf-Gal80>0n4r tau^{R406W} flies in LD and DD conditions. (A) Average daily activity histograms for three days of LD conditions are shown for 5-day old UAS control and Elav, Pdf-Gal80>0n4r tau^{R406W} flies. Both genotypes exhibit bimodal activity rhythms. Vertical bars represent activity counts displayed in 30-min bins (mean ± SEM). (B) Quantification of daytime and night-time activity for Elav>0n4r tau^{R460W}, Elav, Pdf-Gal80>0n4r tau^{R406W} and control flies. (C) Quantification of daytime and night-time sleep for Elav>0n4r tau^{R460W}, Elav, Pdf-Gal80>0n4r tau^{R406W} flies and control flies. n = 32-106 flies per genotype from 2-6 independent experiments. (D) Average daily activity histograms and representative double-plotted actograms for nine days of DD conditions are shown for 5-day old UAS control and Elav, Pdf-Gal80>0n4r tau^{R406W} flies. UAS control flies exhibit robust rhythms of daytime activity and night-time inactivity, with a period of nearly 24 h. Elav, Pdf-Gal80>0n4r tau^{R406W} flies exhibit weak rhythms with a normal period. (E) Power is severely reduced in Elav>0n4r tau^{R460W} and Elav, Pdf-Gal80>0n4r tau^{R406W} flies relative to controls. (F) No differences in period length of rhythms are found in Elav>0n4r tau^{R460W} and Elav, Pdf-Gal80>0n4r tau^{R406W} flies relative to controls. (G) No differences in activity levels in DD are found in Elav>0n4r tau^{R460W} and Elav, Pdf-Gal80>0n4r tau^{R406W} flies relative to controls. n = 32-96 flies per genotype from 2-6 independent experiments. Box plots show median with second and third quartiles and 10th and 90th percentiles. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. (B,E,G) Data were lognormally distributed. Multiple comparisons between different genotypes by 1-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C,F) Data were neither normally nor lognormally distributed. Multiple comparisons between different genotypes by Kruskal-Wallis ANOVA and post-hoc Dunn's tests.

The average circadian period of tim, repo-Gal80>2n4r tau^{WT} flies was 0.4 h longer in 5-day old flies and 0.7-0.8 h longer in 25-day old flies than in age-matched controls. The period-lengthening effects were similar in tim, repo-Gal80>2n4r tau^{WT} and tim>2n4r tau^{WT} flies (Figure 3.12C). Regardless of age, activity levels in DD in tim, repo-Gal80>2n4r tau^{WT} flies did not significantly differ from controls of the same age (Figure 3.12D). Thus, suppressing 2n4r tau^{WT} expression in tim-positive glial cells rescues the hyperactivity evoked by pan-clock 2n4r tau^{WT} expression. This is surprising because 2n4r tau^{WT} expression exclusively in PDF neurons is sufficient to cause hyperactivity (Figure 3.8D). However, it is possible that the repo-Gal80 transgene also affects Gal4 activity in clock neurons, which may contribute to the milder phenotype in tim, repo-Gal80>2n4r tau^{WT} flies compared to tim>2n4r tau^{WT} flies.

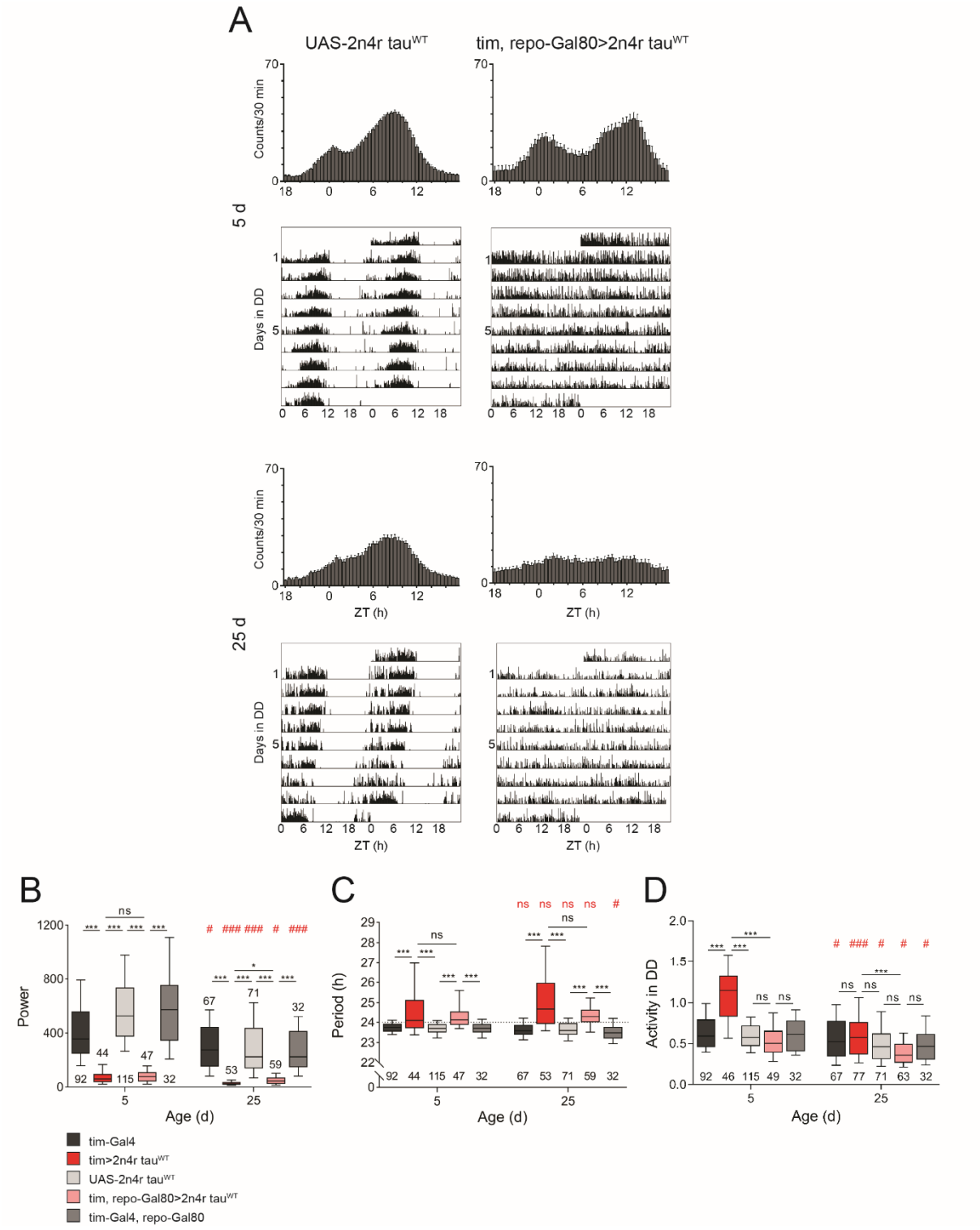

A
UAS-2n4r tau^WT
tim, repo-Gal80>2n4r tau^WT
Counts/30 min
5 d
Days in DD
ZT (h)
25 d
B
Power
Age (d)
C
Period (h)
Age (d)
D
Activity in DD
Age (d)
tim-Gal4
tim>2n4r tau^WT
UAS-2n4r tau^WT
tim, repo-Gal80>2n4r tau^WT
tim-Gal4, repo-Gal80

Figure 3.12: Circadian locomotor behaviour in tim, repo-Gal80>2n4r tau^{WT} flies in DD conditions. (A) Average daily activity histograms and representative double-plotted actograms for nine days of DD are shown for 5- and 25-day old UAS control (left column) and tim, repo-Gal80>2n4r tau^{WT} (right column) flies. Flies were entrained in standard LD cycles for three days and then monitored in DD for nine days. UAS control flies exhibit robust rhythms of daytime activity and night-time inactivity, with a period of just under 24 h. tim, repo-Gal80>2n4r tau^{WT} flies exhibit weak rhythms with a long period (>24 h). (B) Substantial reduction in power is found in tim>2n4r tau^{WT} and tim, repo-Gal80>2n4r tau^{WT} flies relative to controls. See Table 3.1 for the percentage of rhythmic flies. (C) Lengthened free-running period is found in tim>2n4r tau^{WT} and tim, repo-Gal80>2n4r tau^{WT} flies relative to controls. (D) Large increase in activity levels is found in 5-day old tim>2n4r tau^{WT} flies relative to controls. No differences in activity levels are found in tim, repo-Gal80>2n4r tau^{WT} flies and 25-day old tim>2nr tau^{WT} flies relative to controls. Graphs show second and third quartiles and 10th and 90th percentiles. n = 32-115 flies per genotype/age from 2-6 independent experiments. ns $p>0.05$; **$p<0.001$; ***$p<0.0001$. (B,D) Data were lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C) Data were neither normally nor lognormally distributed. Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.

3.3.14 Tau expression in adult clock neurons is sufficient to disrupt circadian behaviour in DD conditions

Finally, we sought to investigate whether expressing 2n4r tau^{WT} in all clock cells during adulthood (using the TARGET system; McGuire et al., 2003) is sufficient to disrupt locomotor activity rhythms. Tim, tub-Gal80^{ts}>2n4r tau^{WT} flies were raised and maintained at 20 °C (where Gal80^{ts} is active and blocks tau expression). Activity rhythms of 5-day old tim, tub-Gal80^{ts}>2n4r tau^{WT} flies were first measured in DD for six days at 20 °C (following three days in LD) and then at 30 °C (where Gal80^{ts} becomes inactivated and permits tau expression) for six days. At 20 °C, tim, tub-Gal80^{ts}>2n4r tau^{WT} flies showed robust behaviour, consistent with no tau expression. After shifting the flies to 30 °C, their overall rhythmicity substantially reduced and their average circadian period lengthened by 1 h compared with controls. Intriguingly, we found shifting control flies to 30 °C signifcantly increased their overall rhythmicity and shortened their free-runing period. On the other hand, shifting tim, tub-Gal80^{ts}>2n4r tau^{WT} flies to 30 °C had no effect on their overall rhythmcity, but signifcantly lenghtend their free-running period (Figure 3.13). These results show that short-term 2n4r

tau^{WT} expression in adult clock neurons is sufficient to disrupt normal circadian locomotor activity rhythms.

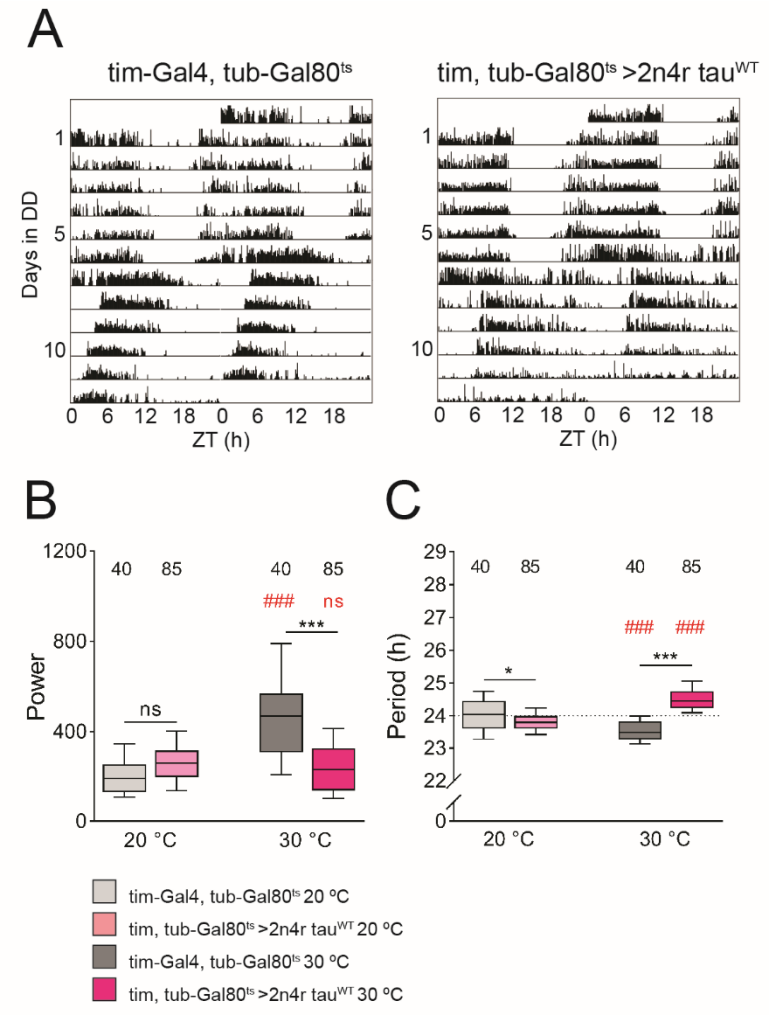


Figure 3.13: Behavioural arrhythmia can be induced by 2n4r tau^{WT} expression in the adult clock system. (A) Representative double-plotted actograms are shown for tim-Gal4, tub-Gal80^{ts} (left) and tim, tub-Gal80^{ts}>2n4r tau^{WT} (right) flies. Flies were maintained at 20 °C (permissive temperature) until after eclosion and for three days in LD (not shown) and the first six days in DD. The temperature was then increased to 30 °C (restrictive temperature) for six days. (B) 2n4r tau^{WT} expression in adult clock neurons reduces rhythmicity. (C) 2n4r tau^{WT} expression in adult clock neurons prolongs the average circadian period. Graphs show second and third quartiles and 10th and 90th percentiles. n = 40-85 flies per genotype/age from 2 independent experiments. ns p>0.05; *p<0.05; ***p<0.0001; two-tailed Student's t-tests with log-transformed data for (B); Mann-Whitney U-tests for (C). Number symbols (in red) show differences between the same genotype at 20 °C and 30 °C.

3.4 Discussion

3.4.1 Pan-neuronal tau expression affects activity and sleep in LD

Circadian rhythm and sleep-wake cycle disturbances are common in AD. However, it is not clear whether tau abnormalities are sufficient to cause them. To answer this question, we have looked at the effect on circadian behaviour of pan-neuronally expressing human tau in the fly brain. We found that regardless of age, pan-neuronal full-length human tau (wild-type or R406W) expression (using Elav-Gal4) had an effect on circadian locomotor behaviour in both LD and DD conditions (Figure 3.1, Figure 3.2 and Figure 3.3). In LD conditions, both tau-expressing and control flies showed strong bimodal activity rhythms, with elevated basal activity in tau-expressing flies (Figure 3.1A). Pan-neuronal tau expression produced a substantial increase in activity levels during the night (the inactive phase) (Figure 3.1B). Similar findings reported in Tg4510 mice (express human 0n4r tau^{P301L} under the control of the forebrain-specific CaMKII promoter), which show a substantial increase in daytime activity (the inactive phase as mice are nocturnal) (Stevanovic et al., 2017). Thus, both *Drosophila* and mouse models of tauopathy recapitulate the elevated activity during the inactive phase and shift towards a higher percentage of total activity occurring during the inactive phase found in individuals with AD (Satlin et al., 1995; Volicer et al., 2001; Harper et al., 2004).

Although detailed analysis of sleep was beyond the scope of this investigation, we showed tau-expressing flies slept less – we saw a small daytime sleep loss and a large night-time sleep loss (Figure 3.2). The night-time activity and sleep phenotypes in tau-expressing flies were neither age-dependent nor progressive (Figure 3.1 and Figure 3.2). Currently, sleep has not been examined in 3xTg-AD mice (express human APP, PS1 and tau) or in transgenic tau mice. The reduction in sleep in AD patients and in AD animal models may contribute to the observed memory deficits, because many studies have shown the important role of sleep in memory consolidation (for review see Dickelmann and Born, 2010). Several studies have shown sleep loss in *Drosophila* and mouse models of β-amyloidosis (Zhang et al., 2005; Roh et al., 2012; Gerstner et al., 2017). Thus in AD, where both tauopathy and β-amyloidosis are present, they may be an additive effect in disrupting sleep. Gerstner et al. (2017) have

shown that pan-neuronal human Aβ expression in *Drosophila* causes dramatic daytime and night-time sleep loss. Thus, pan-neuronal expression of neither human tau nor Aβ in *Drosophila* recapitulates the increased propensity to sleep during the day reported in AD patients (Bonanni et al., 2005; Musiek et al., 2015).

3.4.2 Pan-neuronal tau expression causes behavioural arrhythmia in DD

Pan-neuronal 2n4r tau^{WT} and 0n4r tau^{R406W} expression caused similar progressive behavioural arrhythmia, as evidenced by reduced overall rhythmicity and an increased arrhythmic subpopulation in DD conditions (Figure 3.3A,B and Table 3.1). In contrast, we found that pan-neuronal 0n4r tau^{WT} expression failed to induce behavioural arrhythmicity (Supplementary Figure 3.2A,B). This is surprising because 2n4r tau^{WT}, 0n4r tau^{R406W} and 0n4r tau^{WT} have all been shown to cause neurodegeneration when expressed ectopically in the developing *Drosophila* brain and visual system (Wittmann et al., 2001; Jackson et al., 2002; Grammenoudi et al., 2008; Kosmidis et al., 2010). Because the changes in circadian behaviour (in both LD and DD conditions) are already present in young flies (~5-days old), they precede the onset of other traditionally established AD phenotypic markers, including neurodegeneration. As previous work using the same model has established neurodegeneration first appears in 10-day old flies, with a progressive increase in 25- and 50-day old flies (Wittmann et al., 2001). In DD conditions, Tg4510 mice show disrupted activity rhythms (Stevanovic et al., 2017). Thus, both our *Drosophila* model and Tg4510 mice recapitulate the dampening of circadian behaviour found in AD patients (Volicer et al., 2001; Coogan et al., 2013). However, PLB2$_{Tau}$ mice (express human 2n4r tau^{P301L} and 2n4r tau^{R460W} under the control of the forebrain-specific CaMKII promoter) show normal activity rhythms, despite abnormalities in sleep EEGs (Koss et al., 2016). We have shown that pan-neuronal expression of 2n4r tau^{WT} and 0n4r tau^{R406W} moderately shortens lifespan (Supplementary Figure 3.1). This is consistent with previous reports (Wittmann et al., 2001; Gorsky et al., 2016).

Pan-neuronal wild-type tau expression in *Drosophila* has been previously associated with locomotor activity deficits (Ali et al., 2011). In addition, motor deficits are a frequent occurrence in mouse models of tauopathy. However, these are mostly rescued by restricting

tau expression to the forebrain (under the control of the CaMKII promotor) (Koss et al., 2016). We found normal levels of activity in Elav>0n4r tau^{R406W} flies up to 35-days of age (Figure 3.3D). This indicates that the behavioural arrhythmicity is not an artifact of reduced activity levels. Interestingly, we found age-induced hyperactivity in Elav>2n4r tau^{WT} flies (activity levels were normal in young flies, but elevated in aged flies) (Figure 3.3D). Therefore, Elav>2n4r tau^{WT} expression reverses the age-related decline in levels of activity normally observed in *Drosophila.* Furthermore, we found age-independent hyperactivity in Elav>0n4r tau^{WT} flies (Supplementary Figure 3.2D). Thus, tau expression can affect the overall amount of activity independently of the distribution of activity; for example, Elav>0n4r^{R406W} flies are arrhythmic but have normal activity levels, whereas Elav>0n4r tau^{WT} flies are rhythmic but are hyperactive. These results show isoform-specific effects of tau to produce behavioural arrhythmicity and hyperactivity when expressed pan-neuronally. However, AD patients do not usually differ from healthy controls concerning overall activity levels (van Someren et al., 1996; Harper et al., 2004). Although AD patients often exhibit other behavioural symptoms including restlessness, wandering, agitation and pacing, and perhaps these manifest as hyperactivity in our *Drosophila* model. To the best of our knowledge, this is the first study to show that human tau expression makes flies hyperactive in constant conditions.

3.4.3 Adult-onset pan-neuronal tau expression is insufficient to trigger circadian abnormalities

We next investigated whether adulthood restricted ubiquitous neuronal tau expression (using Elav-GS) is sufficient to cause circadian behavioural deficits. We found that neither 2n4r tau^{WT} nor highly toxic phosphorylation-resistant 2n4r tau^{S11A} expression had any effect on circadian locomotor behaviour in either LD or DD conditions (Figure 3.4 and Supplementary Figure 3.3). The lack of a phenotype in ElavGS>2n4r tau^{WT} and ElavGS>2n4r tau^{S11A} flies is likely due to insufficient tau expression levels, it is possible that increasing the concentration of RU486, longer exposure to RU486 or introducing additional copies of Gal4/UAS might induce a phenotype. On the other hand, Tabuchi et al. (2015) showed that adulthood restricted pan-neuronal Aβarctic expression (via Elav-GS) reduces and fragments daytime and night-time sleep. We found that under similar circumstances both 2n4r tau^{WT}

and 2n4r tau^{S11A} were insufficiently potent to produce a sleep loss phenotype (Supplementary Figure 3.3). However, this discrepancy could be related to differences in the genetic background or experimental protocol. During the course of this work, Dissel et al. (2017) showed that adulthood restricted ubiquitous tau expression (using Daughterless-GS) reduces daytime sleep. This inconsistency could be due to differences in the expression levels of the Elav-GS and Daughterless-GS drivers.

3.4.4 Tau expression in the clock system affects activity and sleep in LD

Next, we investigated how tau expression restricted to the clock system would affect circadian locomotor behaviour. Human tau (wild-type or R406W) expression in the clock system affects activity and sleep in LD conditions. Tau expression in PDF neurons (using Pdf-Gal4), or in *timeless* clock neurons (using tim-Gal4), caused a substantial increase in night-time activity and decrease in night-time sleep in an age-independent manner (Figure 3.5C, Figure 3.6C and Figure 3.7D,H). Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} expression produced similar night-time activity and sleep phenotypes (Supplementary Figure 6B,D). The elevated night-time activity in Pdf>tau flies is not caused by loss of PDF signalling as *Pdf^{01}* mutants (which lack the PDF peptide) have an advanced evening peak of activity, which results in an increase in daytime activity (Renn et al., 1999). Tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} expression produced similar night-time activity and sleep phenotypes in young flies. As the flies aged, levels of night-time activity and sleep were well maintained in tim>0n4r tau^{R406W} flies, but not in tim>2n4r tau^{WT} flies. As a result, the night-time activity and sleep phenotypes were milder in tim>2n4r tau^{WT} flies than in tim>0n4r tau^{R406W} flies (Supplementary Figure 5B,D).

Elav>2n4r tau^{WT} and Pdf>2n4r tau^{WT} expression caused similar night-time activity and sleep phenotypes (Supplementary Figure 8B,D). In 5-day old flies, we found similar night-time activity and sleep phenotypes in Elav>0n4r tau^{R406W} and Pdf>tau^{R406W} flies. Whereas, in 25-day old flies, we found a stronger night-time activity phenotype in Pdf>0n4r tau^{R406W} flies compared to Elav>0n4r tau^{R406W} flies. Elav>0n4r tau^{R460W} and Pdf>0n4r tau^{R460W} expression produced similar night-time sleep phenotypes (Supplementary Figure 8F,H).

Furthermore, suppression of tau expression exclusively in the PDF neurons rescued the night-time activity and sleep phenotypes in flies pan-neuronally expressing tau (Figure 3.11B,C). Thus, tau acts in the PDF neurons to promote night-time activity and suppress night-time sleep. We found most parental controls showed a small, but significant, age-related reduction in night-time activity, but no age-related differences in daytime activity (Figure 1B,C, Figure 5B,C and Figure 6B,C). However, other studies have reported the opposite (Metaxakis et al., 2014). Most parental controls showed no age-related differences in daytime and night-time sleep (Figure 2B,D and Figure 7C,D,G,H). On the other hand, previous studies have reported an age-related reduction in both daytime and night-time sleep (Koh et al., 2006; Vienne et al., 2016).

Different tau proteins (2n4r tau^{WT} and 0n4r tau^{R406W}) differentially affected daytime activity and sleep when expressed in the clock system. Both Pdf>2n4r tau^{WT} and tim>2n4r tau^{WT} expression had no effect on daytime activity (Figure 3.5B). In contrast, tim>0n4r tau ^{R460W} expression affected daytime activity in an age-dependent manner; in young flies daytime activity was reduced, whereas in aged flies daytime activity was increased. A similar, but weaker, daytime activity phenotype was observed in Pdf>0n4r tau^{R460W} flies. Tim>0n4r tau^{R460W} and Pdf>0n4r tau^{R406W} flies showed a significant age-related reduction in daytime activity levels (Figure 3.6B). Whereas, no age-related differences in daytime activity levels were seen in tim>2n4r tau^{WT}, Pdf>2n4r tau^{WT} and control flies (Figure 3.5B and Figure 3.6B). However, an increase in activity during the active phase as a function of age has not been previously reported in either AD patients or in AD mice models. Tim>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} expression had no effect on daytime sleep. In comparison, tim>0n4r tau^{R460W} expression produced a small gain in daytime sleep in young flies and a large loss of daytime sleep in aged flies. Similarily, Pdf>2n4r tau^{WT} expression produced a small gain in daytime sleep in young flies. In both tim>0n4r tau^{R406W} and Pdf>2n4r tau^{WT} flies levels of daytime sleep decreased with age (Figure 3.7). We found sleep gain does not always coincide with reduced activity in tau-expressing flies. For example, in 5-day old tim>2n4r tau^{WT} flies levels of daytime activity were normal, but levels of daytime sleep were increased (Figure 3.5B and Figure 3.7C). On the other hand, sleep loss does not always coincide with increased activity. For example, in 25-day old Elav>2n4r tau^{WT} flies levels of daytime activity were normal, but levels of daytime sleep were reduced (Figure 3.1B and

Figure 3.2C). Taken together, these data show tau expression in the clock system resulted in age-dependent daytime activity and sleep phenotypes, and age-independent night-time activity and sleep phenotypes. In both tim>tau and Pdf>tau flies the daytime activity and sleep phenotypes were weaker than the night-time activity and sleep phenotypes. Therefore, tau expression restricted to the clock system (in young flies) better recapitulates the sleep-wake cycle disturbances characteristic of AD (increased daytime naps, reduced and fragmented night-time sleep and increased night-time wakefulness) than pan-neuronal tau expression, which produced daytime and night-time sleep loss (Figure 3.2). Thus, tau expression in the clock neurons promotes daytime sleep and tau expression in other parts of the brain blocks these sleep-prompting effects. These data suggest that the sleep-wake cycle disturbances in AD are, at least in part, due to tau abnormalities in the SCN.

3.4.5 Tau expression in the clock system is sufficient to produce behavioural arrhythmia in DD

Surprisingly, flies were rhythmic when we expressed tau only in the PDF neurons. Whereas, when we expressed tau in all clock cells, the flies became behaviourally arrhythmic. Neither Pdf>2n4r tau^{WT} nor Pdf>0n4r tau^{R460W} expression caused progressive behavioural arrhythmia (Figure 3.8A,B, Fig 3.9A,B and Supplementary Figure 3.6E). Tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} expression had similar effects on circadian behavioural rhythms. We found a similar age-related decline in overall rhythmicity in tau-expressing and control flies (Figure 3.8D, Figure 3.9D and Supplementary Figure 5E). Flies expressing tau in the *timeless* clock neurons, but not the tim-positive glial cells, were more rhythmic than flies expressing tau in all clock cells as evidenced by increased overall rhythmicity (in 25-day old flies, but not in 5-day old flies) and a reduced arrhythmic sub-population (Figure 3.12B and Table 3.1). These results suggest glia are involved in regulating locomotor activity rhythms (Suh et al., 2007; Ng et al., 2011). It has been proposed glial cells regulate PDF transport or release from sLNvs projections (Ng et al., 2011), it is possible tau may interfere with these processes. Furthermore, pan-clock tau expression had more a more severe effect on circadian behaviour than pan-neuronal tau expression (Supplementary Figure 7), it is possible that this is due, at least in part, to tau expression in tim-positive glial cells.

Suppression of tau expression exclusively in the PDF neurons was insufficient to rescue the behavioural arrhythmia in flies pan-neuronally expressing tau (Figure 3.11D,E). Thus, tau-evoked behavioural arrhythmia in DD is independent of the PDF neurons. Another possibility is that soluble tau migrates into the PDF neurons. However, this is unlikely as tau failed to migrate into the PDF neurons and induce night-time activity and sleep phenotypes in LD conditions (Figure 3.11B,C). Taken together, these experiments highlight the contribution of non-PDF clock neurons in mediating tau-evoked circadian behavioural arrhythmia. Clock neurons communicate with non-clock output neurons to give rise to robust locomotor activity rhythms (reviewed in Dubowy and Sehgal, 2017). For example, DN1 neurons make synaptic connections with a group of cells in the pars intercerebralis (PI) that express the neuropeptide, diuretic hormone 44 (DH44). DH44 is essential for normal free-running behaviour (Cavanaugh et al., 2014; King et al., 2017). It is possible that tau mediates behavioural arrhythmia in DD by disrupting the communication of the DN1 neurons or other non-PDF clock neurons with output neurons, such as DH44-positive PI cells. Several studies have shown pan-clock Aβ expression produces behavioural arrhythmicity. On the other hand, Aβ expression exclusively in the PDF neurons is insufficient to cause circadian behavioural arrhythmia (Chen et al., 2014; Long et al., 2014). Taken together, these findings suggest the primary target for both tau- and Aβ-evoked behavioural arrhythmia is the non-PDF clock neurons. Similarly, other studies have found a blockage in the communication between the central clock and output neurons in both a mice model of HD (Pallier et al., 2007) and AD patients (Wu et al., 2007; Cermakian et al., 2011).

3.4.6 Tau expression in the clock system prolongs the free-running period

Tau expression restricted to the clock system lengthened the free-running period (Figure 3.8, Figure 3.9 and Table 3.1). Tim>tau and Pdf>tau expression produced similar period-lengthening effects (Figure 3.8C, Figure 3.9C, Supplementary Figure 3.5F and Supplementary Figure 3.6F). Period lengths in tim>tau flies varied greatly (Figure 3.8C, Figure 3.9C and Table 3.1). These long-period rhythms are not caused by the loss of PDF signalling, because killing the PDF neurons (using hid) results in short-period rhythms. Similarily, flies mutant for PDF and the PDF receptors show short-period rhythms (Renn et al., 1999). However, we see the opposite in our tau-expressing flies. The period-lengthening effects in Pdf>tau flies were not

caused by blockade of chemical synaptic transmission, because Pdf>TNT flies exhibit robust behaviour in DD (Umezaki et al., 2011). Pan-neuronal tau expression had no effect on the period length of rhythms (Fig 3.3C and Supplementary Fig 3.2C). The period length of rhythms was normal in Elav, Pdf-Gal80>0n4r tau^{R460W} flies (Fig 3.11F). We show tau expression exclusively in the PDF neurons has a period-lengthening effect. Whereas, tau expression in the non-clock neurons blocks the period-lengthening effect of tau expression in the PDF neurons. Similarly, Tg4510 mice show long-period rhythms (Stevanovic et al., 2017). However, period lengths were normal in PLB2 mice (Koss et al., 2016).

3.4.7 Tau expression causes hyperactivity in DD

Tau expression in the clock system produces hyperactivity in DD (Figure 3.8D and Figure 3.9D). Different tau isoforms have differential effects on levels of activity in DD. Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} expression produced a similar increase in activity levels. As the flies aged, the elevated activity levels were stable in Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} flies (Supplementary Figure 3.6G). Parental controls exhibited a small, but significant, age-related reduction in levels of activity, which was inhibited by PDF>tau expression (Figure 3.8D, Figure 3.9D and Supplementary Figure 3.6G). Tau expression exclusively in the PDF neurons has an activity-promoting effect in DD conditions. Tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} expression differentially effected levels of activity in DD. In 5-day old flies, Tim>2n4r tau^{WT} expression produced a mild increase in activity levels, whereas tim>0n4r tau^{R406W} expression produced a strong increase in activity levels. As the flies aged, the elevated activity levels were well maintained in tim>0n4r tau^{R460W} flies, but not in tim>2n4r tau^{WT} flies (Figure 3.8D, Figure 3.9D and Supplementary Figure 3.5G). Thus, tim>2n4r tau^{WT} expression results in a substantial age-related reduction in activity levels in both LD (restricted to the dark phase) and DD conditions (Figure 3.5C and Figure 3.8D). Tau expression in the non-PDF clock neurons blocks the activity-promoting effects of tau expression in the PDF neurons in aged flies. Differences in activity levels in tim>tau flies cannot be attributed to the high proportion of arrhythmic flies, because activity levels in rhythmic and arrhythmic flies did not significantly differ (data not shown).

We found isoform-specific effects of ubiquitous neuronal tau expression on overall daily locomotor activity in DD. Elav>2n4r tau^{WT} expression resulted in age-induced hyperactivity, whereas Elav>0n4r tau^{R406W} expression had no effect on activity levels (Figure 3.3D). These results suggest that tau expression, in other parts of the brain, blocks the activity-promoting effects of tau expression in the PDF neurons. For example, communication between the master pacemaker and the brain regions involved in locomotion control (i.e. the ellipsoid body) could be inhibited by tau. In DD conditions, Tg4510 mice show normal daily activity levels (Stevanovic et al., 2017). Currently, activity levels in constant conditions have not been measured in other transgenic tau mice. It is possible that in mouse models of tauopathy, tau expression in the SCN has an activity-promoting effect, but this effect is inhibited by tau expression in other parts of the brain. It is worth noting that tim>2n4r tau^{S11A} expression caused behavioural arrhythmia without affecting activity levels (Supplementary Figure 3.4). This lends further support to the idea that tau expression can affect the overall amount of activity, independently of the distribution of activity.

Collectively, these results show pan-clock human tau expression causes progressive behavioural arrhythmia and hyperactivity and prolongs the free-running period in DD. In contrast, human tau expression exclusively in PDF neurons produces hyperactivity and lengthens the free-running period, but is insufficient to induce progressive behavioural arrhythmia in DD (Figure 3.8 and Figure 3.9).

3.4.8 Circadian and sleep disruption is due to neuronal dysfunction in the clock cells

Because tau expression in the clock system causes abnormalities in circadian locomotor behaviour (in both LD and DD conditions), we investigated the status of the master pacemaker neurons. We found no obvious loss of PDF neurons in either Pdf>tau or tim>tau flies up to 25-days of age (Figure 3.10). This indicates that circadian behavioural rhythm degradation in tim>tau flies occurs without neuronal death in the master pacemaker. These results support the idea that tau expression outside of the PDF neurons is of primary importance in tau-evoked behavioural arrhythmia in DD. This also suggests that the circadian behavioural abnormalities in Pdf>tau flies (elevated night-time activity and night-time sleep loss in LD and hyperactivity in DD) are due to dysfunction, rather than

degeneration, in the master pacemaker neurons. A growing body of evidence shows that human tau expression causes neuronal dysfunction in *Drosophila* (Mudher et al., 2004; Chee et al., 2005; Mershin et al., 2010; Papanikolopoulou et al., 2010). Recent work undertaken in our laboratory has demonstrated that expression of human wild-type or mutant tau in the fly eye produces neuronal and synaptic dysfunction in the absence of neuronal death (Chapter 2).

Recent work by Julienne et al. (2017) has shown electrophysiological abnormalities in the lLNvs coincide with behavioural arrhythmicity in a *Drosophila* model of PD. Specifically, the lLNvs are hyperactive and do not show a day/night difference in firing rate. We suggest that electrophysiological abnormalities in the PDF neurons underlie tau-evoked elevated night-time activity, daytime sleep gains and night-time sleep loss in LD and hyperactivity in DD. Whereas, electrophysiological abnormalities in the non-PDF clock neurons (possibly the DN1s) underlie tau-evoked progressive behavioural arrhythmia in DD. In support of this, Sheeba et al. (2008) showed that hyperexcitation of the lLNvs neurons leads to sleep loss in *Drosophila*. Similarly, in a *Drosophila* model of human Aβ toxicity, daytime sleep loss coincides with hyperexcitability of the lLNvs neurons (Gerstner et al., 2017). Therefore, it is possible that electrophysiological abnormalities in the clock neurons are common to both tau and Aβ pathology. Flies were not aged past 35 days, degeneration of PDF neurons may occur in older flies. Furthermore, it is likely that there are changes in the ultrastructure of the clock neurons in tau-expressing flies; like which have been reported in several different neuronal populations in tau-expressing mice (Yoshiyama et al., 2007; Polydoro et al., 2009; Sydow et al., 2011; Jackson et al., 2017). However, this was beyond the scope of this investigation.

In summary, we show human tau expression (either wild-type or R406W) in *Drosophila* recapitulates the sleep-wake cycle and circadian rhythm disturbances characteristic of AD. In this study, we show a role for the PDF neurons in tau-evoked elevated night-time activity, daytime sleep gains and night-time sleep loss in LD. We further establish a role for the non-PDF clock neurons and tim-positive glial cells in tau-evoked progressive behavioural arrhythmicity in DD. Thus, distinct groups of cells mediate different tau-evoked circadian behavioural abnormalities (Figure 3.14). We also show the circadian behavioural deficits do

not coincide with structural degeneration of the PDF neurons. Additionally, tau expression exclusively in the PDF neurons lengthens the circadian free-running period and makes the flies hyperactive in DD. Finally, we show tau expression in adult clock neurons is sufficient to induce behavioural arrhythmia. Our fly model provides an opportunity to study the mechanisms that underlie AD-linked circadian behavioural abnormalities. Further understanding, will facilitate the development of novel therapeutics that could improve wellbeing and clinical outcome in AD patients.

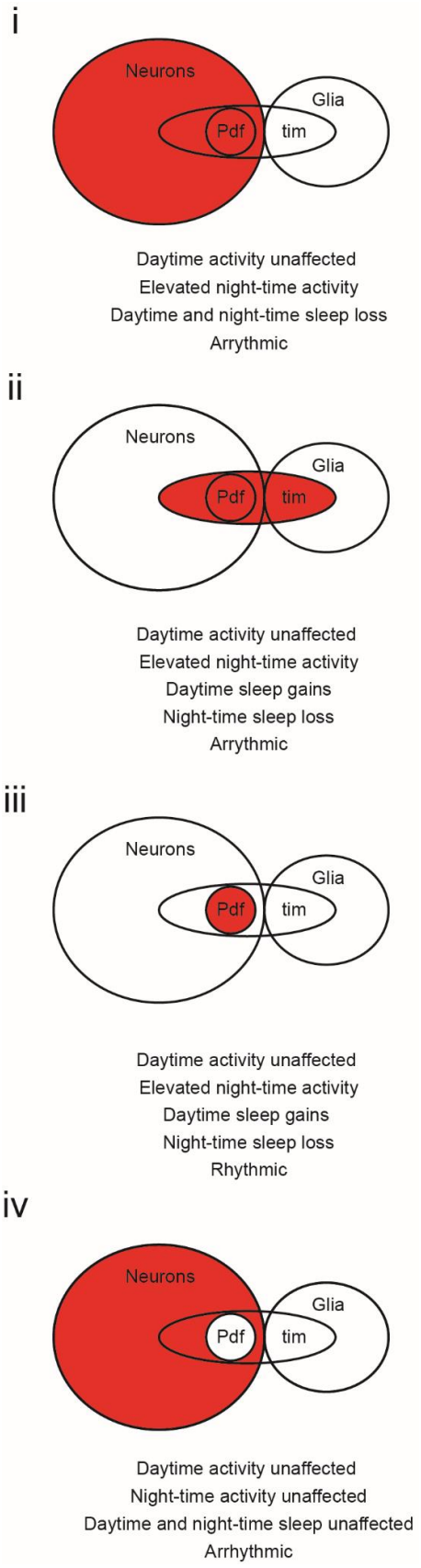


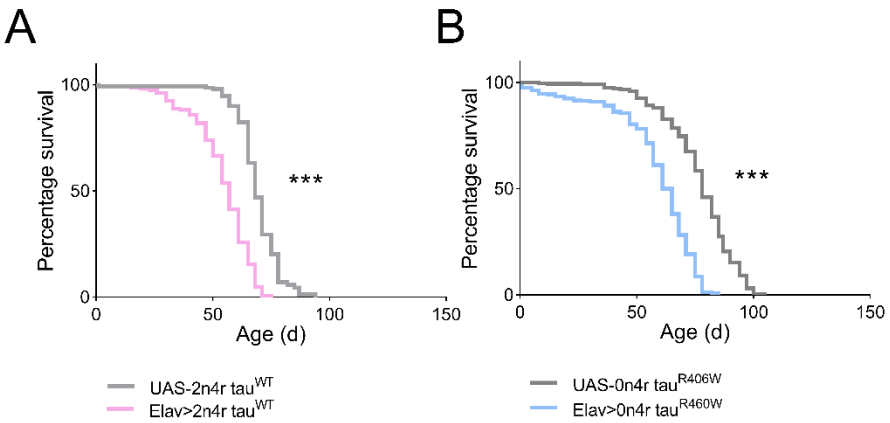
Figure 3.14: Tau expression in the fly brain results in circadian behavioural abnormalities in LD and DD conditions. (i) Scheme showing tau expression in all neurons results in elevated night-time activity (age-independent) and daytime and night-time sleep loss (age-independent) in LD conditions, and progressive behavioural arrhythmia in DD conditions. (ii) Scheme showing tau expression in all clock cells results in elevated night-time activity (age-independent), daytime sleep gains (age-dependent) and night-time sleep loss (age-independent) in LD conditions, and progressive behavioural arrhythmia and hyperactivity in DD conditions. (iii) Scheme showing tau expression in PDF neurons results in elevated night-time activity (age-independent), daytime sleep gains (age-dependent) and night-time sleep loss (age-independent) in LD conditions, but no progressive behavioural arrhythmia in DD conditions. (iv) Scheme showing tau expression in all neurons apart from PDF neurons has no effect on night-time activity and daytime and night-time sleep, but causes progressive behavioural arrhythmia in DD conditions. Tau expression in **ii** and **iii** also lengthens the free-running period.

Genotype	Age (d)	n	Power		Period (h)		Rhythmic percentage	Activity in DD		Survival percentage	Colour
			median	mean ± SEM	median	mean ± SEM		median	mean ± SEM		
Elav-Gal4	5	60	574	634 ± 43	23.7	23.7 ± 0.04	100	0.66	0.69 ± 0.04	94	
Elav-Gal4	25	84	330	410 ± 34	23.6	23.6 ± 0.10	100	0.46	0.51 ± 0.03	93	
UAS-2n4r tau^{WT}	5	115	529	597 ± 31	23.7	23.7 ± 0.03	100	0.57	0.60 ± 0.02	96	
UAS-2n4r tau^{WT}	25	71	226	293 ± 28	23.6	23.6 ± 0.06	100	0.46	0.49 ± 0.03	92	
Elav-Gal4/UAS-2n4r tau^{WT}	5	71	156	237 ± 29	23.5	23.5 ± 0.05	98	0.71	0.74 ± 0.03	89	
Elav-Gal4/UAS-2n4r tau^{WT}	25	97	58	118 ± 15	23.9	24.0 ± 0.15	84	0.61	0.83 ± 0.06	88	
UAS-0n4r tau^{R406W}	5	88	454	527 ± 34	23.6	23.8 ± 0.02	99	0.66	0.68 ± 0.04	95	
UAS-0n4r tau^{R406W}	25	54	346	382 ± 35	23.5	23.8 ± 0.05	98	0.41	0.46 ± 0.04	89	
Elav-Gal4/UAS-0n4r tau^{R406W}	5	96	81	142 ± 18	23.6	23.9 ± 0.09	95	0.69	0.71 ± 0.03	86	
Elav-Gal4/UAS-0n4r tau^{R406W}	25	79	65	81 ± 8	23.4	24.0 ± 0.12	86	0.50	0.59 ± 0.04	59	
UAS-0n4r tau^{WT}	5	32	575	625 ± 47	575	23.8 ± 0.05	100	0.77	0.80 ± 0.05	100	
UAS-0n4r tau^{WT}	25	44	281	342 ± 35	281	24.0 ± 0.06	100	0.91	0.89 ± 0.04	98	
Elav-Gal4/UAS-0n4r tau^{WT}	5	46	601	643 ± 59	601	23.8 ± 0.06	100	1.31	1.44 ± 0.10	96	
Elav-Gal4/UAS-0n4r tau^{WT}	25	44	529	546 ± 66	529	24.2 ± 0.14	100	1.53	1.59 ± 0.07	92	
Elav-GeneSwitch-Gal4/UAS-2n4r tau^{WT} (vehicle control)	12	46	452	564 ± 50	23.4	23.5 ± 0.04	100	0.29	0.31 ± 0.02	96	
Elav-GeneSwitch-Gal4/UAS-2n4r tau^{WT} (RU486)	12	68	475	538 ± 35	23.3	23.5 ± 0.04	100	0.33	0.34 ± 0.02	93	
Elav-GeneSwitch-Gal4/UAS-2n4r tau^{S11A} (vehicle control)	12	16	142	302 ± 44	23.7	23.7 ± 0.06	100	0.23	0.25 ± 0.04	89	
Elav-GeneSwitch-Gal4/UAS-2n4r tau^{S11A} (RU486)	12	29	222	284 ± 45	23.8	23.8 ± 0.05	100	0.27	0.30 ± 0.04	85	
tim-Gal4	5	92	335	447 ± 33	23.8	23.8 ± 0.03	100	0.59	0.66 ± 0.03	96	
tim-Gal4	25	67	275	317 ± 32	23.6	23.6 ± 0.06	100	0.53	0.59 ± 0.02	87	
tim-Gal4/2n4r tau^{WT}	5	46	60	74 ± 7	24.1	24.7 ± 0.25	95	1.15	1.10 ± 0.06	100	
tim-Gal4/2n4r tau^{WT}	25	77	24	30 ± 2	24.7	25.1 ± 0.35	70	0.57	0.61 ± 0.04	96	
Pdf-Gal4	5	71	508	560 ± 25	23.8	23.8 ± 0.02	100	0.55	0.58 ± 0.02	96	

Pdf-Gal4	25	60	228	303 ± 33	23.8	23.8 ± 0.06	100	0.42	0.50 ± 0.04	89	
Pdf-Gal4/2n4r tau^{WT}	5	55	519	533 ± 29	24.5	24.5 ± 0.05	100	1.00	1.07 ± 0.05	98	
Pdf-Gal4/2n4r tau^{WT}	25	31	199	230 ± 25	24.5	24.5 ± 0.06	100	0.90	0.97 ± 0.07	97	
tim-Gal4/UAS-0n4r tau^{R406W}	5	88	68	86 ± 7	24.2	24.2 ± 0.13	98	0.81	0.82 ± 0.03	97	
tim-Gal4/UAS-0n4r tau^{R406W}	25	50	37	50 ± 5	24.7	24.9 ± 0.29	98	0.84	0.93 ± 0.05	98	
Pdf-Gal4/UAS-0n4r tau^{R406W}	5	109	189	233 ± 16	24.3	24.3 ± 0.03	100	0.83	0.85 ± 0.02	97	
Pdf-Gal4/ UAS-0n4r tau^{R406W}	25	27	183	183 ± 20	24.3	24.3 ± 0.06	100	0.99	1.03 ± 0.05	84	
Elav-Gal4, Pdf-Gal80	5	48	556	604 ± 63	23.4	23.4 ± 0.04	100	0.90	0.94 ± 0.07	100	
Elav-Gal4, Pdf-Gal80/UAS-0n4r tau^{R406W}	25	32	67	105 ± 21	23.7	23.8 ± 0.14	100	0.71	0.85 ± 0.08	91	
Tim-Ga4, repo-Gal80	5	32	575	600 ± 52	23.7	23.7 ± 0.06	100	0.61	0.62 ± 0.04	100	
Tim-Ga4, repo-Gal80	25	32	226	278 ± 31	23.5	23.5 ± 0.09	100	0.47	0.49 ± 0.04	100	
Tim-Ga4, repo-Gal80/UAS-2n4r tau^{WT}	5	49	78	87 ± 10	24.1	24.5 ± 0.20	96	0.50	0.54 ± 0.03	100	
Tim-Ga4, repo-Gal80/UAS-2n4r tau^{WT}	25	63	45	53 ± 5	24.3	24.4 ± 0.21	95	0.35	0.39 ± 0.02	98	
tim-Gal4, tub-Gal80^{ts} (20 °C)	3-5	40	189	216 ± 17	24.1	24.0 ± 0.10	100	0.87	0.95 ± 0.05	93	
tim-Gal4, tub-Gal80^{ts} (30 °C)	3-5	40	468	468 ± 34	23.5	23.6 ± 0.08	100	0.76	0.75 ± 0.03	93	
tim-Gal4, tub-Gal80^{ts}/UAS-2n4r tau^{WT}(20 °C)	3-5	85	257	265 ± 13	23.8	23.8 ± 0.03	100	0.75	0.80 ± 0.03	99	
tim-Gal4, tub-Gal80^{ts}/UAS-2n4r tau^{WT}(30 °C)	3-5	85	229	245 ± 14	24.5	24.6 ± 0.08	100	0.75	0.77 ± 0.02	99	
UAS-2n4r tau^{S11A}	5	33	412	517 ± 61	23.6	23.6 ± 0.07	100	0.47	0.53 ± 0.04	96	
UAS-2n4r tau^{S11A}	25	25	335	342 ± 32	23.6	23.6 ± 0.06	100	0.43	0.46 ± 0.04	96	
tim-Gal4>UAS-2n4r tau^{S11A}	5	53	39	54 ± 6	23.7	24.1 ± 0.25	82	0.43	0.60 ± 0.05	98	
tim-Gal4>UAS-2n4r tau^{S11A}	25	37	22	31 ± 4	24.5	25.4 ± 0.80	62	0.37	0.46 ± 0.04	93	

Table 3.1: Power (PN) and period length of rhythms was determined by Lomb-Scargle periodogram analysis. Flies were defined as rhythmic based upon the presence of a peak above the 0.05 significance line (usually at 12.5 PN). In flies with complex rhythms (more than a single peak above the 0.05 significance line), the largest peak was used for analysis. n: number of flies tested per genotype/age. Rhythmic percentage: number of rhythmic flies/ number of tested flies as a percentage. Activity in DD:

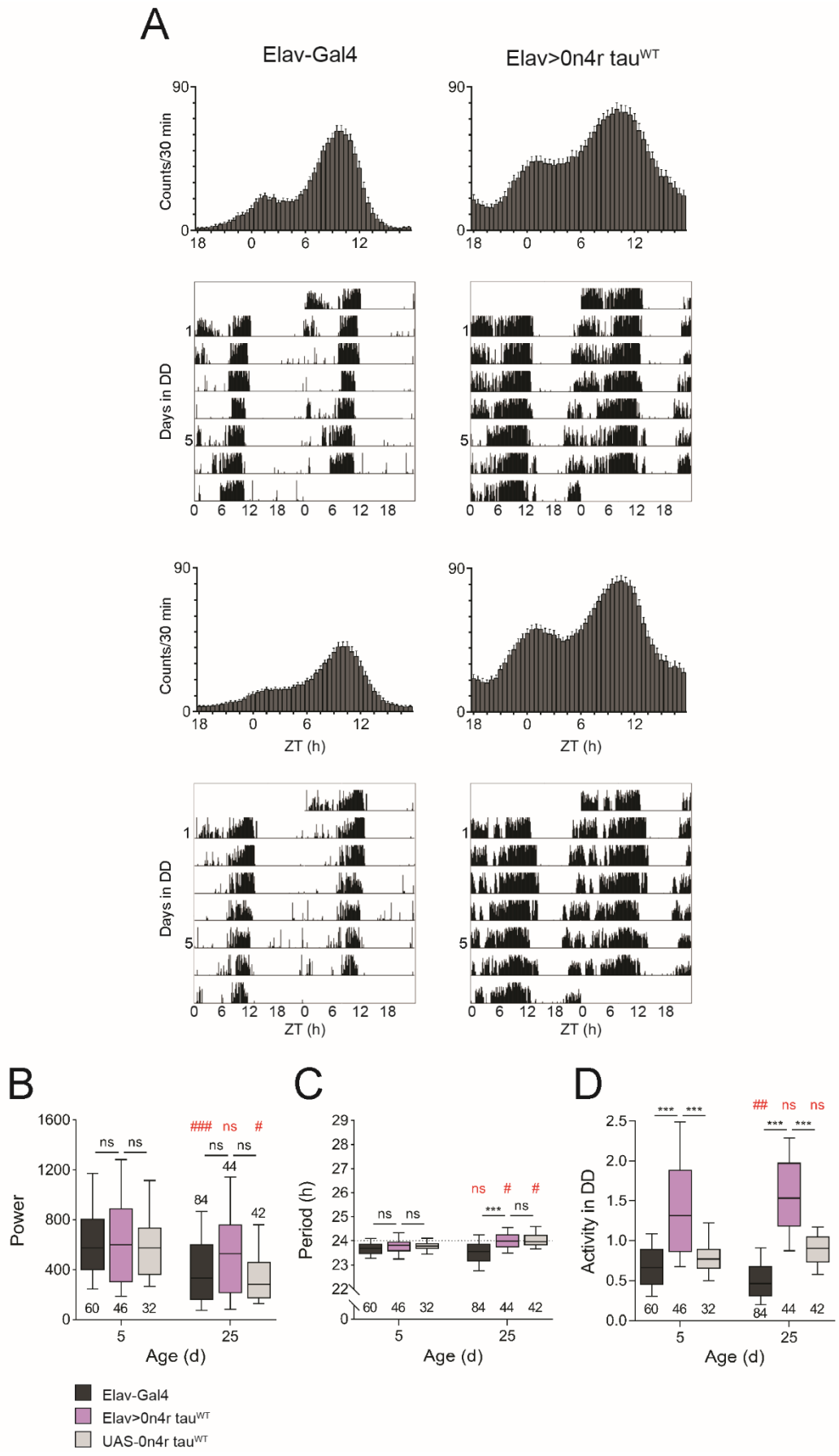
activity counts per min across 24 h. Survival percentage: number of flies which survived to the end of the experiment/ number of flies which started the experiment as a percentage. Colour corresponds to the colour used in the graphs.



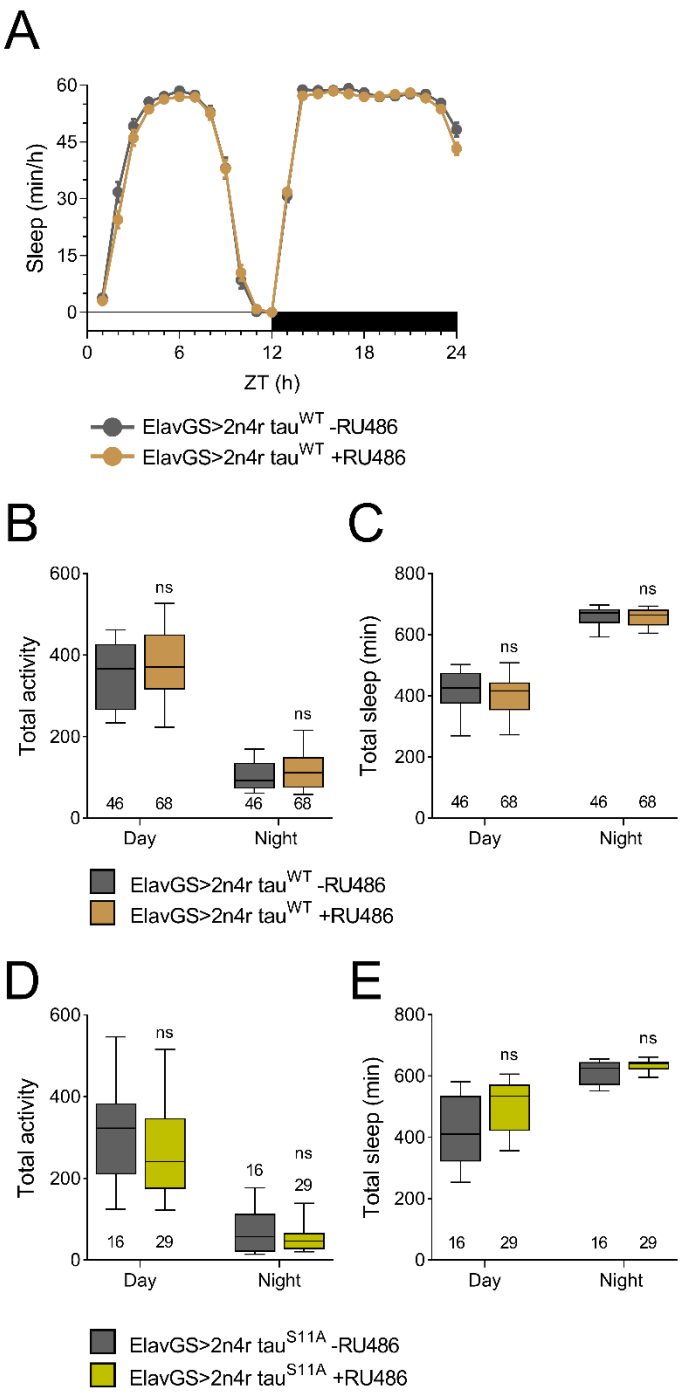
C

Genotype	Median lifespan (d)	Percentage change
UAS-2n4r tau^{WT}	68	
Elav>2n4r tau^{WT}	57	-16
UAS-0n4r tau^{R406W}	78	
Elav>0n4r tau^{R406W}	63	-20

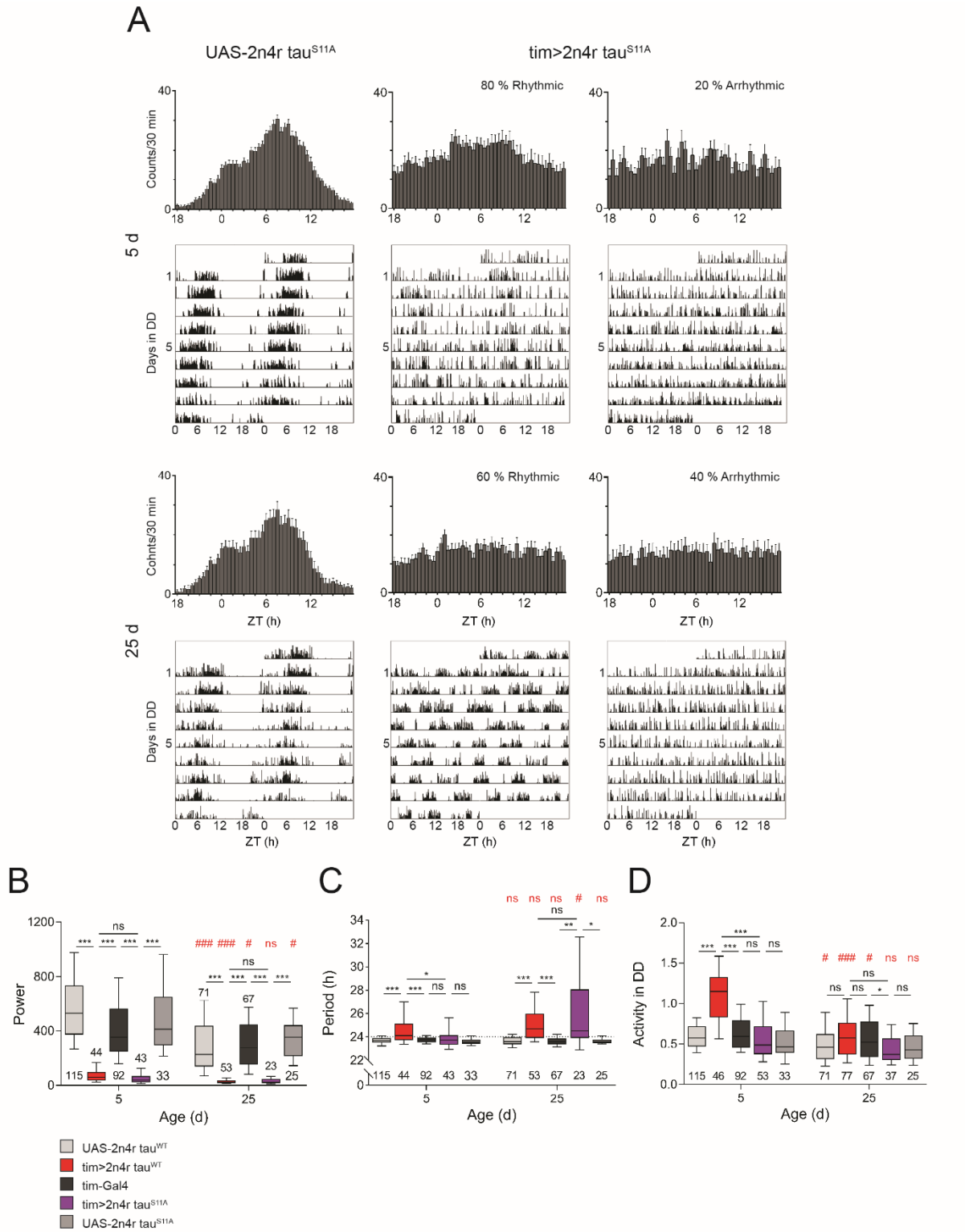
Supplementary Figure 3.1: Pan-neuronal tau expression significantly reduces lifespan. Survival curves of tau-expressing and control adult male flies raised and maintained at 25 °C. (A) Elav>2n4r tau^{WT} flies show a significant reduction in lifespan compared with the UAS control. (B) Elav>0n4r tau^{R406W} flies show a significant reduction in lifespan compared with the UAS control. ***p<0.0001; Log-rank (Mantel-Cox) test. (C) Median lifespan for tau-expressing and control flies. 400 flies per genotype were tested.


A
Elav-Gal4
Elav>0n4r tauWT
Counts/30 min
Days in DD
ZT (h)
B
Power
Age (d)
C
Period (h)
D
Activity in DD
Elav-Gal4
Elav>0n4r tauWT
UAS-0n4r tauWT

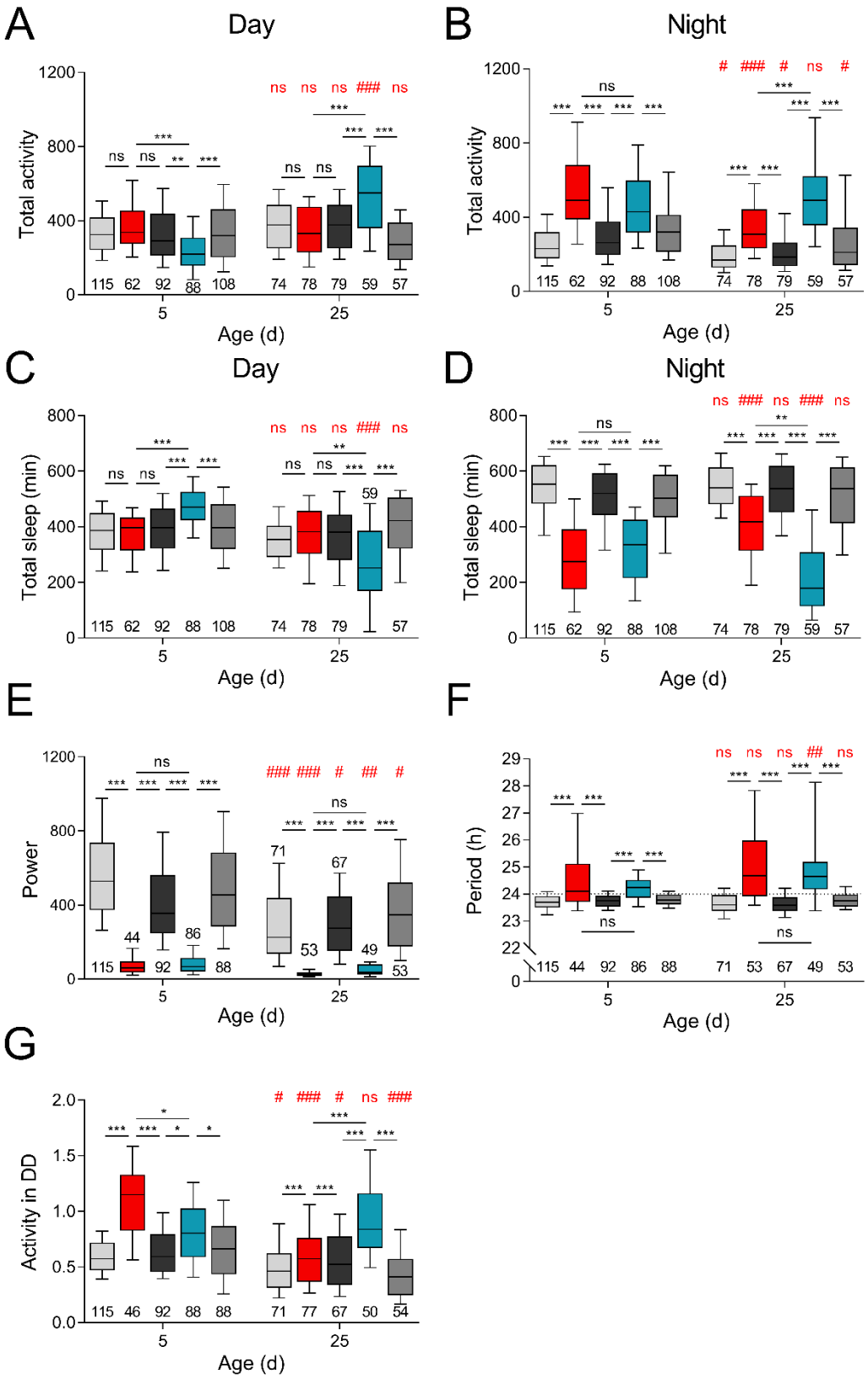
Supplementary Figure 3.2: Normal locomotor behaviour in Elav>0n4r tau^{WT} flies. (A) Average daily activity histograms and representative double-plotted actograms for seven days of DD conditions are shown for 5- and 25-day old Gal4 control (left column) and Elav>0n4r tau^{WT} (right column) flies. Flies were entrained in standard LD cycles for three days and then monitored in DD for seven days. Gal4 control flies maintain robust rhythms of daytime activity and night-time inactivity, with a period of just under 24 h. Elav>0n4r tau^{WT} flies exhibit robust rhythms with a normal period. (B) No differences in power are found in Elav>0n4r tau^{WT} flies relative to controls. (C) No differences in period length are found in Elav>0n4r tau^{WT} flies relative to controls. (C) Substantial increase in activity levels in DD is found in Elav>0n4r tau^{WT} flies relative to controls. Graphs show median with second and third quartiles and 10th and 90th percentiles. n = 32-84 flies per genotype/age from 2-6 independent experiments. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. (B,D) Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C) Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.



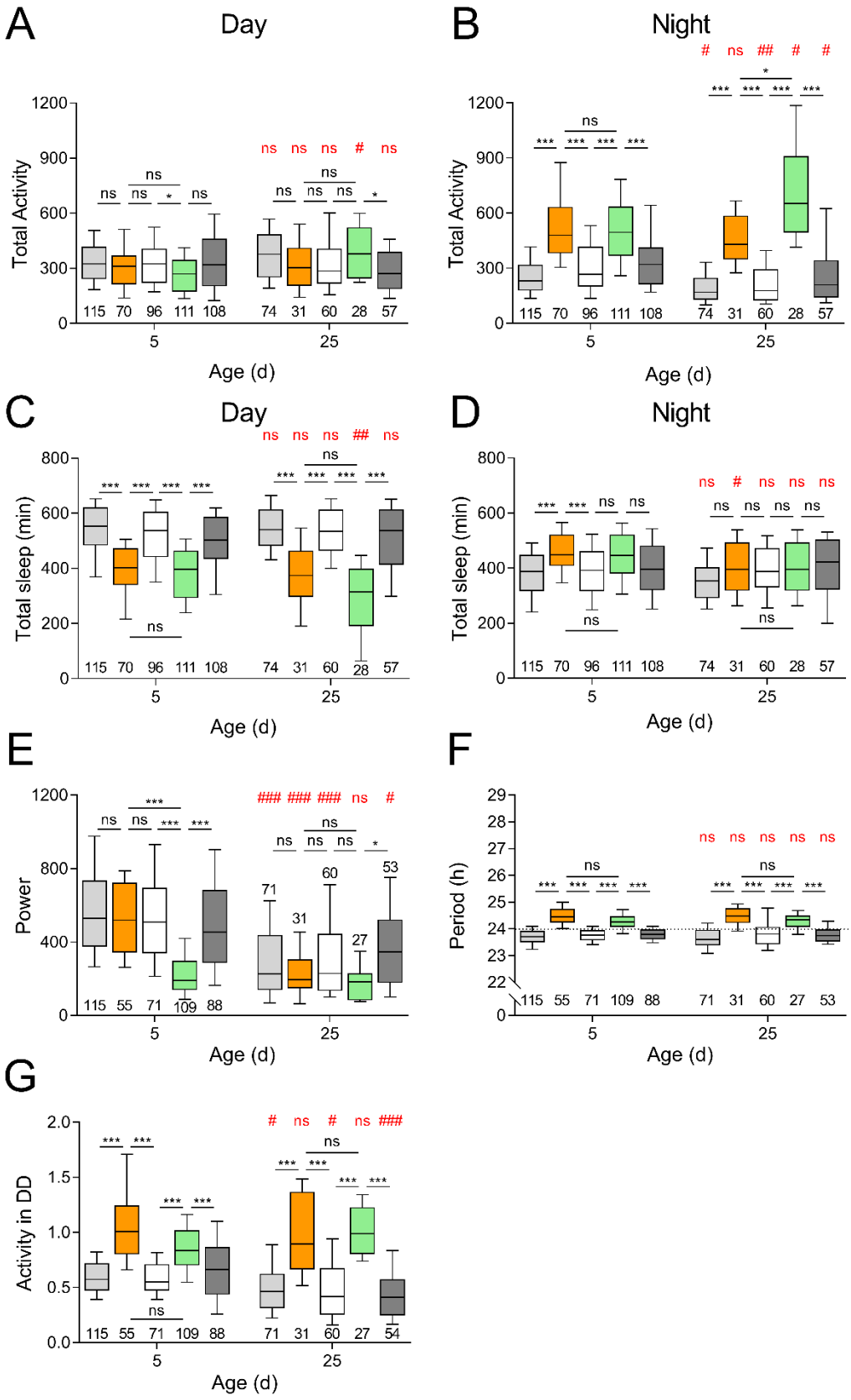
Supplementary Figure 3.3: Activity and sleep in ElavGS>2n4r tau^{WT} and ElavGS>2n4r tau^{S11A} flies. (A) Average daily sleep profile for three days of LD conditions are shown for RU486- and vehicle-fed ElavGS>2n4r tau^{WT} flies. Mean ± SEM. (B) Quantification of daytime and night-time activity for RU486- and vehicle-fed ElavGS>2n4r tau^{WT} flies. (C) Quantification of daytime and night-time sleep for RU486- and vehicle-fed ElavGS>2n4r tau^{WT} flies. n = 46-68 flies per group from 3-4 independent experiments. D) Quantification of daytime and night-time activity for RU486- and vehicle-fed ElavGS>2n4r tau^{S11A} flies. (E) Quantification of daytime and night-time sleep for RU486- and vehicle-fed ElavGS>2n4r tau^{S11A} flies. Graphs show median with second and third quartiles and 10th and 90th percentiles. n = 16-29 flies per group from a single experiment. ns p>0.05; two-tailed Student's t-tests with log-transformed data for (B,D); Mann-Whitney U-tests for (C,D).


A
UAS-2n4r tauS11A
tim>2n4r tauS11A
80 % Rhythmic
20 % Arrhythmic
60 % Rhythmic
40 % Arrhythmic
Counts/30 min
Cohnts/30 min
5 d
25 d
Days in DD
ZT (h)
B
Power
Age (d)
C
Period (h)
Age (d)
D
Activity in DD
Age (d)
UAS-2n4r tauWT
tim>2n4r tauWT
tim-Gal4
tim>2n4r tauS11A
UAS-2n4r tauS11A

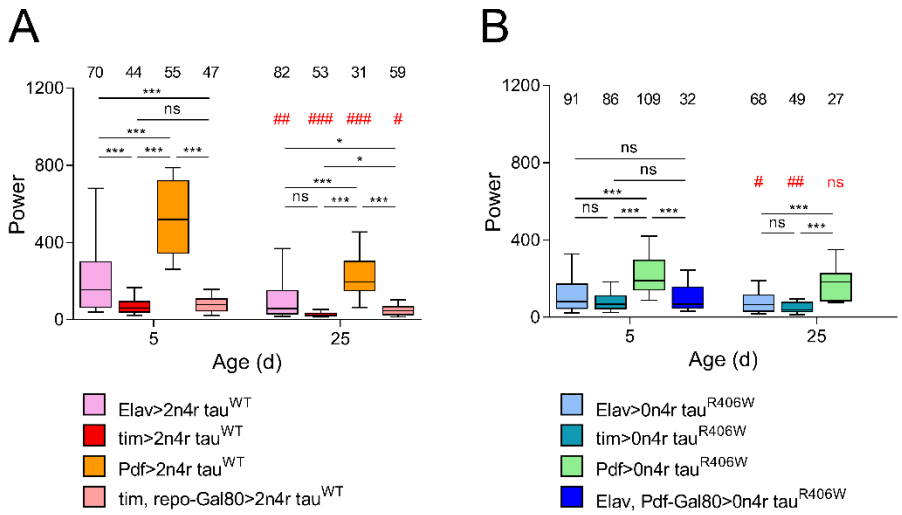
Supplementary Figure 3.4: Phospho-resistant 2n4r tau^{S11A} expression in the clock system disrupts normal circadian locomotor rhythms. (A) Average daily activity histograms and representative double-plotted actograms for nine days of DD conditions are shown for 5- and 25-day old UAS control (left column) and tim>2n4r tau^{S11A} (right column) flies. Flies were entrained in standard LD cycles for three days and then monitored in DD for nine days. UAS control flies maintain robust rhythms of daytime activity and night-time inactivity, with a period of just under 24 h. 5-day old tim>2n4r tau^{S11A} flies show very weak rhythms with a normal period or are arrhythmic. 25-day old tim>2n4r tau^{S11A} flies show very weak rhythms with a long period (>24 h) or are arrhythmic. (B) Severely reduced power is found in tim>2n4r tau^{WT} and tim>2n4r tau^{S11A} flies relative to controls. See Table 3.1 for the percentage of rhythmic flies. (C) No differences in period length are found in 5-day old tim>2n4r tau^{S11A} flies relative to controls. Increase in period length is found in tim>2n4r tau^{WT} and 25-day old tim>2n4r tau^{S11A} flies relative to controls. (D) Substantial increase in activity levels in DD is found in 5-day old tim>2n4r tau^{WT} flies relative to controls. No differences in activity levels in DD are found in tim>2n4r tau^{S11A} and 25-day old tim>2n4r tau^{WT} flies relative to controls. Graphs show median with second and third quartiles and 10th and 90th percentiles. n = 25-115 flies per genotype/age from 1-6 independent experiments. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. (B,D) Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C) Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.


A
Day
Total activity
Age (d)
B
Night
Total activity
Age (d)
C
Day
Total sleep (min)
Age (d)
D
Night
Total sleep (min)
Age (d)
E
Power
Age (d)
F
Period (h)
Age (d)
G
Activity in DD
Age (d)

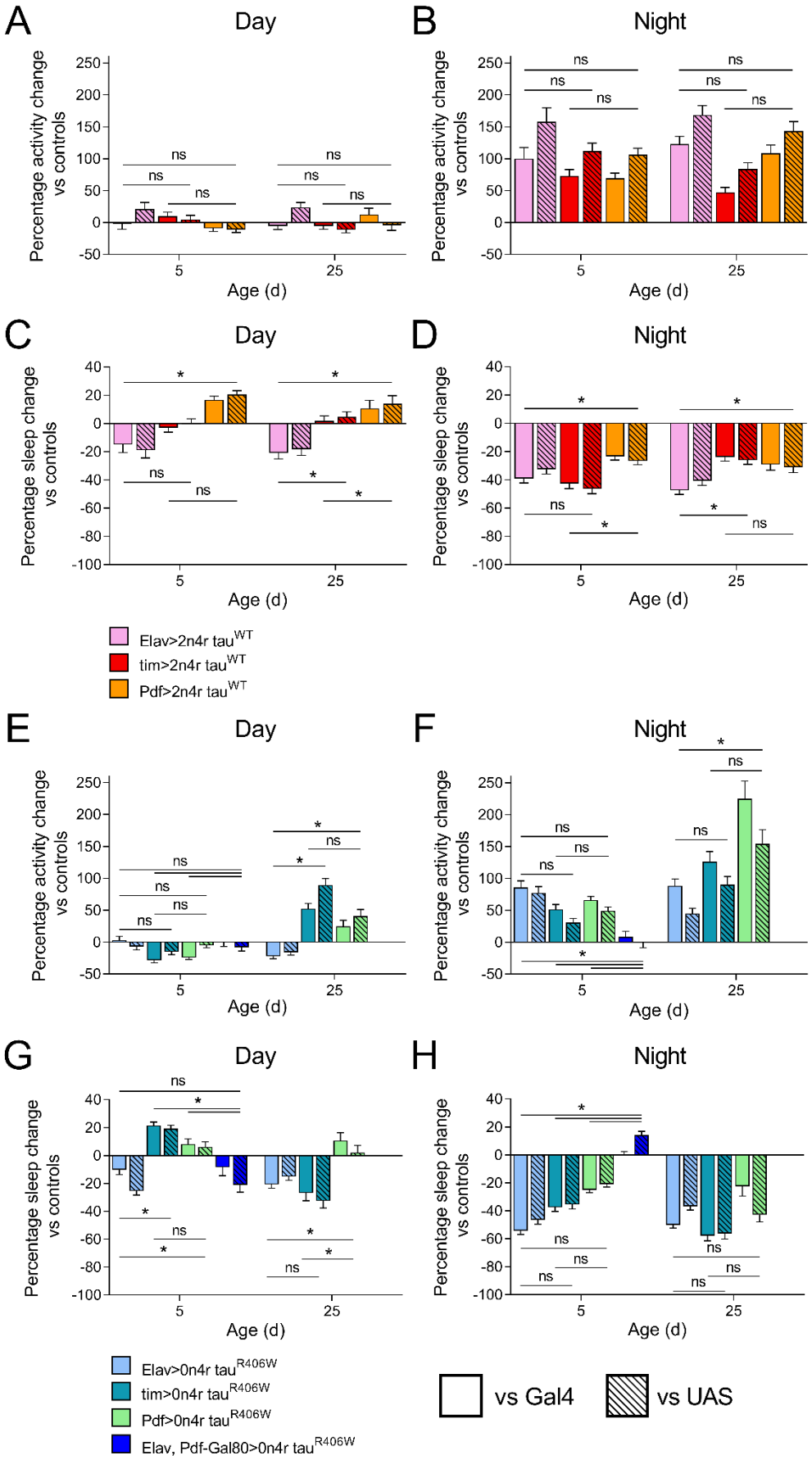
Supplementary Figure 5: Effects of tim>2n4r tau^{WT} and tim>0n4r tau^{R460W} expression on circadian locomotor behaviour. (**A-B**) Quantification of daytime and night-time activity. Reduced daytime activity is found in 5-day old tim>0n4r tau^{R460W} flies relative to age-matched tim>2n4r tau^{WT} flies. Increased daytime activity is found in 25-day old tim>0n4r tau^{R460W} flies relative to age-matched tim>2n4r tau^{WT} flies. No differences in night-time activity are found between 5-day old tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} flies. Increased night-time activity is found in 25-day old tim>0n4r tau^{R406W} flies relative to age-matched tim>2n4r tau^{WT} flies. (**C-D**) Quantification of daytime and night-time sleep. Increased daytime sleep is found in 5-day old tim>0n4r tau^{R460W} flies relative to age-matched tim>2n4r tau^{WT} flies. Reduced daytime sleep is found in 25-day old tim>0n4r tau^{R460W} flies relative to age-matched tim>2n4r tau^{WT} flies. No differences in night-time sleep are found between 5-day old tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} flies. Reduced night-time sleep is found in 25-day old tim>0n4r tau^{R406W} flies relative to age-matched tim>2n4r tau^{WT} flies. n = 57-115 flies per genotype/age from 3-6 independent experiments. (**E**) No differences in power are found between tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} flies. (**F**) No differences in period length are found between tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} flies. (**G**) Increased activity levels in DD are found in 5-day old tim>2n4r tau^{WT} flies relative to age-matched tim>0n4r tau^{R460W} flies. In contrast, increased levels of activity in DD are found in 25-day old tim>0n4r tau^{R406W} flies relative to age-matched tim>2n4r tau^{WT} flies. n = 46-115 flies per genotype from 3-6 independent experiments. All graphs show median with second and third quartiles and 10th and 90th percentiles. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. (A,B,E,G) Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C,D,F) Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.


A
Day
Total Activity
Age (d)
B
Night
Total Activity
Age (d)
C
Day
Total sleep (min)
Age (d)
D
Night
Total sleep (min)
Age (d)
E
Power
Age (d)
F
Period (h)
Age (d)
G
Activity in DD
Age (d)

Supplementary Figure 6: Effects of Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R460W} expression on circadian locomotor behaviour. (A-B) Quantification of daytime and night-time activity. No differences in daytime and night-time activity between Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R460W} flies, with the exception of increased night-time activity in 25-day old Pdf>0n4r tau^{R460W} flies compared to age-matched Pdf>2n4r tau^{WT} flies. (C-D) Quantification of daytime and night-time sleep. No differences in daytime and night-time sleep between Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R460W} flies. n = 28-115 flies per genotype from 2-6 independent experiments. No differences in power (E), period length (G) and activity in DD (G) are found between Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R460W} flies. n = 27-115 flies per genotype from 2-6 independent experiments. All graphs show median with second and third quartiles and 10th and 90th percentiles. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001. (A,B,E,G) Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data. (C,D,F) Multiple comparisons between different genotypes of the same age (asterisks in black) by Kruskal-Wallis ANOVA and post-hoc Dunn's tests. Comparisons between different ages of the same genotype (number symbols in red) by Mann-Whitney U-tests.



Supplementary Figure 7: Comparing the effects on circadian rhythmicity of expressing 2n4r tau^{WT} (A) or 0n4r tau^{R460W} (B) to various extents. Elav-Gal4: all neurons; tim-Gal4: all clock cells; Pdf-Gal4: PDF clock neurons; tim-Gal4, repo-Gal80: all clock neurons; Elav-Gal4, Pdf-Gal80: all neurons except PDF neurons. (A) n = 31-82 flies per genotype from 2-6 independent experiments. (B) n = 27-109 flies per genotype from 2-4 independent experiments. All graphs show median with second and third quartiles and 10th and 90th percentiles. ns p>0.05; *p<0.05; **p<0.001; ***p<0.0001.Multiple comparisons between different genotypes of the same age (asterisks in black) and different ages of the same genotype (number symbols in red) by 2-way ANOVA and post-hoc Tukey HSD tests with log-transformed data.


A
Day
B
Night
Percentage activity change vs controls
Percentage sleep change vs controls
Age (d)
ns
*
C
D
E
F
G
H
Elav>2n4r tau^WT
tim>2n4r tau^WT
Pdf>2n4r tau^WT
Elav>0n4r tau^R406W
tim>0n4r tau^R406W
Pdf>0n4r tau^R406W
Elav, Pdf-Gal80>0n4r tau^R406W
vs Gal4
vs UAS

Supplementary Figure 8: Comparing the effects on activity and sleep of expressing 2n4r tau^{WT} (A-D) or 0n4r tau^{R046W} (E-H) to various extents. (A-B) Percentage change in daytime and night-time activity between experimental genotypes (Elav>2n4r tau^{WT}, tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT}) in comparison to relevant Gal4 and UAS control genotypes. (C-D) Percentage change in daytime and night-time sleep between experimental genotypes (Elav>2n4r tau^{WT}, tim>2n4r tau^{WT} and Pdf>2n4r tau^{WT}) in comparison to relevant Gal4 and UAS control genotypes. (E-F) Percentage change in daytime and night-time activity between experimental genotypes (Elav>0n4r tau^{R406W}, tim>0n4r tau^{R406W}, Pdf>0n4r tau^{R406W} and Elav, Pdf-Gal80>0n4r tau^{R406W}) in comparison to relevant Gal4 and UAS control genotypes. (G-H) Percentage change in daytime and night-time sleep between experimental genotypes (Elav>0n4r tau^{R406W}, tim>0n4r tau^{R406W}, Pdf>0n4r tau^{R406W} and Elav, Pdf-Gal80>0n4r tau^{R406W}) in comparison to relevant Gal4 and UAS control genotypes. All graphs show mean ± SEM. ns $p>0.05$; *$p<0.05$. Multiple comparisons between experimental genotypes by 2-way ANOVA and post-hoc Tukey HSD tests (the most conservative p value is shown).

Chapter 4: Investigating the spatial resolution of *Drosophila* motion vision through optomotor behaviour in a flight simulator system

The work presented in this chapter was previously published in Juusola, M., Dau, A., Song, Z., Solanki, N., Rien, D., Jaciuch, D., Dongre, S. A., Blanchard, F., de Polavieja, G. G., Hardie, R. C.; Takalo, J. (2017). Microsaccadic sampling of moving image information provides *Drosophila* hyperacute vision. eLife. **Figure 10, Figure Supplement 10 and Appendix 10.** All experiments in this chapter were performed by myself. This chapter was written by myself.

4.1 Introduction

The *Drosophila* compound eye is composed of ~750 unit eyes called ommatidia arranged in a hexagonal array. Each ommatidium is physically separate from its neighbours, containing a lens and eight photoreceptors. The outer R1-R6 photoreceptors are arranged in a trapezoid and the inner R7+R8 photoreceptors lie centrally, on top of one another. Because of the *Drosophila* eyes neural superposition principal, R1-R6 photoreceptors from neighbouring ommatidia point in the same direction. Pooling the output of R1-R6 photoreceptors that point in the same direction increases sensitivity (~sixfold) without loss of spatial resolution (Paulk et al., 2013).

Visual acuity is the minimum resolvable angle. The acuity of a *Drosophila* eye is limited by its photoreceptor spacing, which essentially matches its interommatidial angle (~4.5°). For example, to be able to resolve a black-and-white grating, one ommatidium must look at a black stripe and the neighbouring ommatidium must look at the adjacent white stripe. If a single ommatidium looks at both a black and a white stripe it will be seen as a single grey stripe. To resolve two objects requires at least three ommatidia, one for each of the objects and one for the gap between the objects (Land, 1997). If a single ommatidium looks at two objects they will be seen as a single object. Animals view the world through a pattern of slow fixations with fast saccades that shift the direction of gaze. These moments may be made by either the eye, head or body. According to the classic ideas, animals would keep the gaze still during fixation to avoid motion blur resulting from photoreceptors' slow

integration time. However, fast photoreceptor adaptation could cause perceptual fading during fixation. Therefore, it was thought that photoreceptor sampling would occur only when the gaze is stabilised, whereas during saccades the animal would be effectively blind. Thus, eyes would sample information through a series of more or less stationary images (Land, 1999).

However, recent findings in our lab suggest that photoreceptors in fact exploit saccadic light modulation to enhance visual acuity. Juusola et al. (2017) found photoreceptors encoding capacity is maximised to fast high contrast bursts that are similar to saccadic light input. Photoreceptors transfer two-to-four times more information to fast high contrast bursts than to Gaussian white-noise and naturistic stimuli. This high information transfer rate is because of the darker intervals in the bursty light stimulus, which relieve photoreceptors refractory microvilli. Thus, they are able to approach close to 100 percentage encoding efficiency. The authors further found that rapid photomechanical contractions reduce motion-blur during saccades and reduce adaptation during fixation to improve the resolvability of saccadic visual inputs beyond the theoretical motion-blur limit. Collectively, refractory sampling and photomechanical contractions actively modulate light input, and thereby reduce adaption during gaze stabilisation that could otherwise fade vision. Jointly these processes allow photoreceptors to encode visual information in both space and time. Therefore, when the authors incorporated refractory sampling and photomechanical contractions into a biophysically-realistic model, it predicted *Drosophila* see ≥ four-fold finer detail than suggested by the classic theories. To test this experimentally, we investigated the spatial resolution of *Drosophila* motion vision through optomotor behaviour in a flight simulator system. We show *Drosophila* possess hyperacute motion vision and in accordance with the model predictions respond to patterns with ~1° spatial resolution.

4.2 Material and Methods

In all experiments, 1- to 3-day-old wild-type “Berlin” female flies were used. Flies were kept in bottles at a low population density to prevent overcrowding and maximise size. Following cold-anaesthesia (lasting three-five minutes) a small triangle-shaped copper-wire hook was glued (Loctite UV glue) to a fly’s head and thorax. Flies were prepared in the late afternoon

(four-six pm). After preparation, flies were left overnight individually in small moist chambers with sugar to recover. A bespoke flight simulator system was used to study *Drosophila*'s optomotor behaviour (Wardill et al., 2012). A fly was connected by its small triangular hook to a small metal clamp. The metal clamp was then connected to the torque meter (Tang and Guo, 2001). A fly connected to the torque meter was placed inside the centre of a white cylinder. Inside it, we placed a laser printed transparency containing a black-and-white vertical grating. The grating forms a continuous (360°) scene around the fly. Bright homogenous illumination of the arena was provided by a ring–shaped light tube (Philips, 350-900 nm). The scene was rotatable by the use of a stepping motor. We used an eight second stimulus which consisted of two seconds of counterclockwise rotation and two seconds of clockwise rotation separated by two seconds when the arena was still – preceded and concluded by one second when the arena was still. The eight second stimulus was repeated ~20 times. Data from any flies who failed to fly for the entire duration of the experiment (~three minutes) was excluded. At least 10 flies were tested per experiment. All experiments were performed in a dark room at ~23 °C.

4.3 Results and Discussion

We investigated the visual acuity of *Drosophila* motion vision through their optomotor behaviour in a flight simulator system. *Drosophila* were connected to a torque meter in the centre of a white cylinder. Inside it, we placed a black-and-white vertical grating which formed a continuous (360°) scene around the fly. The scene was rotatable via the use of a stepping motor. We measured flies yaw torque responses to an eight second stimulus – preceded and concluded by a one second pause, the panorama was rotated anticlockwise for two seconds then clockwise for two seconds, separated by a two second pause - which was repeated ~20 times. When presented with scene rotations, flies attempt to follow them producing yaw torque responses (optomotor responses to the left or to the right), to attempt to minimise retinal slippage. The strength of the fly's response is thought to be proportional to the strength of their perception. For example, if a fly can comfortably resolve the grating they will be able to accurately follow the scene rotations. Whereas, if a fly is unable to resolve the grating they will fail to follow the scene rotations, resulting in much smaller optomotor responses (Gotz, 1964; Heisenberg and Buchner, 1977).

We recorded optomotor responses of wild-type flies to black-and-white vertical gratings of three different spatial resolutions (wavelength: 1.16°, 2.88° and 14.4°), rotating slowly at 75°/s. The flies responded most strongly to the 14.4° bar panorama. The white cylinder alone (no apparent visual features) did not evoke a response, and thus provided the baseline response level. The 3.9° bar panorama evoked a strong response. The maximum response (peak-to-peak) to the 3.9° bar panorama was 70 % of the maximum response to the 14.4° bar panorama. Similarly, the 1.2° bar panorama also evoked a strong response. The maximum response to 1.2° bar panorama was 50 % of the maximum response to 14.4° bar panorama. The response to both the 3.9° and 1.2° bar panoramas was significantly stronger than the response to the white panorama (Figure 4.1). The population response of 10 flies is shown in Figure 4.1, whereas, the responses of individual flies is shown in Figure 4.2. Responses vary in strength between different flies. However, all flies tested responded to the 3.9° and 1.2° bar panoramas – much stronger response than to the white panorama. The strength of the responses to the different patterns was consistent amongst all the flies – response to 14.4° bars > 3.9° bars > 1.2° bars > no bars (Figure 4.2).

It is not possible to test optomotor responses of flies to black-and-white vertical gratings rotating at <75°/s, because the stepping motor is unable to rotate the arena smoothly and uniformly at <75°/s. It is also not possible to test patterns with a wavelength <1°, because printers are unable to print on transparent film at high enough resolution. These results demonstrate flies possess hyperacute (<4.5° interommatidial angle) motion vision and see at least four-fold finer detail than previously thought.

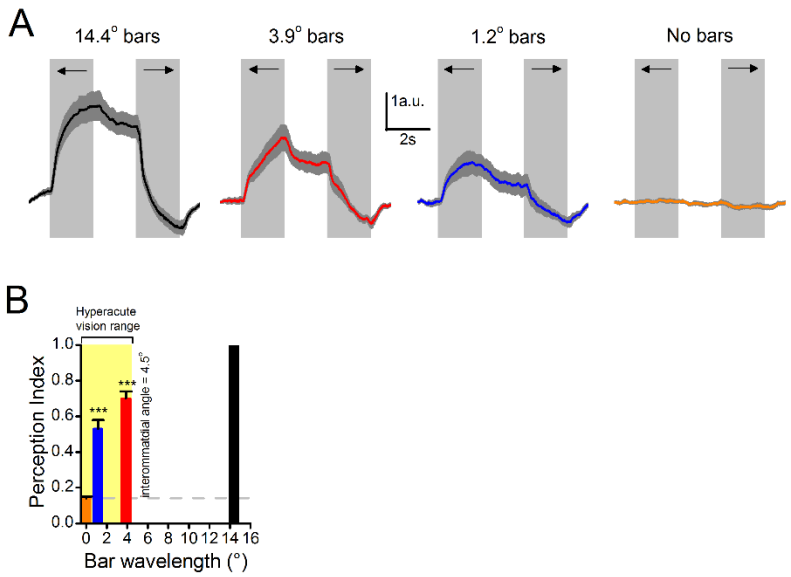


Figure 4.1: Optomotor behaviour in the flight simulator indicates *Drosophila* possess hyperacute motion vision. (A) Optomotor responses of tethered flying *Drosophila* to black-and-white vertical gratings of three different spatial resolutions (wavelength; 14.4°, 3.9° and 1.2°) and the white cylinder alone rotating slowing at 75°/s. The 14.4°, 3.9° and 1.2° bar panoramas evoke strong optomotor responses. The white panorama does not evoke an optomotor response. (B) Flies optomotor responses are strongest to the 14.4° bar panorama. The response to the white panorama established the baseline response level. The response to 3.9° and 1.2° bar panoramas is significantly stronger than the response to the white panorama. ***p<0.001; 1-way ANOVA followed by post-hoc Tukey HSD tests. Mean of 10 flies. Individual flies are shown in Figure 4.2

Next, we asked if we could find the limit of *Drosophila* motion vision through optomotor behaviour in the flight simulator system. We recorded optomotor responses of wild-type flies to black-and-white vertical gratings of three different spatial resolutions (wavelength: 1.16°, 2.88° and 14.4°), rotating at 75, 150, 300 or 400°/s. Flies responded very strongly to the 14.4° bar panorama rotated at all the different speeds tested. Increasing the speed of the 14.4° bar panorama had little effect on the size of the response. In contrast, increasing the speed caused a dramatic reduction in strength of the response to the 3.9° bar panorama. Strong response to the 3.9° bar panorama rotating at 75°/s and 150°/s – 70 % of the maximum response to the 14.4° bar panorama . Weak response to the 3.9° bar panorama rotating at 300°/s – 20 % of the maximum response to the 14.4° bar panorama. No response to the 3.9° bar panorama rotating at 400°/s – does not significantly differ from the response to the white panorama. Increasing the speed also caused a dramatic reduction

in the strength of the response to the 1.2° bar panorama. Strong response to the 1.2° bar panorama rotating at 75 – 60 % of the maximum response to the 14.4° bar panorama. However, no response to the 1.2° bar panorama rotating at 150, 300 or 400°/s – does not significantly differ from the response to the white panorama rotating at the same speed (Figure 4.3).

Very weak response to the white panorama, which was similar at all the different speeds tested – 10 % of the maximum response to the 14.4° bar panorama. It is not spontaneous activity because it is consistent and coupled to the optomotor stimuli. Therefore, we performed two control experiments to ensure there were no unbeknown visual features in the white cylinder that was affecting optomotor behaviour. First, we tested the optomotor response of wild-type flies to the 14.4° bar panorama rotating at 75°/s in darkness (surrounding ring-shaped light turned off). The 14.4° bar panorama in darkness evoked a very weak response, similar to the response evoked by the lit white panorama. Next, we tested the optomotor response of blind hdc^{JK910} flies (photoreceptors lack the neurotransmitter, histamine) (Burg et al., 1993) to the 14.4° bar panorama and white panorama rotating at 75°/s. As expected the responses of hdc^{JK910} flies to the 14.4° bar panorama and white panorama were essentially identical. Responses of hdc^{JK910} flies to either the 14.4° bar panorama or the white panorama were similar to the response of wild-type flies to the white panorama. Thus, the weak optomotor responses evoked by the white panorama are not due to inadvertent visual features, but probably due to airflow on the halters (Figure 4.4) (Mureli and Fox, 2015).

Drosophila have been used to study vision for over a hundred years (Paulk et al., 2012), but to date no study has tested the limit of the spatial resolution of *Drosophila* motion vision. This is probably because of the long held belief that *Drosophila* possess coarse vision, as determined by the 4.5~ interommatidial angle (Land, 1997). Buchner (1976) used a trackball system coupled with microscope-mediated local grating stimulation to probe *Drosophila* motion vison through optomotor behaviour. However, they did not test whether flies responded to a grating with a spatial resolution of <4.5°. Moreover, it is reasonable to assume the microscope-mediated local grating and panoramic high resolution black-and-white vertical gratings used in this study evoke very different neural responses.

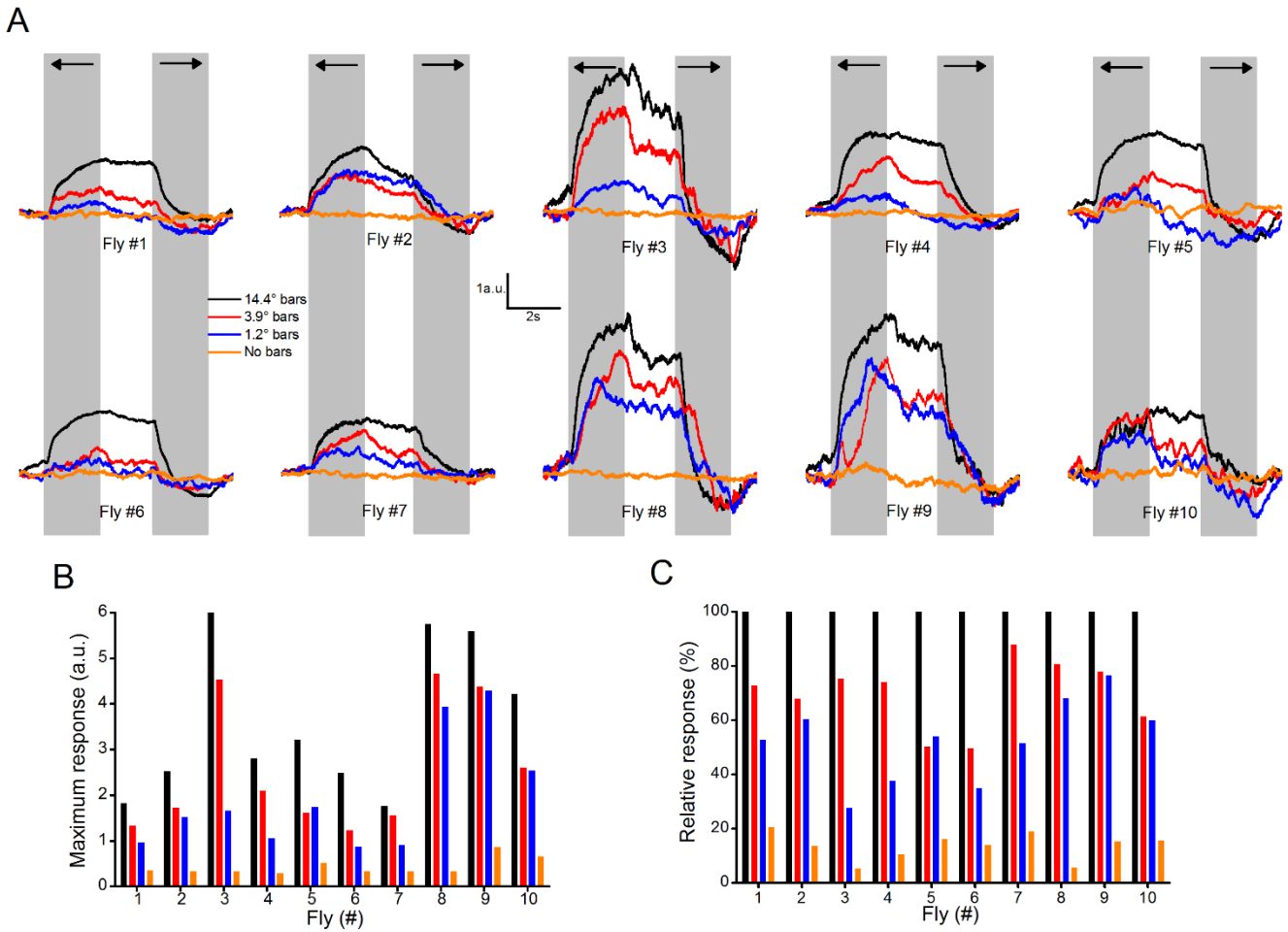


Figure 4.2: Optomotor behaviour in the flight simulator shows *Drosophila* see hyperacute visual patterns. (A) Optomotor responses of individual flies to black-and-white vertical gratings of three different spatial resolution (wavelength: 14.4°, 3.9° or 1.2°) and the white cylinder alone rotating slowly at 75°/s. All flies respond to the 3.9° and 1.2° bar panoramas. Whereas, no flies respond to the white panorama. (B) Variation in the strength of the maximum optomotor response (peak-to-peak) between different flies. All flies response was strongest to the 14.4° bar panorama. (C) Relative response to the 3.9° bar panorama varied between 50-90 % of the response to the 14.4° bar panorama. Relative response to the 1.2° bar panorama varied between 30-80 % of the response to the 14.4° bar panorama. The mean response of the 10 flies is shown in Figure 4.1.

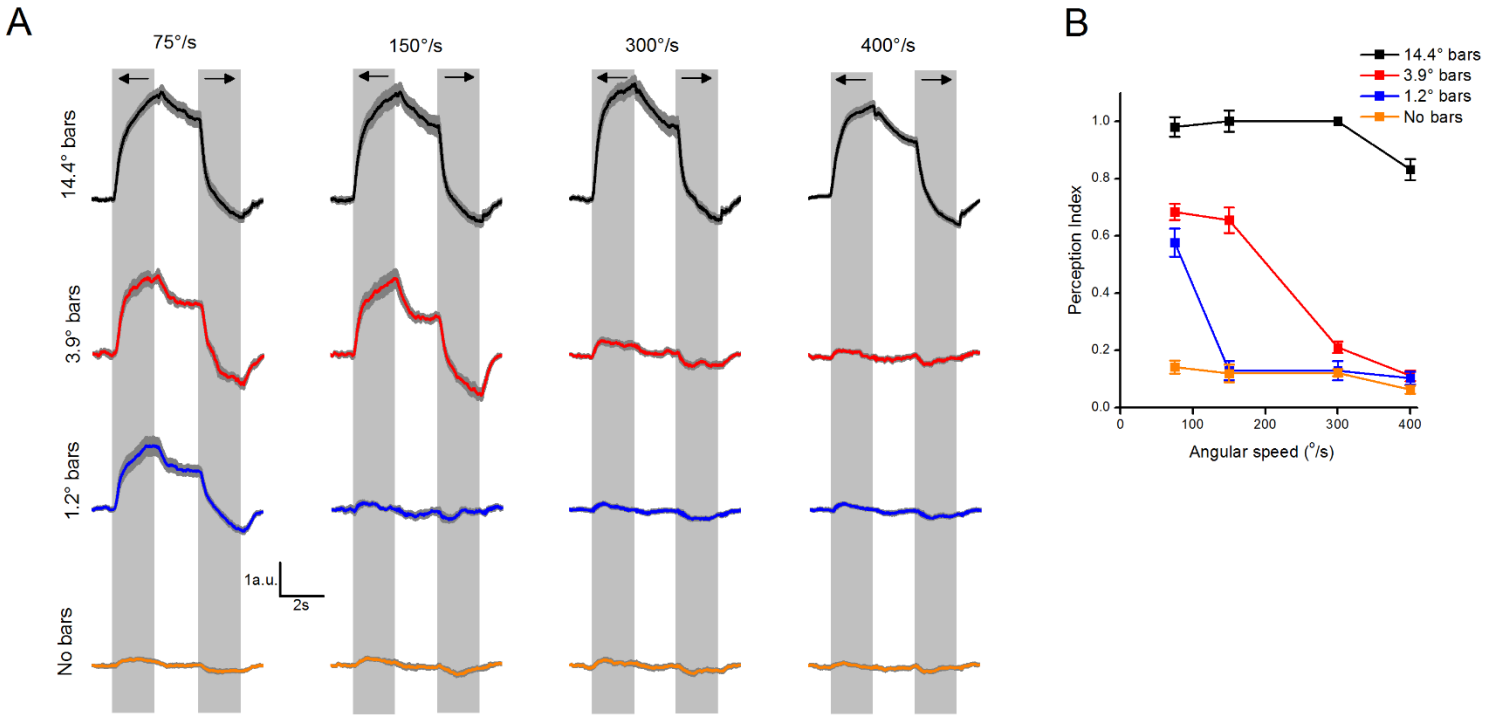


Figure 4.3: Optomotor behaviour in a flight simulator system reveals the limit of *Drosophila* hyperacute motion vision. (A) Optomotor responses of tether flying *Drosophila* to black-and-white vertical gratings of three different spatial resolutions (wavelength; 14.4, 3.9, or 1.2°) or the white cylinder alone rotating at 75, 150, 300 or 400°/s. Strong optomotor response to the 14.4° bar panorama at all different speeds tested. Increasing the speed dramatically reduces the optomotor response to the 3.9° and 1.2° bar panoramas. Strong optomotor response to the 3.9° bar panorama rotating at 75°/s and 150°/s but not 300°/s and 400°/s. Strong optomotor response to the 1.2° bar panorama rotating at 75°/s but not 150, 300 or 400°/s. (B) Optomotor response is strongest to the 14.4° bar panorama rotating at 300°/s (perception index = 1). Flies respond to the 3.9° bar panorama rotating at 75 and 150°/s, and 300°/s but not 400°/s. Flies respond to the 1.2° bar panorama rotating at 75°/s but not 150, 300 or 400°/s. Very weak responses to the white panorama, which therefore provides the baseline response level. Mean of 20 flies.

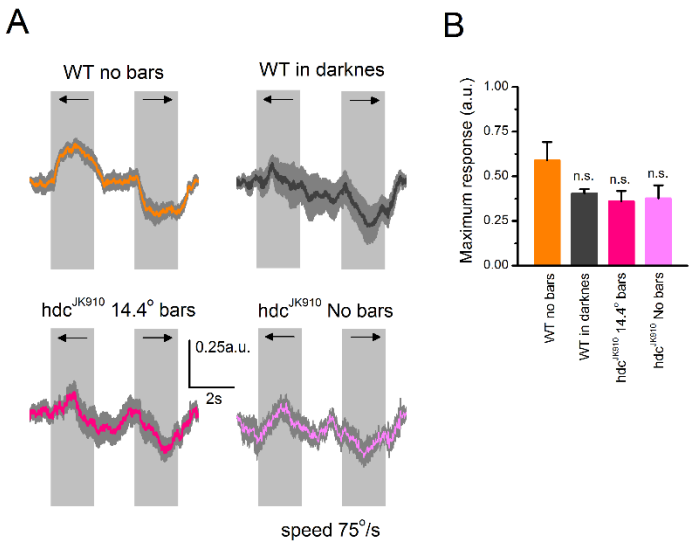


Figure 4.4: Control measurements of optomotor behaviour in the flight simulator system. (A) Very weak response of wild-type flies to the white panorama. Similar weak response of wild-type flies to the 14.4° bar panorama in darkness. Also similar weak responses of blind *hdc^{JK910}* flies to the 14.4° bar and white panoramas. (B) The maximum responses of wild-type flies to the 14.4° bar panorama in darkness and *hdc^{JK910}* flies to the 14.4° bar or white panorama does not significantly differ from the maximum response of wild-type flies to the white panorama. Mean of 15-20 flies. ns $p>0.05$; 1-way ANOVA followed by post-hoc Tukey HSD tests.

Chapter 5: Final Discussion

Tauopathies are a group of diseases characterised by the aggregation of hyperphosphorylated tau protein into neurofibrillary tangles (NFTs) and slow and progressive neurodegeneration. Abnormal post-translational modifications (PTMs), misfolding and aggregation of tau are common to all tauopathies. However, the brain regions the aggregates deposit in and their constituent tau isoform vary according to the disease. Different tauopathies have distinct clinical profiles, which range from cognitive to motor deficits (reviewed in Wang and Mandelkow, 2016). The vast majority of research has focused on NFT formation and neuronal death, because of the long held belief that the NFTs exert neurotoxicity.

However, mounting evidence suggests that the NFTs are relatively inert and instead the soluble tau species are neurotoxic. Human histopathological studies have shown clinical symptoms precede aggregate formation and neuronal death (reviewed in Arendt et al., 2009). Thus, a period of cellular dysfunction often precedes cell loss. This offers an exciting opportunity for therapeutic intervention before unrepairable neuronal damage. Therefore, a current priority is to develop tools and techniques to identify affected individuals earlier. As such, further understanding of the mechanisms underlying tau-mediated neuronal dysfunction is urgently needed. To achieve this we need to develop animal models that faithfully recapitulate the early stages of tauopathies, in which one is able to express different tau proteins in specific neuronal populations and robustly assay neuronal function and death.

Many transgenic tau mice have been produced that express various different tau proteins to varying extents in the brain. These transgenic tau mice exhibit a myriad of behavioural abnormalities and neuronal and synaptic dysfunction (Kimura et al., 2007; Yoshiyama et al., 2007; Polydoro et al., 2009; Hoover et al., 2010; Sydow et al., 2011; Tai et al., 2012). However, it is a confusing and contradictory picture as to whether the neuronal dysfunction in transgenic tau mice coincides with neuronal death, precedes neuronal death, or occurs in the absence of neuronal death. Because different transgenic tau mice use different

expression systems and express different tau proteins, it is difficult to make direct comparisons between them.

A key question is whether different 4r tau isoforms cause similar results when studied in isolation in the same neuronal population. In Chapter 2 to answer this question we expressed different human tau proteins in the *Drosophila* eye (using the Gal4/UAS system) and monitored age-related changes in function and structure. The *Drosophila* eye permits rapid and reproducible measures of function using electrophysiology (electroretinograms (ERGs)) or behaviour (optomotor responses), and structure using the corneal neutralisation technique.

We show that 0n4r tau expression in the developing eye causes progressive adult-onset neuronal dysfunction as shown by the progressive loss of the ERG response (Figure 2.1) and abnormal optomotor behaviour in the flight simulator system (Figure 2.5), without neuronal death as shown by the normal number and organisation of photoreceptor rhabdomeres (Figure 2.2). On the other hand, 2n4r tau expression in the developing eye causes progressive age-related neuronal dysfunction as shown electrophysiologically (Figure 2.3) and behaviourally (Figure 2.5), which precedes neuronal death as shown by the progressive loss of photoreceptor rhabdomeres (Figure 2.4). Thus, lGMR>2n4r tau expression produces temporally separable neuronal dysfunction and neuronal death.

lGMR>0n4r tau and lGMR>2n4r tau expression differentially affect the ERG response in young flies. We found normal ERG responses and optomotor behaviour in the flight simulator system in 0n4r tau-expressing flies up to 5-days of age (Figure 2.1 and Figure 2.5). Whereas, we found tiny ON transients in ERG responses in 2n4r tau-expressing flies early in life, indicating severe synaptic transmission defects (Figure 2.3). Thus, synaptic transmission is more sensitive to 2n4r tau expression than 0n4r tau expression. These results suggest tau influences synaptic transmission via its N-terminal domain, as has been suggested by others (Zhou et al., 2017). On the other hand, lGMR>0n4r tau and lGMR>2n4r tau expression produces a similar effect on the ERG response in aged flies (30-day old). ERG responses showed a lack of transients (reflect synaptic transmission) and a substantial reduction in depolarization (reflect the phototransduction cascade) in aged tau-expressing flies (Figure

2.1 and Figure 2.3). These results suggest isoform-specific effects are more dramatic early in the disease process.

We further show that 2n4r tau expression in the adult *Drosophila* eye causes progressive neuronal dysfunction as evidenced by the progressive loss of the ERG response (Figure 2.7), without neuronal death as evidenced by the normal number and organisation of photoreceptor rhadomeres (Figure 2.9). Thus, adult 4r tau-mediated structural degeneration, unlike function degeneration, requires a neurodevelopmental component. We further suggest a role for tau phosphorylation, by the PAR1 kinase, in tau-mediated neuronal dysfunction. Tau with mutations at the PAR1 phosphorylation sites is unable to cause neuronal dysfunction in the *Drosophila* eye (Figure 2.10). Our results suggest tau is an important downstream mediator of Aβ-dependent neuronal dysfunction. Aβ42 expression alone is insufficient to cause neuronal dysfunction, but coexpression of Aβ42 with 2n4r tau^{WT} potently exacerbates tau-mediated neuronal dysfunction (Figure 2.11).

Circadian rhythm disruption is common in tauopathies. The vast majority of individuals with Alzheimer's Disease (AD), Frontotemporal dementia (FTD) or Parkinson's Disease (PD) will experience circadian rhythm disturbances, typically early in the disease process. In fact, circadian disruption often occurs before the onset of traditional clinical symptoms, such as memory and motor deficits. Manifestation varies according to the disease, but typically night-time activity levels and daytime sleepiness are increased and night-time sleep levels are reduced (for review see Musiek et al., 2015). It is important to delineate the effects of tau and Aβ pathology on circadian dysfunction in AD, where they are both present. Animal models that are susceptible to sophisticated genetic manipulation and recapitulate the key features of circadian dysfunction in the human disease are urgently needed. *Drosophila* are a very well established model for studying circadian rhythms and sleep (reviewed in Dubowy and Sehgal, 2017).

Pan-neuronal 2n4r tau^{WT} and 0n4r tau^{R406W} expression (using Elav-Gal4) causes a shortened lifespan. Elav>2n4r tau^{WT} and Elav>0n4r tau^{R406W} expression produced a similar reduction in lifespan (Supplementary Figure 3.1). Pan-neuronal 2n4r tau^{WT} and 0n4r tau^{R460W} expression causes a substantial increase in night-time activity levels and a severe reduction in night-

time sleep levels in LD conditions. The night-time activity and sleep phenotypes in Elav>2n4r tau^{WT} and Elav>0n4r tau^{R406W} flies are similar. The night-time activity and sleep phenotypes are neither progressive nor age-dependent (Figure 3.1 and Figure 3.2).

Pan-neuronal 2n4r tau^{WT} and 0n4r tau^{R406W} expression produces progressive behavioural arrhythmia in DD conditions (Figure 3.3). Pan-neuronal 2n4r tau^{WT}, but not 0n4r tau^{R406W}, expression causes age-induced hyperactivity in DD (Figure 3.3). Whereas, Elav>0n4 tau^{WT} expression produces hyperactivity in an age-independent manner (Supplementary Figure 3.2). Typically overall activity levels are unaffected in AD and FTD patients (van Someren et al., 1996; Harper et al., 2004). We are the first to show human tau expression is capable of producing hyperactivity in constant conditions. However, many studies have found hyperactivity in mouse models of β-amyloidosis in free-running conditions (Ambree et al., 2006; Gil-Bea et al., 2007; Gorman and Yellon, 2010; Roh et al., 2012). Pan-neuronal 0n4r tau^{WT}, unlike 2n4r tau^{WT} and 0n4r tau^{R406W}, expression fails to cause progressive behavioural arrhythmia (Supplementary Figure 3.2). Collectively, these results show isoform-specific effects of ubiquitous neuronal tau expression to induce progressive behavioural arrhythmia and hyperactivity. These data show tau influences the amount of activity independently of the distribution of activity.

Next, we investigated the effects of restricting tau expression to the clock system, either in all clock cells (using tim-Gal4) or exclusively in PDF clock neurons (using Pdf-Gal4). PDF clock neurons are considered to be the master pacemaker as they synchronise the oscillations of all the clock neurons, so that they oscillate in phase with each other (Stoleru et al., 2005; Lear et al., 2009; Yoshii et al., 2009; Yao and Shafter, 2014). Tim>tau and Pdf>tau expression produces increased night-time activity levels and daytime sleep levels, and reduced night-time sleep levels (Figure 3.5, Figure 3.6 and Figure 3.7). Tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} expression produce similar night-time activity and sleep phenotypes (Supplementary Figure 3.5). Additionally, Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} expression also produce similar night-time activity and sleep phenotypes (Supplementary Figure 3.6). Elav>, tim> and Pdf>tau expression cause similar night-time activity and sleep phenotypes (Supplementary Figure 3.8).

Tim>2n4r tau^{WT} and tim>0n4r tau^{R406W} expression differentially affect daytime activity and sleep (Supplementary Figure 3.5). Tim>2n4r tau^{WT} expression has no effect on daytime activity and sleep (Figure 3.5 and Figure 3.7). Whereas, tim>0n4r tau^{R406W} expression produces age-dependent daytime activity and sleep phenotypes (Figure 3.6 and Figure 3.7). Similarily, Pdf>2n4r tau^{WT} and Pdf>0n4r tau^{R406W} expression differentially affect daytime activity and sleep (Figure 3.5, Figure 3.6, Figure 3.7 and Supplementary Figure 3.6). Thus, 2n4r tau^{WT} and 0n4r tau^{R406W} expression in the clock system produces isoform-specific effects on daytime activity and sleep, but not on night-time activity and sleep. Regardless of isoform, tau has a more significant effect on activity and sleep levels during the night, than the day. These data show tau expression can influence levels of daytime activity and sleep independently of levels of night-time activity and sleep. Thus, these results suggest distinct neural circuits control daytime and night-time activity and sleep levels.

Tau (2n4r and 0n4r isoforms) expression in all clock neurons causes progressive behavioural arrhythmia, prolongs the period length of rhythms and induces hyperactivity in free-running conditions. On the other hand, tau (2n4r and 0n4r isoforms) expression exclusively in the PDF neurons lengthens the free-running period and produces hyperactivity, but is insufficient to trigger progressive behavioural arrhythmia in DD conditions (Figure 3.8 and Figure 3.9). Thus, tau expression in all the clock neurons is the most clock-restricted domain that produces progressive behavioural arrhythmia in DD. The prolonged free-running period suggests changes in relative synchronisation between clocks. We found similar period-lengthening effects and hyperactivity in tim>tau and Pdf>tau flies (Figure 3.8, Figure 3.9, Supplementary Figure 3.5 and Supplementary Figure 3.6). We found no overt neuronal loss in Pdf>tau and tim>tau flies (Figure 3.10). Thus, the circadian behavioural deficits are likely due to neuronal dysfunction rather than neuronal death.

We further suggest a role for tim-positive glial cells in tau-mediated behavioural arrhythmicity. Flies expressing tau in the *timeless* clock neurons are more rhythmic than flies expressing in all the clock cells, including tim-positive glial cells (Figure 3.12). Others have shown a role of glial cells in regulating locomotor activity rhythms in *Drosophila* and mammals (Suh et al., 2007; Ng et al., 2007).

Collectively, these results show distinct groups of neurons mediate different tau-evoked circadian behavioural abnormalities. The PDF neurons mediate tau-evoked elevated night-time activity, daytime sleep gains and night-time sleep loss and in LD and tau-evoked hyperactivity in DD. In support of this, blocking tau expression exclusively in the PDF clock neurons rescues the elevated night-time activity and night-time sleep loss phenotype in tau-expressing flies (Figure 3.11). Whereas, non-PDF clock neurons mediate tau-evoked progressive behavioural arrhythmia in DD. As such, blocking tau expression only in the PDF neurons is insufficient to rescue the progressive behavioural arrhythmia in tau-expressing flies (Figure 3.11). Taken together, these results show both the extent of tau expression and the identity of the tau species influence the circadian behavioural deficits. These results underlie the different manifestations of circadian rhythm disruption in distinct tauopathies, which are characterised by aggregates of unique tau species in specific brain regions.

We show tau expression in specific groups of neurons has differential effects on circadian behaviour, which influence one another. For example, tau expression exclusively in the PDF neurons has an activity-promoting effect and period-lengthening effect in DD. Whereas, flies pan-neuronally expressing tau have normal activity levels and period lengths in DD. Therefore, tau expression in the non-PDF neurons blocks the activity-promoting effect and period-lengthening effect of tau expression in the PDF neurons. Thus, abnormal levels of tau proteins in different neuronal populations can have opposing effects. Therefore, it is possible that widespread tau expression in animal models may mask AD-linked circadian behavioural deficits. These results suggest, in tauopathies, the circadian behavioural deficits are as a result of interactions between the effects of tau abnormalities in distinct groups of neurons. In tauopathies, the clinical symptoms are contributed to by the identity of the tau species and its location. These results indicate that targeting tau abnormalities in specific brain regions may have dramatic effects on the clinical profile, despite the presence of tau abnormalities in other brain regions. Taken together, these results suggest that different therapeutic strategies may need to be developed for distinct tauopathies. To do this successfully, further understanding of the identify and location of tau species in tauopathies is required. Our model provides a platform to investigate the effects of expressing different tau species to varying extents on circadian locomotor behaviour.

Taken together, we show human tau expression in Drosophila is able to cause neuronal dysfunction in independent neuronal populations, in both the peripheral nervous system and central nervous system, with distinct properties. We show both 2n4r and 0n4r isoforms of tau cause neuronal and synaptic dysfunction in both photoreceptors and clock neurons as assayed both electrophysiologically and behaviourally. Tau-mediated neuronal dysfunction in both photoreceptors and clock neurons occurs in the absence of neuronal death as assayed using confocal microscopy. Thus, tau-mediated neuronal dysfunction is separable from neuronal death.

In this thesis, we present two *Drosophila* models of tauopathy that recapitulate neuronal dysfunction in the absence of neuronal death seen in the early stages of the human disease. However, there are some key differences between the two models. The effects of human tau on retinal function and structure are progressive and age-related. Whereas, the effects of human tau on circadian locomotor behaviour are predominately neither progressive nor age-related. We demonstrate isoform-specific effects of tau-mediated neuronal dysfunction when expressed in either the *Drosophila* eye or clock system. The work presented in this thesis, will provide a platform for the future dissection of the mechanisms underlying tau-mediated neuronal dysfunction in the early stages of the disease process.

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