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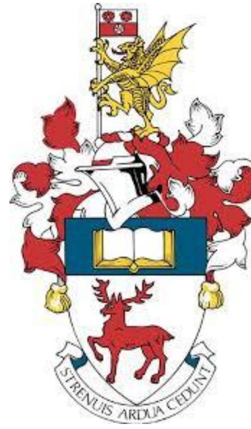


**Investigating how ageing contributes to tauopathy: implications for Alzheimer's Disease**

by

**Megan Abbie Sealey**

Thesis for the degree of Doctor of Philosophy

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# ABSTRACT

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**Investigating how ageing contributes to tauopathy: implications for Alzheimer's Disease**

Megan Abbie Sealey

Age is the single biggest risk factor for Alzheimer's Disease (AD). It is currently unclear how cellular changes that occur during ageing predispose people to the formation of the tau and Aβ pathologies found in AD. Recently, the same cellular pathways that are implicated in ageing have also been found to be altered in AD. These pathways include those involved in the maintenance of protein turnover such as mTORC1 signalling and autophagy. To understand how ageing contributes to the risk of developing AD we sought to analyse how these pathways change with age and how they can impact on the tau pathology found in AD.

To initially understand age-related changes in tau, different tau isoforms of human tau were expressed in *Drosophila* to assess age-related changes in tau-mediated phenotypes and pathologies. Htau$^{0N3R}$ expression caused reduced lifespan and deficits in age-related climbing ability compared with controls. Htau$^{0N4R}$ induced less severe effects on both longevity and climbing than htau$^{0N3R}$ flies. Specific phosphorylation sites were also found to be altered, with age, in htau$^{0N3R}$ but not in htau$^{0N4R}$ flies, implicating phosphorylation in age-related phenotypes. Both isoforms of htau were found to accumulate with age, suggesting dysfunction in pathways that regulate protein turnover. In addition, htau$^{0N3R}$ flies were assessed for changes in circadian rhythms. Expression of htau$^{0N3R}$ in the central clock neurons induced slowing of the circadian clock. htau$^{0N3R}$ was also demonstrated to induce deficits in short term memory tested using a new high throughput adaptation of a learning and memory assay.

To understand how pathways involved in regulation of protein turnover are altered in normal ageing, components of both mTORC1 signalling and autophagy pathways were assessed biochemically for age-related changes in human cortical brain tissue resected from differently aged patients undergoing neurosurgery. The mTORC1 signalling pathway was found to be less active with age and this was correlated with increased levels of autophagy and reduced total and phospho tau levels. Genetic up-regulation of autophagy in htau$^{0N3R}$ *Drosophila* similarly resulted in reduced deficits in locomotion and memory, together with reduced age-related accumulation of total and phospho htau$^{0N3R}$ protein. These studies demonstrate the importance of pathways that regulate protein turnover, such as mTORC1 signalling and autophagy, in both ageing and disease. Understanding how these pathways become dysregulated in disease has the potential to lead to new therapeutic targets for AD.

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# DECLARATION OF AUTHORSHIP

I, Megan Sealey

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

**Investigating how ageing contributes to tauopathy: implications for Alzheimer's Disease**

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed:

Date:

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# Definitions and Abbreviations

| **3R** | 3-repeat |
|---|---|
| **3xTg** | Triple transgenic |
| **4E-BP1** | Eukaryotic initiation factor E-binding protein 1 |
| **4R** | 4-repeat |
| **AD** | Alzheimer's Disease |
| **ADNP** | Activity-dependent neuroprotective protein |
| **AL** | Antennal lobe |
| **ALS** | Amyotrophic lateral sclerosis |
| **AMPA** | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| **AN** | Antennal nerve |
| **ANOVA** | Analysis of variance |
| **APOE** | Apoliproprotein |
| **APP** | Amyloid precursor protein |
| **APS** | Aversive phototaxic suppression |
| **Atg** | Autophagy-related gene |
| **ATP** | Adenosine triphosphate |
| **AVP** | Arginine vasopressin |
| **Aβ** | Amyloid beta |
| **BBB** | Blood brain barrier |
| ***C. elegans*** | *Caenorhabditis elegans* |
| **CBD** | Corticobasal degeneration |
| **CHO** | Chinese hamster ovary |
| **CK1** | Casein kinase 1 |
| **CRH** | Cortico-releasing hormone |
| **CRY** | CRYPTOCHROME |
| **DAM** | *Drosophila* activity monitoring system |
| **Dbt** | Double time |
| **DNA** | Deoxyribonucleic acid |
| **dnc**$^{ML}$ | Null mutant of dunce |
| **dtau** | *Drosophila* tau |
| **dTOR** | *drosophila* Target of Rapamycin |
| **EDTA** | Ethyenediamineetetracacetic acid |
| **eEF2** | Eukaryotic translation elongation factor 2 |
| **eEF2K** | Eukaryotic elongation factor 2 kinase |
| **EGFP** | Enhanced green fluorescent protein |
| **eIF4E** | Eukaryotic translation initiation factor 4 E |
| **eIF4G1** | Eukaryotic translation initiation factor 4 gamma 1 |
| **Elav** | Pan-neuronal driver |
| **FRET** | Fluorescence resonance energy transfer |
| **FTDP-17** | Frontotemporal dementia with Parkinsonism linked to chromosome 17 |
| **GAL4** | Yeast transcription activator protein |
| **GAL80**$^{ts}$ | Temperature-sensitive repressor of GAL4 |
| **GSK-3** | Glycogen synthase kinase |
| **HCl** | Hydrogen chloride |
| **htau**$^{0N3R}$ | Human tau 0N3R isoform |
| **htau**$^{0N4R}$ | Human tau 0N4R isoform |
| **LTP** | Long term potentiation |
| **MARK** | Microtubule affinity-regulating kinase |

| | |
|---|---|
| **MB** | Mushroom bodies |
| **MBC** | Mushroom body calyx |
| **MES** | 2-(N-morpholino)ethanesulfonic acid |
| **mRNA** | Messenger ribonucleic acid |
| **mtDNA** | Mitochondrial DNA |
| **mTOR** | mammalian Target of Rapamycin |
| **NaCl** | Sodium chloride |
| **NAP** | NAPVISPQ peptide |
| **NFT** | Neurofibrillary tangles |
| **NMDA** | N-methyl-D-aspartate |
| **NMJ** | Neuromuscular junction |
| **PAD** | Phosphatase-activating domain |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **Pdf** | Pigment dispersing factor |
| **PER** | PERIOD |
| **PHF** | Paired helical filaments |
| **PiD** | Pick's Disease |
| **PIKK** | Phosphatidylinositol 3-kinase-related kinase |
| **PKA** | Protein kinase A |
| **PP1** | Phosphatase-1 |
| **PP2A** | Phosphatase-2A |
| **PP5** | Phosphatase-5 |
| **PSP** | Progressive supranuclear palsy |
| **RNAi** | RNA interference |
| **ROS** | Reactive oxygen species |
| **rpm** | Revolutions per minute |
| ***S. cerevisiae*** | *Saccharomyces cereviciae* |
| **S6K** | Ribosomal protein S6 kinase |
| **SCN** | Suprachiasmatic nuclei |
| **SDS** | Sodium dodecyl sulfate |
| **Ser** | Serine |
| **sgg** | Shaggy |
| **Shot** | Short stop |
| **SYA** | Sugar yeast agar diet |
| **TBE** | Tris/Borate/EDTA |
| **TBS** | Tris-buffer saline |
| **Thr** | Threonine |
| **TIM** | TIMELESS |
| **TORC** | Target of rapamycin complex |
| **TSC** | Tuberous sclerosis protein |
| **TUNEL** | Terminal deoxynucleotidyl transferase dUTP nick end labelling |
| **UAS** | Upstream activating sequence |
| **ULK1/Atg1** | Autophagy activating kinase 1 |
| **UPS** | Ubiquitin proteasome system |
| **VIP** | Vasoactive intestinal polypeptide |
| **WT** | Wild-type |

# Chapter 1: Introduction

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## 1.1 Alzheimer's Disease

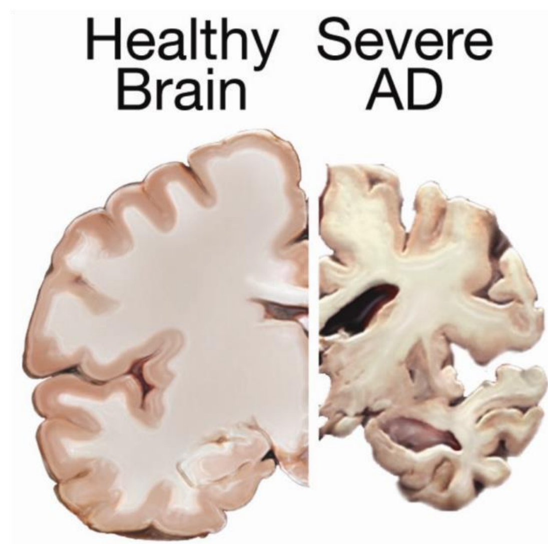
Alzheimer's Disease (AD), first described in 1907 by Alois Alzheimer (Stelzmann et al., 1995) is the most common cause of dementia in the world. There are an estimated 46.8 million people with dementia worldwide and this number is set to double to 74.7 million by 2030 (Prince et al., 2015). Age is the biggest risk factor for AD and therefore the ageing global population is driving this striking increase in AD cases. Understanding why age increases the risk of AD is vital to understanding the mechanisms that underlie the disease and it is this which will form the main focus of the research presented here.

AD begins as minor deficits in short term memory, such as, forgetting recent names and faces or regularly misplacing items. These symptoms progress over time to patients being unable to carry out daily activities and a worsening of cognitive abilities. Patients often go on to develop deficits in orientation, language and decision making (Yiannopoulou & Papageorgiou, 2013). Other symptoms such as changes to sleep/wake cycles and disruptions to circadian rhythms have also been found to manifest early in AD patients (Prinz et al., 1982, Hatfield et al., 2004). Due to the highly debilitating nature of this disease and the need for high levels of care, the total estimated worldwide cost of dementia in 2015 is $818 billion (Prince et al., 2015). This will be a continuing burden on the global economy unless a disease-modifying treatment is found.

Currently, there is no cure for AD and there are very few effective treatments. The treatments that are available are restricted to treating the symptoms of AD. One type of treatment consists of the cholinesterase inhibitors that target the loss of the neurotransmitter acetylcholine. Cholinesterase inhibitors inhibit acetylcholinesterase which is an enzyme that degrades the neurotransmitter acetylcholine, thereby increasing the levels of acetylcholine in the synaptic cleft. Cholinesterase inhibitors that are on the market for treatment of AD are donepezil (Pfizer), rivastigmine (Novartis) and galantamine (Birks, 2006). An alternative treatment for AD is memantine. This is an N-methyl-D-aspartate (NMDA) receptor antagonist and believed to protect cholinergic neurons from excitotoxicity (Rogawski & Wenk, 2003). In addition, antispsychotic and antidepressant treatments are also used to treat the behavioural and psychological symptoms of the disease. These drugs have shown some benefits to patients in mild to moderate forms of AD but not in late stages or in severe AD. These drugs are also not disease-modifying and therefore will only delay the decline in cognitive abilities. With AD on the rise and there being a lack of disease-modifying treatments, there is a drive to understand the pathogenesis of the disease so that new therapeutic interventions can be found.

The first clues to understanding the pathogenesis of AD lie in the changes that have been found in AD brains. One major difference in AD is that the brain shows increased atrophy (Figure 1-1a). In addition two main pathologies are found in the brains of people with AD, namely extracellular plaques (Figure 1-1b) made up of amyloid beta (Aβ) protein (Masters et al., 1985) and intracellular neurofibrillary tangles (NFTs) (Figure 1-1c) made up of tau protein (Grundke-Iqbal et al., 1986a). Understanding how these pathologies form in AD is a key focus of AD research. This thesis will form part of this research and will investigate how ageing contributes to the accumulation of these pathologies in AD, with a specific interest in how ageing impacts on tau pathology.

A

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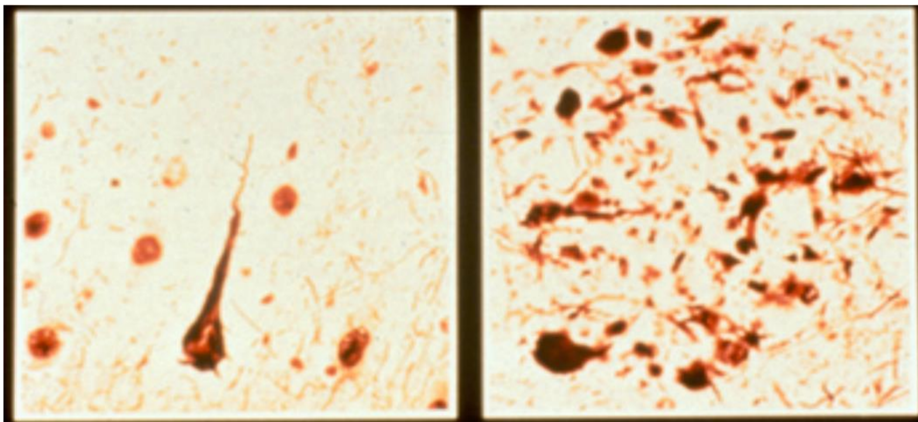


Figure 1-1. The pathological hallmarks of AD.
A) Atrophy of the brain in AD compared to control brain B) An intracellular neurofibrillary tangle made up of hyperphosphorylated tau. C) Extracellular plaques made up of Aβ protein. (Image s courtesy of National Institute on Aging/ National Institute of Health).

## 1.2 The pathological hallmarks of AD: Aβ

Although the main focus of this thesis will be on tau pathology, the pathogenesis of tau is inherently linked to Aβ and therefore requires some introduction. Aβ has long been thought to be a key initiator of AD. This hypothesis was based on studies conducted on rare genetic forms of the disease that cause between 1-5% of all AD cases. Some of these genetic forms of the disease are caused by mutations in the amyloid precursor protein (APP) gene (Levy et al., 1990), which Aβ is cleaved from (Haass et al., 1992). In addition, mutations in genes that affect APP processing, such as presenilin 1 and 2 have also been found to cause genetic forms of AD (Scheuner et al., 1996). The finding that these mutations cause genetic forms of the disease has led to the generation of the amyloid cascade hypothesis. This postulates that the pathogenesis of AD is initiated by increased levels of Aβ. Aβ then aggregates to form oligomers that are thought to cause detrimental effects to a range of cellular processes and functions including synaptic function, axonal transport and the proteasome. These deficits then accumulate to produce progressive injury to synapses and neurons and, over time, are thought to induce oxidative stress (Hardy & Selkoe, 2002).

How tau becomes involved in this cascade of events is currently unclear. However, there is evidence to suggest that tau is required to mediate Aβ toxicity. Aβ has been demonstrated to induce deficits in long term potentiation (LTP) when applied to mouse hippocampal neurons. However, one such study demonstrated that when Aβ oligomers were added to hippocampal slices from tau knockout mice Aβ no longer induced deficits in LTP (Shipton et al., 2011). Studies have also investigated reducing Aβ pathology by using antibodies that target Aβ. This study demonstrated that Aβ immunisation significantly reduced plaque pathology from the brains of mice (Maeda et al., 2007). Therefore, immunisation against Aβ was investigated as a potential therapeutic intervention and was taken forward into clinical trials. However, clinical trials utilising immunisation against Aβ have been largely unsuccessful. One such study showed that immunisation against Aβ in AD patients was found not to improve clinical prognosis, although it did lead to the removal of plaques from the brain (Holmes et al., 2008). A follow-up investigation revealed that although immunisation against Aβ cleared the plaques, tau-containing NFTs remained (Boche et al., 2010). These studies collectively demonstrate the importance that tau has in mediating Aβ toxicity in AD. Therefore, understanding the roles that tau plays in mediating disease is of paramount importance to unravelling the mechanisms that underlie AD.

## 1.3 The pathological hallmarks of AD: Tau

Tau was first discovered in 1975 in porcine brain (Weingarten et al., 1975). Tau was found to localise to axons (Binder et al., 1985) and bind to tubulin inducing microtubule assembly, in vitro (Kempf et al., 1996). Interest in the role of tau in dementia gathered pace after the discovery that genetic mutations in tau cause certain types of Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (Hutton et al., 1998, Poorkaj et al., 1998, Spillantini et al., 1998). These diseases are characterised by the accumulation of pure tau pathology, whereas AD exhibits both Aβ and tau pathology. These studies provide clear evidence that mutations in tau can directly cause dementia, fuelling investigations in to how tau is potentially involved in AD. Since then there have been a wealth of studies that have demonstrated tau's involvement in not just AD but in a number of other diseases, collectively known as tauopathies. However, to understand how tau becomes pathological in disease, it is important to first consider how tau normally functions in the human brain.

The human microtubule-associated protein tau (MAPT) gene, localised to chromosome 17q21.1 is encoded by a single gene composed of 16 exons (Neve et al., 1986). Alternative splicing of exons 2, 3 and 10 results in 6 different isoforms of tau (Figure 1-2) all of which are found in the adult human brain. These isoforms range in size from 352 to 441 amino acids long and are between 45 kDa- 65 kDa in size (Buée et al., 2000). These different isoforms can be separated into two main types, namely 3-repeat (3R) and 4-repeat (4R) isoforms. This refers to the number of tandem repeat sequences contained within the carboxyl terminal (C-terminal). These are the microtubule binding repeats that bind to tubulin. The number of which is determined by alternative splicing of exon 10. The alternative splicing of exons 2 and 3 determines the expression of the N-terminal repeats with different isoforms containing 0,1 or 2 repeats (Himmler et al., 1989). The N-terminal repeats are thought to be involved in interacting with the plasma membrane/cytoskeletal proteins and also in signal transduction (Brandt et al., 1995).

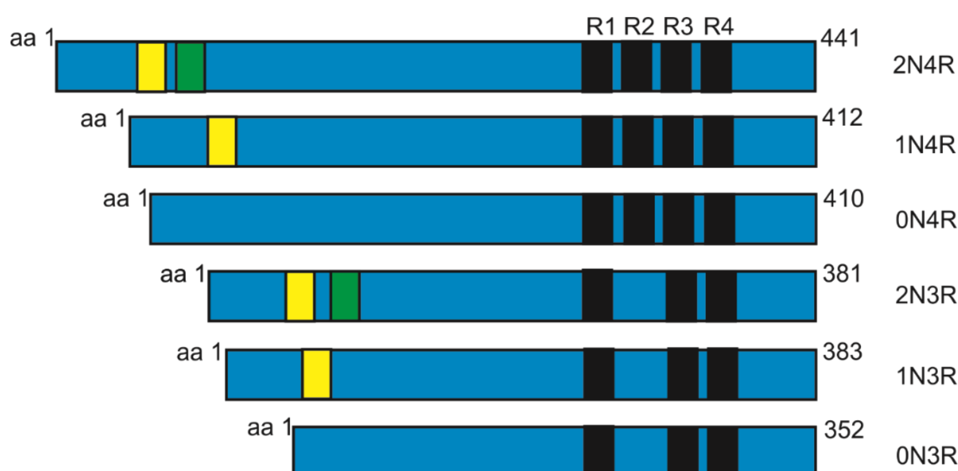



Figure 1-2. The MAPT gene is alternatively spliced into 6 tau isoforms in the human brain.
The different isoforms are referred to as either 3R or 4R tau, depending on the number of microtubule binding repeats (R1-4) in the C-terminus. 3R and 4R isoforms of tau are further classified depending on the presence of N-terminal inserts (0N, 1N and 2N).

The different isoforms of tau have been implicated in different diseases, collectively referred to as tauopathies, that develop tau pathology. For example, in some diseases, such as AD, both 3R- and 4R-isoforms make up the tau pathology, whereas Pick's Disease (PiD) is characterised by pathology made up of 3R-isoforms. Progressive supranuclear palsy (PSP) and Corticobasal degeneration (CBD) are examples of tauopathies that contain only 4R-isoforms (Sergeant et al., 2005). This suggests that the different isoforms have different roles in disease. It is possible that the different isoforms have different physiological roles as well. For example, the shortest isoform of tau (0N3R) is the only tau isoform expressed in foetal brain, whereas all six isoforms are expressed in adulthood (Kosik et al., 1989). There is also evidence to suggest that different tau isoforms are differentially expressed in different subsets of neurons, for example only 3R-isoforms are found in granular cells of the dentate gyrus (Goedert et al., 1989). The alternative expression of the N- and C-terminal repeats potentially underlies the differences in functions of the different isoforms. For example, it has been demonstrated that 4R-isoforms bind to and stabilise microtubules more strongly than 3R-isoforms (Goode et al., 2000, Panda et al., 2003). These studies demonstrate distinct differences between tau isoforms and how this may underlie the different tauopathies. However, more research is required to understand the differences in 3R- and 4R-isoforms and how they are implicated in the different tauopathies.

Table 1. List of tauopathies and the isoforms of tau that are found in the pathology of each disease.

| Disease | Isoform of tau |
|---|---|
| Alzheimer's Disease (AD) | 3R & 4R isoforms |
| Amyotrophic lateral sclerosis/ parkinsonism-dementia complex of Guam (ALS) | 3R & 4R isoforms |
| Argyrophilic grain disease (AGD) | 4R isoforms |
| Corticobasal degeneration (CBD) | 4R isoforms |
| Dementia pugilistica | 3R & 4R isoforms |
| Down's Syndrome | 3R & 4R isoforms |
| Familial British dementia | 3R & 4R isoforms |
| Frontal lobe degeneration | Loss of tau protein |
| Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) | 3R & 4R isoforms |
| Globular glial tauopathy (GGT) | 4R isoforms |
| Hippocampal tauopathy in cerebral ageing | 3R & 4R isoforms |
| Neurofibrillary tangle-only dementia (NFT-dementia)/ | 3R & 4R isoforms |
| Niemann-Pick disease type C | 3R & 4R isoforms |
| Parkinsonism with dementia of Guadeloupe | 3R & 4R isoforms |
| Pick's Disease (PiD) | 3R isoforms |
| Postencephalitic parkinsonism | 3R & 4R isoforms |
| Progressive supranuclear palsy (PSP) | 4R isoforms |

## 1.4 Mechanisms of tau pathogenesis

### 1.4.1 Tau phosphorylation

This thesis will investigate how ageing impacts on tau pathogenesis, therefore, it is important to understand the different mechanisms in which tau becomes pathogenic in disease. These mechanisms, among others, include phosphorylation, misfolding and aggregation. Phosphorylation is thought to be an early event in tau pathogenesis occurring prior to misfolding and aggregation (Noble et al., 2013). Tau was first identified as a phospho protein in 1977 (Cleveland 1977). Interest in tau phosphorylation and its relevance to pathology grew with the discovery that the paired helical filaments (PHF)s found in AD are made up of phosphorylated tau (Grundke-Iqbal et al., 1986a, Grundke-Iqbal et al., 1986b). PHFs are thought of as the predecessor step to the formation of NFTs and will be discussed further below. It has been demonstrated that phosphorylation is involved in regulating tau's ability to bind to microtubules. For example, dephosphorylation of tau, in vitro, causes increased polymerisation of microtubules (Lindwall & Cole, 1984). This suggests that phosphorylation of tau inhibits its ability to bind to microtubules.

In AD, tau has been found to be highly phosphorylated relative to control brains. Studies in human AD brain have demonstrated that the tau found in PHFs is phosphorylated 3-4

fold more than that of tau in control brains (Ksiezak-Reding et al., 1992). This suggests an integral role of tau phosphorylation in the pathogenesis of AD. In addition, there are over 85 potential phosphorylation sites of tau, approximately 10 of which are phosphorylated in tau in control brains (Hanger et al., 2007). Whereas in AD, 45 sites have been reported to be phosphorylated (Noble et al., 2013). These sites are mostly found in the proline-rich domain in tau or flanking the microtubule binding domains, as depicted in Figure 1-3. Specific sites of phosphorylation have been identified as being important not only in the functions of tau but also in how tau becomes pathogenic in AD.

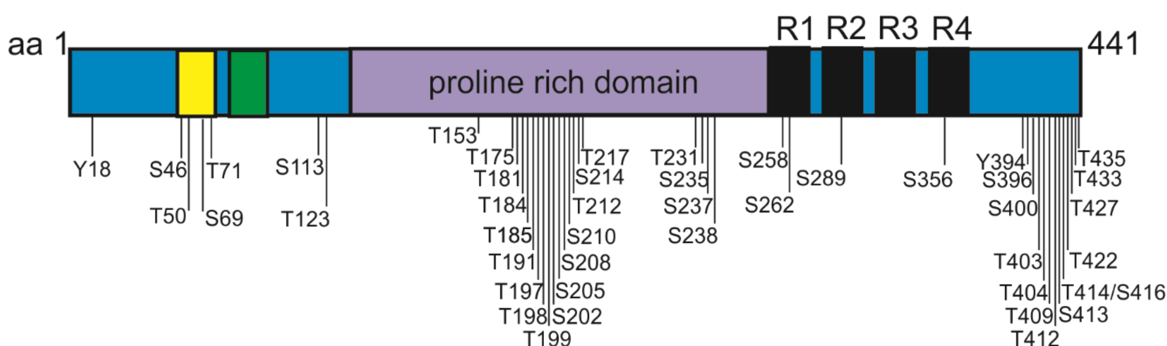



Figure 1-3. The 45 phosphorylation sites that have been shown to be phosphorylated in AD brain (Noble et al., 2013).

Specific phosphorylation sites on tau have been found to be regulated by a number of different kinases and phosphatases in the human brain. The kinases known to phosphorylate tau can be broadly separated into two main classes. The first class, the proline-directed kinases, include glycogen synthase kinase-3 (GSK-3) (Hanger et al., 1992), cyclin-dependent kinase 5 (cdk5) (Baumann et al., 1993) and 5' adenosine monophases-activated protein kinase (AMPK) (Thornton et al., 2011). The second main class of kinases that are known to phosphorylate tau are the non-proline-directed kinases that include casein kinase 1 (CK1) (Hanger et al., 2007), microtubule affinity-regulating kinases (MARKs) (Drewes et al., 1995) and cyclic AMP-dependent protein kinase A (PKA) (Andorfer & Davies, 2000). Tyrosine kinases such as Fyn (Lee et al., 2004) have also been implicated in phosphorylating tau. In addition to kinases that phosphorylate tau, phosphatases that de-phosphorylate tau have also been implicated in regulating tau phosphorylation. These include the protein phosphatases -1 (PP1), -2A (PP2A) and -5 (PP5) (Liu et al., 2005). The dysregulation of these various kinases and phosphatases is thought to be key in the pathogenesis of AD.

One such kinase that is thought to be dysregulated in tauopathies is GSK-3. GSK-3 is known to phosphorylate 26 of the 45 of the sites phosphorylated in AD brains (Noble et al., 2013). There are two isoforms of GSK-3 found in mammals, namely GSK-3α and

GSK-3β (Doble & Woodgett, 2003). GSK-3β has been found to co-localise with NFTs in AD brain (Yamaguchi et al., 1996, Pei et al., 1997). In addition, the active form of GSK-3β (phosphorylated at Tyr326) has been found to be increased in the frontal cortex of AD brains. These studies demonstrate that GSK-3 activity is increased in AD. Additionally in mice, overexpression of GSK-3β induces hyperphosphorylation of tau and subsequent neurodegeneration in the hippocampus (Lucas et al., 2001), suggesting a role of GSK-3β in mediating tau-toxicity. Others have also demonstrated that inhibition of GSK-3β can reduce tau phosphorylation and alleviate tau-mediated toxicity. For example, lithium, which is a GSK-3β inhibitor has been used to reduce tau phosphorylation and in addition caused decreased neurodegeneration in a mutant tau mouse model (Noble et al., 2005). Similarly, studies in flies that overexpress a human 3R-isoform ($htau^{0N3R}$) found that both lithium and a specific GSK-3β inhibitor (AR-A014418) reduced tau phosphorylation. These studies also reported that this rescued tau-mediated locomotor and axonal transport deficits in lithium/AR-A014418 treated flies (Mudher et al., 2004). These studies collectively demonstrate the importance of GSK-3β in the pathogenesis of tau and highlights it as a possible therapeutic target for the treatment of tauopathies.

### 1.4.2 Misfolding

Phosphorylation is also implicated in causing conformational changes and the misfolding of tau. Tau normally does not have a particularly ordered secondary structure (Schweers et al., 1994). Studies have shown that tau, in solution, has a loose "paperclip" or double hairpin conformation, where the protein folds so that the C-terminus is next to the microtubule binding domain and the N-terminus is folded back towards the C-terminus, as shown in (Figure 1-4) (Jeganathan et al., 2006). It has been demonstrated that phosphorylation induces a tighter folding of this conformation, inducing its reactivity to conformational specific antibodies, such as Alz50 and MC1. Interestingly, these conformational antibodies bind more readily to tau found in AD brains compared to in control brains (Nukina et al., 1988, Weaver et al., 2000), suggestive of misfolding being an important aspect to tau pathogenesis. These antibodies have been demonstrated to bind to a discontinuous sequence of tau which includes the N-terminus and the microtubule binding domain (Carmel et al., 1996). In addition, Alz50 is only reactive to tau that is also phosphorylated at Ser396 (Mondragón-Rodríguez et al., 2008), suggesting that phosphorylation at Ser396 is necessary for conformational changes in tau. In addition, in vitro studies have demonstrated that phosphorylation at specific sites (Ser202/Thr205 and Ser396/Ser404) also induce conformational change in tau. This study suggested that

phosphorylation of these sites induces a tighter folding of tau and renders the tau reactive to the MC1 antibody (Jeganathan et al., 2008). These studies collectively demonstrate the involvement of phosphorylation in causing conformational changes in tau. Some studies have also demonstrated how misfolding of tau can induce toxicity. One study found that misfolding exposed a section of the N-terminus of tau, termed the phosphatase-activating domain (PAD) (Kanaan et al., 2011). This domain triggers a signalling cascade that activates GSK-3 and consequently has been shown to disrupt axonal transport in squid axoplasm. In addition, an antibody that binds specifically to PAD binds more readily to the tau found in AD brains (Kanaan et al., 2011). This study demonstrates the importance of misfolding in the pathogenesis of tau and the toxic-mediated effects of tau on axonal transport.

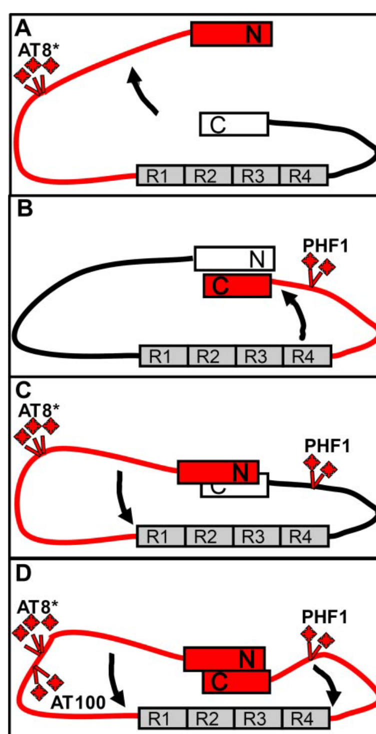



Figure 1-4. "Paperclip" conformation of tau, taken from (Jeganathan et al., 2006). Phosphorylation at AT8 (Ser202/Thr205), PHF-1 (Ser396/Ser404) and AT100 (Thr212/Ser214) sites induces a tighter folded conformation which is reactive to conformational specific antibodies, Alz50 and MC1.

### 1.4.3 Aggregation

In addition to misfolding, tau is also known to aggregate into a number of different species. It is currently unclear what the toxic species of tau are that might mediate the pathogenesis of AD. Tau can be present (in ascending size order) as monomers, dimers, trimers, soluble oligomers, granular insoluble oligomers, filaments, PHFs and NFTs (Cowan & Mudher, 2013), (Figure 1-5). It was originally thought that the toxic species of tau were the larger

species, namely PHFs and NFTs. Evidence that corroborates this includes the spread and accumulation of NFTs across the brain correlating with the severity of dementia of symptoms in AD (Arriagada et al., 1992). Neuronal loss is also found to correlate with NFTs in mutant tau mouse models (Lewis et al., 2000). The human tau expressed in this particular mouse model contains the P301L mutation which has been found to cause FTDP-17. Similar studies where large insoluble aggregates were removed by immunisation against tau improved locomotor phenotypes in P301L mice (Asuni et al., 2007). In addition, using recombinant tau, the P301L mutation has been demonstrated to cause tau filaments to form faster than WT tau (Nacharaju et al., 1999), suggesting a causal role of filaments in dementia. However, there have been studies that also implicate the smaller species of tau in inducing toxicity. In mouse models of tauopathy where tau expression is switched off, memory deficits were found to improve but NFTs were still found to accumulate (SantaCruz et al., 2005, Spires et al., 2006), suggesting a different species is responsible for tau-mediated deficits in memory. In addition, various models of tauopathy including many *Drosophila* models present neurodegeneration and neurodysfunction mediated by tau without the presence of NFTs and PHFs (Wittmann et al., 2001, Mudher et al., 2004, Mershin et al., 2004, Kosmidis et al., 2010). Also, the appearance of oligomeric tau coincides with memory deficits that manifest before any NFTs develops in mouse models of tauopathy (Berger et al., 2007). These studies demonstrate the complexity that different tau species may mediate in disease. The general consensus is that the most likely toxic species in AD are small soluble oligomers, as PHFs and NFTs are not necessary to induce tau-mediated phenotypes. However, it remains unclear whether other species may also confer toxicity as well.

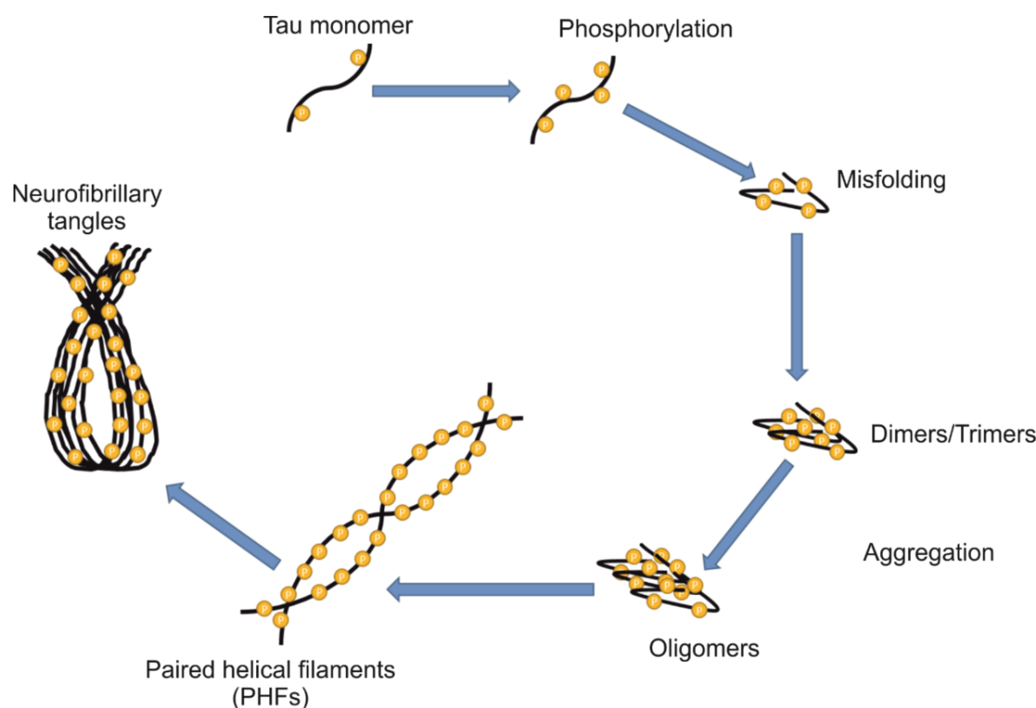



Figure 1-5. The generation of different tau species; phosphorylation, misfolding and aggregation. Tau monomers become increasingly phosphorylated at specific phosphorylation sites, which induces misfolding of tau. Misfolding is hypothesised to preclude aggregation into dimers, trimers and larger aggregates termed oligomers. These are then thought to aggregate to form larger species termed paired helical filaments (PHFs) and neurofibrillary tangles (NFTs).

## 1.5 Mechanisms of tau toxicity

### 1.5.1 Microtubule destabilisation

It has been described above that tau becomes increasingly phosphorylated, misfolded and aggregated in AD but how do these pathogenic alterations in tau lead to tau-mediated toxicity of neurons? Phosphorylation of tau is thought to be key to tau's role in stabilising microtubules. It is hypothesised that in disease, phosphorylation of tau causes decreased binding to microtubules and consequently leads to a breakdown in axonal transport (Figure 1-6) (Cowan et al., 2010). In support of this hypothesis, a reduction in stable microtubules has been demonstrated in AD brain (Hempen & Brion, 1996). Phosphorylation of tau at specific sites has been implicated in reducing the ability of tau to bind to microtubules. These include, among others, phosphorylation at sites Ser262, Thr231, Ser235 and Ser214 (Sengupta et al., 1998, Schneider et al., 1999). For example, site-directed mutagenesis studies have been carried out that mutate Ser262 to either an alanine (rendering the site unphosphorylatable) or to a glutamate (mimicking hyperphosphorylation). Ser262 phosphorylation has been demonstrated, in vitro, to inhibit

the binding of tau to microtubules (Sengupta et al., 1998). Similarly, mimicking phosphorylation of Ser262, Ser293, Ser324 and Ser356 sites caused significantly reduced binding of tau to microtubules (Fischer et al., 2009). Importantly, phosphorylated tau isolated from AD patient brains has been demonstrated to inhibit the assembly of microtubules in vitro. Additionally, when this phosphorylated tau was treated with alkaline phosphatase, the tau was able to induce assembly of the microtubules (Alonso et al., 1994). In addition, studies performed in cells have also demonstrated that phosphorylation is vital in tau's role to bind to microtubules. CHO cells transfected with mutant tau that was unphosphorylatable at Ser396 increased tau's affinity for microtubules and subsequently increased stability of the microtubules (Bramblett et al., 1993). In a different approach, COS cells were transfected with both tau and GSK-3β and found this to also reduce tau's binding to microtubules (Lovestone et al., 1996). These studies demonstrate that phosphorylation of certain sites on tau are important for its role in binding to and stabilising microtubules. Increased phosphorylation of tau has been demonstrated to cause decreased binding of microtubules, leading to microtubule destabilisation.

Microtubule destabilisation has been demonstrated in a number of different tau models. For example, in *Drosophila* where the shortest isoform of tau was expressed ($htau^{0N3R}$) found that $htau^{0N3R}$ did not bind efficiently to the microtubules. It was also found that the hyper-phosphorylated $htau^{0N3R}$ sequesters endogenous *Drosophila* tau (dtau) away from the microtubules by binding to dtau (Figure 1-6). Using electron microscopy, a loss of microtubular structure was demonstrated in $htau^{0N3R}$ expressing flies. It was also found that expression of $htau^{0N3R}$ mediated a breakdown in axonal transport (Mudher et al., 2004, Cowan et al., 2010, Quraishe et al., 2013). Similar findings were also gathered from a mouse model that also expressed $htau^{0N3R}$. This study found a reduction of intact microtubules and loss of axonal transport (Ishihara et al., 1999). These studies demonstrate that phosphorylation of tau can cause microtubule destabilisation and subsequently a breakdown in axonal transport. This would inhibit the necessary transport of materials from the soma to the synapse, potentially triggering the initiation of neurodegeneration.

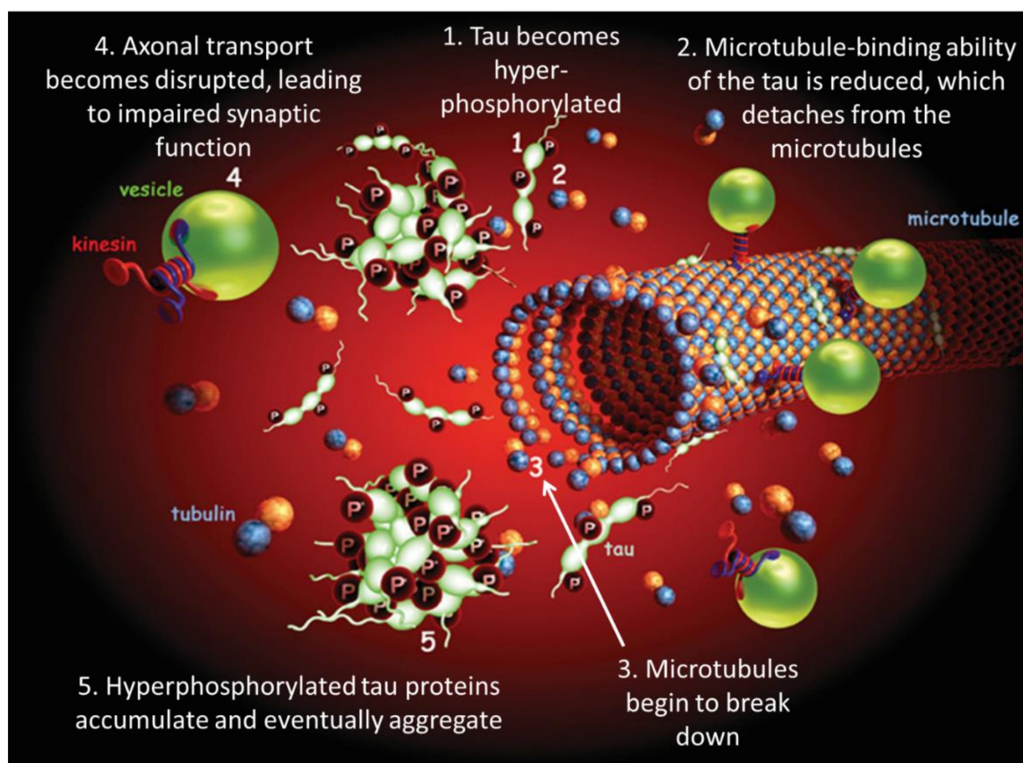



Figure 1-6. Tau microtubule hypothesis adapted from (Cowan et al., 2010).
Tau is hypothesised to cause neurodegeneration through (1) becoming hyperphosphorylated, (2) reducing tau's normal function of binding to microtubules. This (3) destabilises the microtubule network causing (4) axonal transport to become disrupted which leads to synaptic dysfunction. The tau that detaches from the microtubules then (5) accumulates in the cytosol and aggregates.

The implication of tau-mediated microtubule destabilisation in disease has led to therapeutic strategies that target the re-stabilisation of microtubules. For example, Epothilone D is a microtubule stabilising compound which has been demonstrated to increase numbers of intact microtubules, reduce numbers of dystrophic axons and consequently cause improvements in learning and memory in a mouse model of tauopathy (P301S) (Brunden et al., 2010). A different microtubule stabilising agent paclitaxel, has also been found to have similar effects in a different mouse model of tauopathy that expressed $htau^{0N3R}$. This study demonstrated that paclitaxel rescued axonal transport deficits induced by $htau^{0N3R}$ expression and this was correlated with improvement in tau-mediated deficits in locomotion (Zhang et al., 2005). However, agents such as paclitaxel do not easily cross the blood brain barrier (BBB) and are toxic to neurons (Scuteri et al., 2006). Therefore, other agents have been recently investigated that are non-toxic and do permeate the BBB (Heimans et al., 1994). Such an agent is a small peptide, NAPVSIPQ (NAP) which is derived from activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999). NAP has been shown to stabilise microtubules and improve axonal transport in $htau^{0N3R}$ expressing flies (Quraishe et al., 2013). Collectively, these studies demonstrate microtubule destabilisation is key to tau-mediated toxicity and also highlights this mechanism as a promising avenue for new therapeutic targets.

### 1.5.2 Tau propagation

Another mechanism of tau-mediated toxicity is in its propensity to propagate and spread across the brain, affecting a number of different brain regions (Braak & Braak, 1995). It has been demonstrated that NFTs accumulate and propagate across the brain in a characteristic pattern as AD progresses. This is termed as Braak staging and involves 6 different stages (Braak & Braak, 1995). In stages I-II tangles begin to appear in the transentorhinal regions of the brain. This occurs in clinically silent patients, where symptoms have not begun to appear. In stages III-IV, NFTs can also be found in the limbic regions of the brain with AD symptoms beginning to manifest at this stage. In stages V-VI, AD symptoms are fully developed and NFTs are found to have spread to the neocortical structures of the brain (Braak & Braak, 1995). This shows that the spread of NFTs closely correlates with the severity of disease symptoms demonstrating a clear link between tau propagation and the pathogenesis of AD.

But how does tau propagation occur? It has been demonstrated that tau can spread from cell to cell and cause normal tau to become pathogenic. This was demonstrated by injecting tau filaments from mutant (P301S) tau mice into the hippocampus of mice only expressing wild-type (WT) tau. The P301S model, similar to the P301L model, expresses a form of mutant tau that causes FTDP-17. WT mice do not normally develop filaments however, after injection of the P301S human tau filaments, WT mouse tau was also found to aggregate. Interestingly, this did not just occur at the injection site but also in adjacent cells and even in distant cells that were synaptically connected (Clavaguera et al., 2009). This not only demonstrates that tau can spread from cell to cell but pathogenic tau has the ability to convert normal tau. This has been replicated in a model where human mutant (P301L) tau was expressed genetically only in the entorhinal cortex. This study subsequently found tau pathology that had spread to other regions of the brain that were synaptically connected including the dentate gyrus, hippocampus and cingulate cortex (de Calignon et al., 2012). In addition it was found that human tau pathology seeded mouse tau aggregation by binding to mouse tau (de Calignon et al., 2012). This suggests that pathogenic tau can bind directly to normal tau inducing a conformational change in the normal protein, therefore acting prion-like. This has also been demonstrated in cells where intracellular tau fibrils are released and then taken up by adjacent cells. This study used FRET microscopy to demonstrate that tau fibrils could bind to naïve (non-pathogenic) tau inside cells and induce fibrillisation (Kfoury et al., 2012). Therefore, these studies suggest that the spread of pathogenic tau occurs between synaptically connected cells and causes normal tau to become pathogenic through a prion-like templating mechanism.

### 1.5.3 Neurodegeneration

But how does pathogenic tau cause neurodegeneration? This is currently unclear, however there are a number of theories. One theory is that tau causes synapses to become dysfunctional, causing a breakdown in communication between cells, ultimately triggering the neuron to degenerate. This is particularly relevant to AD as synaptic loss is thought to be an early event in neurodegeneration in AD (Terry et al., 1991, Masliah et al., 2001). This may well be caused due to loss of axonal transport mediated by tau-induced microtubule destabilisation, as discussed above. However, it has also been found that phosphorylation of tau causes mis-localisation of tau from axons to the somatodendritic compartment (Hoover et al., 2010) This study, in P301L tau-expressing mice also found that expression of tau caused decreased synaptic expression of AMPA and NMDA receptors (Hoover et al., 2010). Similarly, in a *Drosophila* model, hyperphosphorylated tau was found to cause changes to the morphology, alterations in exo/endocytosis and deficits in transmission at the neuromuscular junction (NMJ) (Chee et al., 2005). These studies demonstrate how tau can be toxic in synapses, potentially being an early event in neurodegeneration.

A different theory of tau-mediated toxicity is through cell-cycle re-entry. A number of different studies have demonstrated increased expression of cell-cycle proteins in neurons prior to degeneration in AD (Busser et al., 1998, Ding et al., 2000). Cell-cycle re-entry has been demonstrated to trigger neuronal apoptosis. In a *Drosophila* model expressing mutant human tau, cell-cycle activation resulted in increased TUNEL staining, indicative of apoptotic cells. Furthermore, this study demonstrated that if cell-cycle is inhibited, TUNEL staining was found to be reduced (Khurana et al., 2006). Caspase activation which activates apoptosis has been found in neurons in AD brains (Stadelmann et al., 1999, Rohn et al., 2001), providing evidence that apoptosis may be occurring in AD neurons. However, a study conducted in a mouse model of tauopathy used 2-photon imaging on living mouse brains to follow caspase- positive cells over time but did not find these neurons to undergo apoptotic neurodegeneration (Spires-Jones et al., 2008) therefore complicating the role that tau has in initiating apoptosis.

Collectively tau has been demonstrated to become increasingly phosphorylated, misfolded and aggregated in AD. These changes in the tau protein are closely linked with loss of function, through loss of the cytoskeletal structure, causing a breakdown in axonal transport and dysfunction of neurons. In addition, misfolding and aggregation are thought to lead to a toxic gain of function, where toxic aggregates cause neurodegeneration. These findings have led to the generation of possible therapeutic interventions that target these

pathogenic alterations in tau. However, it is still unclear what causes tau to become pathogenic initially. To investigate this, it is important to consider the risk factors of AD and how these potentially highlight pathways and mechanisms that cause tau to become pathogenic over time.

## 1.6 Risk factors for AD

Sporadic AD, which accounts for approximately 95% of all AD cases, has a range of genetic and environmental risk factors associated with it, examples of which are listed in Table 2. The greatest risk factor for AD is age, with the vast majority of patients being diagnosed at >65 years of age. Data from the World Alzheimer Report 2015 found that the risk of developing dementia increases exponentially as people age. This report combined many studies from around the world to find that the incidence of dementia doubles every 6.3 years of ageing. It was found that 3.9 people out of 1000 aged between 60-64 had dementia whereas 104.8 people per 1000 had dementia when aged 90+ (Prince et al., 2015). Investigating why ageing increases the risk of AD could lead to a better understanding of how AD is initiated potentially highlighting pathways upstream of tau pathogenesis. Therefore, this study will investigate pathways involved in ageing and how they impact on tau.

However, other risk factors, other than age, associated with AD also highlight certain cellular pathways that are implicated in the mechanisms that underlie the disease, which may also be relevant to tau. One such risk factor is Diabetes mellitus type 2 being diagnosed in middle to later ages. This can lead to approximately a 50% increase in the risk of developing AD (Profenno et al., 2010). This is a metabolic disorder characterised by increased blood glucose levels and insulin resistance. Therefore, implicating the insulin signalling pathway in AD. Other lifestyle risk factors for AD include smoking and obesity with protective factors including increased exercise and a mediterranean diet (Winblad et al., 2016) demonstrating an involvement of metabolic, respiration and nutrient-sensing pathways in AD. Interestingly, these pathways are also pathways that are implicated in ageing, providing more evidence for a strong correlation between ageing and AD.

Table 2. List of risk and protective factors for late onset sporadic AD (Winblad et al., 2016)

| Risk factors for sporadic AD | Protective factors for sporadic AD |
| --- | --- |
| Increasing age | Genetic factors (APOE ε2 allele) |
| Genetic factors (APOE ε4 allele, susceptibility genes CR1, PICALM, CLU, TREM2 etc.) | Psychosocial factors (High education and socioeconomic status, mentally stimulating occupation, rich social network and social engagement) |
| Vascular disease (cardiovascular, cerebral macrovascular and microvascular lesions) | Physical activity |
| Diabetes mellitus type 2 | Light-moderate alcohol intake |
| Obesity | Mediterranean diet |
| Lifestyle factors (sedentary lifestyle, smoking, heavy alcohol consumption) | Vitamins (A, B6, B12 C, D, E and folate) |
| Diet and nutritional factors (saturated fats, hyperchmocysteinaemia, deficiencies in vitamin B6, B12 and folate) | Drugs (antihypertensive drugs, statins, non-steroidal anti-inflammatory drugs) |

## 1.7 Introduction to ageing

To be able to understand why ageing increases the risk of AD it is important to firstly understand the ageing process. There are various theories of ageing that shed light on the potential cellular pathways involved in ageing and age-related disease. Age-related diseases, such as AD, are increasingly prevalent around the globe due to the world's increasingly ageing population. The 20$^{th}$ century has seen a huge leap in average lifespan of people from 33 to 80 years of age. This has been attributed to the various improvements seen in public health, hygiene, nutrition and availability of vaccines and antibiotics. These advances have meant decreases in infantile mortality and improved treatment of a great number of diseases. However, an increased average lifespan has resulted in an ageing population. Between 2015 and 2030 the number of people in the world that will be 60 years and older is predicted to increase by 56%, from 901 million to 1.4 billion people (United Nations 2015).

Ageing can be defined as a progressive decline in function which coincides with decreased fertility and increased probability of disease and mortality (Kirkwood & Austad, 2000). Ageing can also be thought of as a general wearing of bodily functions that reach a peak during development and maturation of an individual and then decline (Viña et al., 2007). Ageing is one of the few processes that affects most organisms - from unicellular organisms to plants, insects and mammals including humans. Understanding the mechanisms that underpin ageing is one of the biggest challenges for scientific research. For a long time, ageing was thought of as an inevitable process that could not be manipulated by extrinsic factors. However, studies on rats in the 1930's disproved this

when McCay et al., (1935) restricted food intake in rats, resulting in extended lifespan (McCay et al., 1935). This was the first convincing piece of evidence that ageing can indeed be regulated. Furthermore, genetic screens were conducted in *C. elegans* (Hamilton et al., 2005) where long lived mutants were identified. These studies highlighted possible candidate pathways involved in the regulation of ageing such as insulin signalling, metabolism, signal transduction, protein turnover and gene expression (Hamilton et al., 2005). Studies such as these have led to a number of different theories of ageing, some of which are also linked to age-related disease.

## 1.8 Theories of ageing

### 1.8.1 Mitochondrial theory of ageing

One such theory of ageing which has also been implicated in AD is the "free-radical theory of ageing". This theory postulates that reactive oxygen species (ROS) accumulate during ageing resulting in increased damage to cells and tissues, eventually leading to the death of the organism (Harman, 1955). As dysfunctional mitochondria are the main source of ROS in the cell, this theory evolved into "the mitochondrial theory of ageing" (Harman, 2009), implicating dysfunctional mitochondria as being integral to the ageing process. For example, age-related reductions in mitochondrial DNA (mtDNA) copy number have been found in the skeletal muscle of rats (Barazzoni et al., 2000). In addition, a study in rhesus monkeys demonstrated an age- related decrease in mitochondrial respiratory complex activities in the cerebral cortex (Bowling et al., 1993). In addition, genes involved in oxidative stress response and DNA damage repair are upregulated in ageing human cortex (Lu et al., 2004). Neurons are particularly susceptible to age-related dysfunctional mitochondria as they require higher levels of adenosine triphosphate (ATP) than other cell types. Therefore, accumulations of dysfunctional mitochondria, with age could well be catastrophic for neurons. Collectively these studies implicate dysfunctional mitochondria in ageing. Furthermore, as dysfunctional mitochondria are a key feature of AD as reviewed in (Castellani et al., 2002) mitochondrial dysfunction is a clear mechanism that connects both ageing and AD. Although mitochondrial dysfunction and ROS generation will not be a focus of the research here, it is inherently linked to other age-related pathways that will be studied here.

### 1.8.2 Dysregulation in protein turnover

An alternative theory of ageing that is also very relevant to tauopathies is that protein turnover becomes increasingly dysregulated with age. Protein turnover is dependent on two opposing processes; protein synthesis and protein degradation. A shift in the balance of these two processes could well underlie the accumulations of damaged proteins that build up in disease. New synthesis of proteins is important to replace proteins that have become damaged, for example, by oxidation. However, protein synthesis is generally though to decline with age (Makrides, 1983), although this has not been directly measured in the human brain. Much of the evidence that protein synthesis declines with age is dependent on findings in *C. elegans*. For example, Stable isotope labelling of mutant long-lived worms found decreased protein synthesis in older worms compared with young controls (Visscher et al., 2016).

Protein degradation is also an important aspect to protein turnover as this allows for damaged proteins to be removed from the cell. A number of pathways in the cell can lead to protein degradation, these include the ubiquitin proteasome system (UPS) and autophagy. Autophagy will be described in more detail below, as it will form part of the focus of this research. The UPS targets proteins for degradation by ubiquitination which acts as a degradation signal to the proteasome. The proteasome contains a hollow cavity in which protease activities act to breakdown proteins. Interestingly it has been found that proteasomal activities decline with age, for example in *Drosophila* (Vernace et al., 2007) and in rodents (Shibatani et al., 1996). Collectively, a down regulation of protein synthesis with age and reduced degradation could potentially lead to accumulation of damaged proteins, such as the Aβ and tau pathologies found in AD. This demonstrates a clear link between pathways that implicated in both with ageing and tauopathies. Therefore, protein turnover is an important avenue of research to understand why ageing increases the risk of AD.

### 1.8.3 Hyperfunction theory

Another theory of ageing that is connected to protein turnover is the hyperfunction theory. This theory postulates that hyperactivity of processes that contribute to development continue later on in life at an inflated level, leading to the accumulation of pathologies (Blagosklonny 2008). These processes include those that regulate protein synthesis, namely the nutrient-sensing pathways such as mammalian Target of Rapamycin (mTOR) and insulin signalling. For example, senescence in cells can be inhibited if treated with rapamycin, an inhibitor of the mTOR pathway (Demidenko et al., 2009). In addition, if

nutrients are reduced by dietary restriction, lifespan can be extended. This has been reproduced in many different species such as *C. elegans* (Walker et al., 2005), *Drosophila melanogaster* (Partridge et al., 2005), rodents and non-human primates as reviewed in (Fontana et al., 2010). It is currently unclear whether dietary restriction can slow ageing in humans, however, there are some studies to suggest this. One such study investigating cardiac function (which normally declines with age) found this to be improved in patients that were calorie restricted (Meyer et al., 2006). The hyper-function theory therefore highlights nutrient sensing pathways in ageing. This theory appears to be only part of the overall ageing process as it does not explain the damage that accumulates in ageing organisms, such as from oxidative stress. A model that integrates both damage accumulation and hyper-function would lead to a more encompassing model of ageing, as shown in (Figure 1-7).

This model of ageing proposed by Bishop et al., (2010) demonstrates an integration of the different theories discussed above. Mitochondria become increasingly dysfunctional with age, producing ROS that damage both proteins and DNA. Increasingly damaged proteins are not cleared away due to decreased clearance through the proteasome and autophagic pathways. Nutrient sensing pathways such as mTOR signalling affect both protein synthesis and autophagic clearance, as will be discussed further below (Bishop et al., 2010).

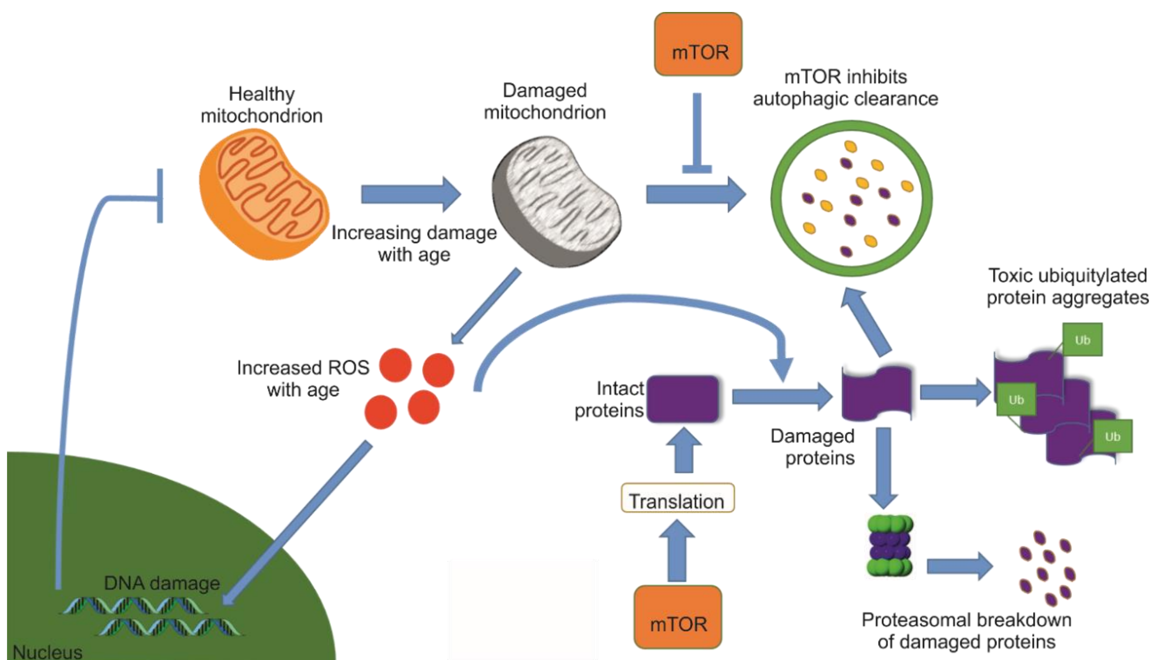



Figure 1-7. Hyperfunction and damage model of ageing, adapted from (Bishop et al., 2010).
This model hypothesises that mitochondrion become increasingly damaged with age, resulting in increased ROS. ROS causes damage to both DNA and proteins. Damaged proteins should be cleared/ broken down by the proteasome or autophagy. However, dysregulation of these clearance pathways could lead to the accumulation of aggregated proteins, such as those found in diseases like AD.

## 1.9 Cellular pathways implicated in ageing

The various theories of ageing have highlighted a number of different pathways, depicted in (Figure 1-8), that potentially regulate ageing. These include those pathways involved in accumulation of damage caused by stress, such as through mitochondrial dysfunction and production of ROS. In addition, pathways involved in regulation of protein synthesis and hyper-function such as insulin signalling and mTOR signalling have also been implicated in ageing. Lastly, those pathways involved in protein clearance, such as autophagy and the UPS are also thought to be important in the ageing process. All of these pathways have been reported to have a demonstrated role in ageing and interestingly are all potentially involved in the pathogeneis of AD. However, this is not an exhaustive list of pathways that are thought to be implicated in both ageing and AD. Others, in particular inflammation are also likely to be involved. In fact inflammation has been found to interact with a number of the pathways depicted here. For example, ROS can trigger inflammatory pathways and the accumulation of damaged mitochondria with ageing is thought to be an important cause of inflammation, as reviewed in (Green et al., 2011). Inflammation has also been inherently linked to mTOR signalling. For example inhibition of mTOR has anti-inflammatory effects in a number of different settings, such as chronic kidney disease and lung infection, reviewed in (Johnson et al., 2013). It is clear that inflammation plays an important role in ageing and interacts with a number of the different pathways here. However ageing related inflammation is outside the scope of the research presented here. This thesis, will focus on the mTOR and autophagy pathways and how these are both implicated in ageing and AD, however it is important to consider that these pathways do not act in insolation and that many other pathways that interact can influence age-related changes in these pathways.

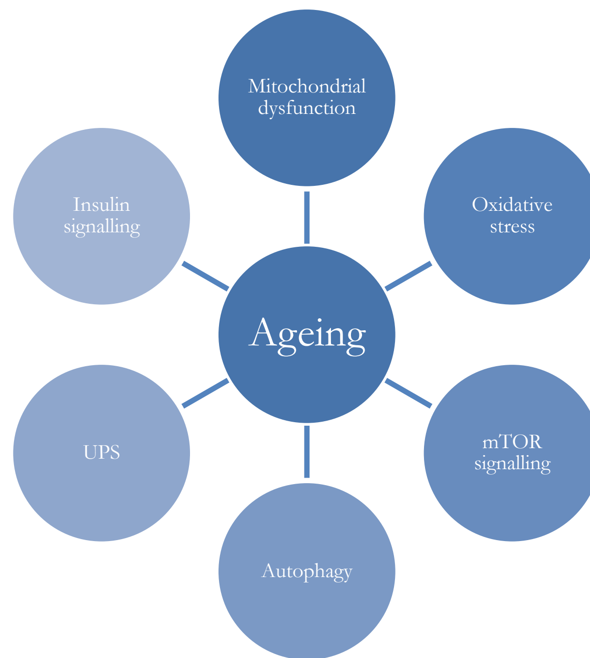



Figure 1-8. Cellular pathways implicated in ageing, including those pathways implicated in the mitochondrial theory, hyperfunction theory and regulating protein turnover.

## 1.10 mTOR signalling

The mTOR signalling pathway is a key regulator of cell growth, responding to a range of environmental cues. This pathway has been found to be important in many human diseases including cancer and also neurodegenerative diseases such as AD. mTOR was identified after the discovery of the antifungal agent and immunosuppressant rapamycin, which was found to inhibit proliferation of mammalian cells. Studies performed in yeast showed that mutations in TOR conferred resistance to the growth inhibition properties of rapamycin (Heitman et al., 1991). mTOR was then purified and found to be the target of rapamycin (Sabatini et al., 1994). mTOR is a serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase-related kinase protein (PIKK) family (Keith & Schreiber, 1995) and is highly conserved across many species including plants (*Arabidopsis thaliana),* *Saccharomyces cerevisiae* (yeast), *C. elegans*, *Drosophila melanogaster* and mammals. mTOR interacts with a number of different proteins to form two different complexes, mTORC complex 1 (mTORC) and 2 (mTORC2). mTOR is highly sensitive to rapamycin when bound in the mTORC1 complex. Whereas mTORC2 signalling is thought as being predominantly rapamycin insensitive although it can also be inhibited by rapamycin after long term exposure, in certain cell types (Sarbassov et al., 2006). mTORC2 signalling has been implicated in regulating the actin cytoskeleton (Sarbassov et al., 2004). However, mTORC1 signalling has been studied in more detail in terms of ageing and neurodegenerative disease and so will be the focus here.

mTORC1 responds to a wide range of stimuli including oxygen levels, amino acids, stress, energy levels and growth factors, among others. mTORC1 signalling can also be activated by insulin signalling. Growth factors and insulin activate the PI3K-Akt signalling cascade. Downstream of the PI3K-Akt cascade is TSC1 and TSC2 that bind to form a complex that negatively regulates mTOR signalling. Once activated Akt will phosphorylate TSC2 (Inoki et al., 2002) which leads to the inactivation of the TSC1/2 complex. TSC2 acts as a GTPase-activating protein (GAP) to promote the inherent GTP hydrolysis activity of the GTPase Rheb for the small GTPase Rheb. Activated GTP-bound Rheb will then bind to and activate mTORC1, reviewed in (Laplante & Sabatini, 2012, Jewell & Guan, 2013).

mTORC1 signalling impacts on a number of different downstream processes such as protein synthesis, autophagy, cell cycle progression, growth and metabolism (Laplante & Sabatini, 2012). The best studied downstream effectors of mTORC1 are the ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Hay & Sonenberg, 2004). It is thought that it is through these two targets that mTOR signalling can act on protein synthesis. S6K is activated by mTORC1 via phosphorylation of the Thr389 site. Activated S6K phosphorylates and activates the 40S ribosomal protein S6. S6K has also been demonstrated to phosphorylate and inhibit the activity of eukaryotic elongation factor 2 kinase (eEF2K) (Wang et al., 2001). eEF2K in turn inactivates eEF2 through phosphorylation of Thr56 (Heise et al., 2014). eEF2 then promotes ribosomal translocation along the mRNA, allowing for translation (Heise et al., 2014). mTORC1 also phosphorylates 4E-BP1 at a number of different serine and threonine residues including Thr70 and Ser65. Phosphorylation of 4E-BP1 inhibits its binding to eIF4E which is a translation initiation factor. If bound, 4E-BP1 inhibits eIF4E from interacting with its binding partners such as eIF4G1 which is involved in a number of different roles in translation of mRNA (Proud, 2014). Collectively these downstream components all converge on mRNA translation with increased activation of the mTORC1 pathway inducing increased mRNA translation.

Another downstream effect of mTORC1 signalling is in regulation of autophagy, through phosphorylation of the ULK-1 autophagy regulatory complex. When mTORC1 is activated it associates with the ULK-1 complex, phosphorylating ULK-1 and Atg13 inactivating them. When mTORC1 is deactivated it dissociates with the ULK-1 complex, resulting in dephosphorylation and initiation of the autophagic pathway. Autophagy will be further described below in Section 1.11.

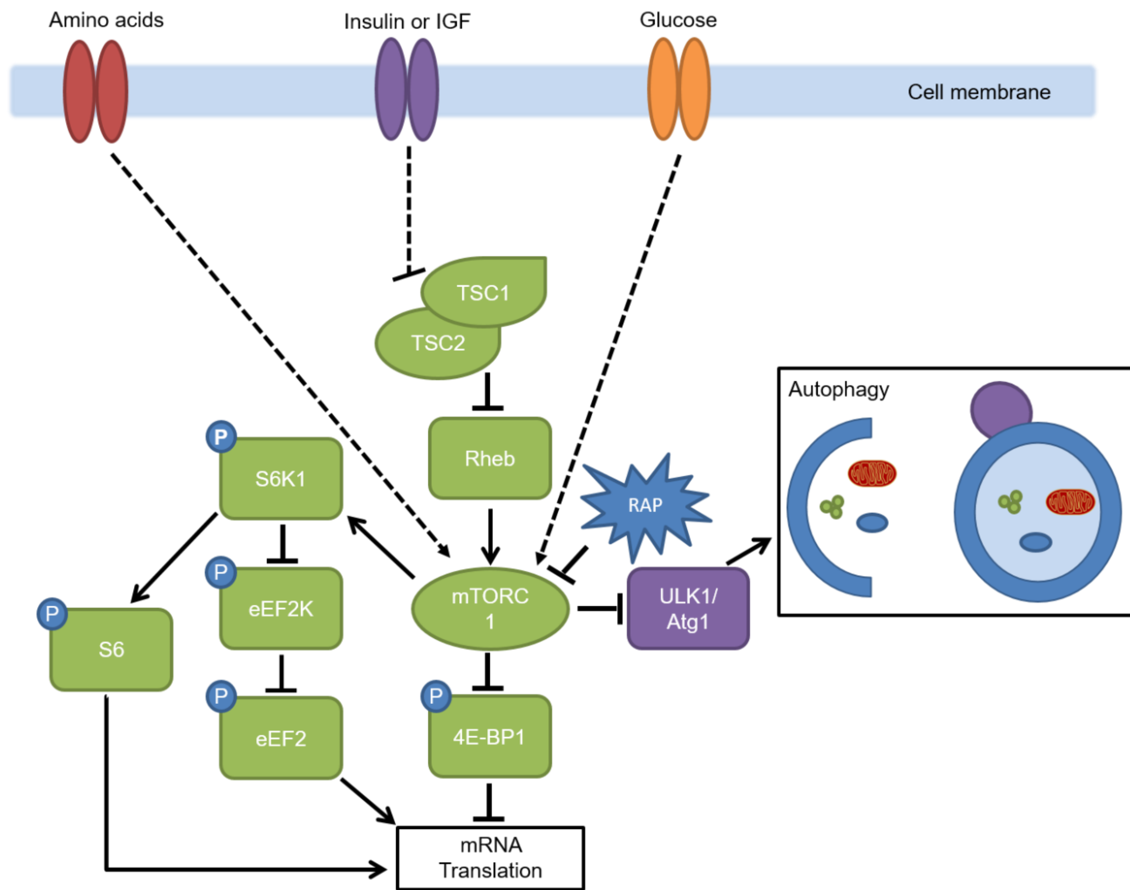



Figure 1-9. Schematic of mTORC1 signalling pathway.
The mTORC1 pathway is activated by a number of different nutrients such as glucose, amino acids and growth factors. Inhibition of TSC1/2 complex allows for activation of the GTPase Rheb which in turn activates mTORC1 complex, phosphorylating downstream components S6K1 and 4E-BP1. These converge on mRNA translation. In addition, mTORC1 activation inhibits the ULK.Atg1 complex, inhibiting autophagy.

### 1.10.1 mTORC1 signalling in ageing

TORC1 signalling has been identified as a pathway that regulates ageing and has also been implicated in age-related diseases such as AD. Therefore, it is possible to suggest that age-related changes in TORC1 signalling could be involved in the mechanisms that underlie AD. TORC1 signalling was first demonstrated to be implicated in ageing when genetic screens in yeast identified long-lived mutants, many of which contained mutations in the TORC1 signalling pathway (Powers et al., 2006). Furthermore, inhibition of the TORC1 signalling pathway either genetically or pharmacologically leads to an increase in lifespan of a number of different species. One such study in *Drosophila* found that overexpression of dTSC1 and dTSC2, which inhibits *Drosophila* TORC1 (dTORC1) signalling resulted in a significant increase in lifespan (Kapahi et al., 2004). In this same study, expression of a dominant negative form of dS6K, a downstream effector of dTORC1, was also found to

increase lifespan in flies. Furthermore, over-expression of the constitutively active form of dS6K caused a decrease in lifespan (Kapahi et al., 2004). Inhibition of the TORC1 signalling pathway in *C. elegans*, using RNAi knockdown of let-363 (homolog of mTOR) and rsks-1 (homolog of S6K) similarly caused an increase in lifespan (Hansen et al., 2007). These findings were also corroborated in mouse models where genetic deletion of S6K1 extended lifespan, specifically in female mice (Selman et al., 2009). This study also found improvements of other age-related physiological characteristics, such as locomotion and bone density, on deletion of S6K1 (Selman et al., 2009). These studies demonstrate that genetic inhibition of the TORC1 signalling pathway, in a number of different species, is associated with an extension in lifespan.

In addition to genetic inhibition the TORC1 pathway has also been inhibited pharmacologically by using rapamycin. Bjedov et al., (2010) found that rapamycin efficiently reduced dTORC1 signalling in *Drosophila* and consequently increased the lifespan of WT female flies in a dose responsive manner. Rapamycin-mediated extension of lifespan was suggested to be due to downregulation of mRNA translation and upregulation of autophagy. This study found that rapamycin mediated an increase in Lysotracker stained lysosomes and autolysosomes in the gut of the flies, indicative of autophagy induction. They also found a significant decrease in the incorporation of $^{35}$S-methionine implying that inhibiting dTORC1 signalling also inhibits translation (Bjedov et al., 2010). Similar findings have also been found in other invertebrates, such as in C. *elegans*, where administering rapamycin increased lifespan and additionally improved oxidative stress resistance (Robida-Stubbs et al., 2012). In addition, rapamycin has also been used to inhibit mTORC1 in mice and found significant increases in lifespan (Anisimov et al., 2010, Miller et al., 2011). Interestingly, one study found that if rapamycin was administered from later in life (mice were fed from 600 days old) this caused an increase in lifespan (Harrison et al., 2009). These studies collectively demonstrate that pharmacological inhibition of mTORC1 signalling, similarly to genetic inhibition, also increases lifespan.

These studies suggest that inhibition of the TORC1 signalling pathway is associated with an extension in lifespan. However, what is not clear is how the TORC1 signalling pathway changes with age. This is important to investigate as this would lead to a better understanding of how ageing contributes to age-related disease. There are very few studies that have investigated how mTORC1 signalling changes with age. Of these studies some have found an increase in mTORC1 signalling with age. For example, increased phosphorylation of S6 was found in the liver of old mice compared with younger mice (Leontieva et al., 2014). Whereas others have found decreased activation of the mTORC1

signalling pathway. Houtkooper et al., (2011) found that phosphorylation of S6K (pS6K) was decreased in both muscle and liver of mice (Houtkooper et al., 2011). There are also very few studies that have investigated how the mTORC1 pathway changes in the brain. However, one study found an age-related decrease in mTORC1 signalling in the hippocampus of WT mice (Yang et al., 2014). As these studies have demonstrated conflicting results it will be important to further assess how mTORC1 signalling changes with age, particularly in the brain. Understanding how mTORC1 signalling changes with age in the brain will provide a better understanding of why this pathway is implicated in age-related diseases such as AD.

### 1.10.2 mTORC1 signalling in AD

The mTORC1 signalling pathway has been implicated in several neurodegenerative diseases, including AD. For example, in human *post-mortem* brains a 3-fold increase in mTOR auto phosphorylation at Ser2481 (indicative of increased mTOR activation) was found in AD brain homogenate compared with control brain. Increased Ser2481 phosphorylation was also found to correlate with progression of Braak staging and co-localised with NFTs. Downstream of mTORC1, p4E-BP1 (Thr70 and Ser65) was also found to be increased with Braak staging progression (Li et al., 2005). This suggests that increased activity of mTORC1 signalling is correlated with an increase in tau pathology in AD patients. A different study found levels of pS6K were elevated in the brains of AD patients (An et al., 2003), indicative of increased mTORC1 signalling. Subsequently, elevated pS6K levels were found to be associated with neurofibrillary tangles in AD brains (An et al., 2004). These studies suggest that increased activity of the mTORC1 pathway are correlated with AD pathology and progression but they do not give an indication of whether this is a cause or effect. Therefore, animal models have been utilised to further understand the connection between AD and TORC1 signalling.

Animal models of AD expressing Aβ and/or tau have been used to further understand the contribution of TORC1 signalling to the disease. Berger et al., (2006) used EGFP-P301L transfected COS-7 cells and treated them with rapamycin. It was found that this reduced levels of the insoluble fraction of tau but not the soluble fraction (Berger et al., 2006). This is suggestive that rapamycin induces clearance of aggregated tau. This same study also used WT and mutant tau expressing *Drosophila*. Rapamycin treatment caused decreased tau-mediated degeneration in the fly eye and caused an increase in lifespan in tau-expressing flies. It was found that rapamycin in this study also decreased total levels of tau (Berger et al., 2006). This could suggest that tau translation and/or clearance is affected by rapamycin-

mediated inhibition of the dTORC1 pathway. Similarly, in another *Drosophila* model that expressed WT human tau, dTORC1 signalling was inhibited both genetically and pharmacologically. This resulted in reduced tau-mediated toxicity in the eye (Khurana et al., 2006). This study also found that activation of TOR by co-expression of Rheb caused increased tau-mediated neurodegeneration (Khurana et al., 2006). These studies have demonstrated that inhibition of the dTORC1 pathway can reduce tau-mediated toxicity. However, tau-toxicity in the *Drosophila* eye is mediated, in part, developmentally (Kramer & Staveley, 2003). Therefore, it is still unclear whether inhibition of the dTORC1 can reduce age-related tau-mediated toxicity.

Studies have also been conducted in mice that have investigated how manipulations of the mTORC1 pathway effect on tau and Aβ-mediated phenotypes and pathologies. For example, Caccamo et al., (2010) using a triple transgenic mouse model of AD, that develop both plaques and NFTs, (3xTg) demonstrated that rapamycin increased the lifespan of these mice. Furthermore, rapamycin treatment reduced both tau and Aβ pathology and improved learning and memory deficits. Rapamycin was also found to cause an increase in autophagy in these animals, suggestive of autophagy being involved in the clearance of the tau and Aβ pathologies (Caccamo et al., 2010). However, as tau levels have been demonstrated to be affected by expression of Aβ it was not possible to elucidate whether mTORC1 could affect tau directly or whether these effects were through Aβ. Subsequent studies have demonstrated that mTORC1 signalling can indeed affect tau directly. Genetic upregulation of the mTORC1 pathway via inhibition of TSC2 was found to decrease total and phospho tau levels in mice (Caccamo et al., 2013). This same study also treated a mutant tau (P301S) mouse model with rapamycin and found an improvement in tau-mediated motor deficits which coincided with decreased tau pathology (Caccamo et al., 2013). This study also found that autophagy was increased in the mice treated with rapamycin but proteasomal activities were unchanged (Caccamo et al., 2013), demonstrating autophagy in mediating mTORCs effect on tau protein. Further studies have also demonstrated that translation may also mediate mTORC1's effect on tau. A study where S6K was genetically downregulated in 3xTg mice rescued tau-mediated deficits in LTP and synaptic deficits, together with improvement of learning and memory deficits (Caccamo et al., 2015). Therefore, in mice inhibition of mTORC1 results in reduced tau and Aβ-mediated phenotypes and pathologies.

The studies, discussed here, demonstrate that inhibition of mTORC1 signalling pathway is associated with extended lifespan and alleviates both Aβ and tau-mediated phenotypes and pathologies. These studies have also demonstrated that both inhibition of translation and

increased autophagy underlie these effects. However, what is currently not known is whether age-related changes in mTORC1 signalling can directly lead to the accumulations of Aβ and tau pathologies in AD.

## 1.11 Autophagy

As discussed above, autophagy has been implicated in mTORC1's role in both ageing and AD. Therefore, autophagy is also an important avenue of research for understanding the association between ageing and AD. Autophagy is a proteolytic process that degrades misfolded proteins and damaged organelles such as mitochondria (Wang & Klionsky, 2011). It involves a multi-step process which is initiated by the formation of the double membrane-bound phagophore which forms around cytoplasm that contains the target proteins and organelles. A number of autophagy-related (Atg) proteins have been identified that regulate this process (Klionsky, 2005), for example Atg1 (ULK-1 in mammals) is required for phagophore formation. In addition, the class III PI-3 kinase, Vps34 and its binding partner Atg6/Beclin-1 is also required for phagophore formation. The phagophore then expands to form the autophagosome. This is in part regulated by the processing of the microtubule-associated protein light chain 3 (LC3). Upon induction of autophagy LC3 is proteolytically cleaved by Atg4 to generate LC3-I. LC3-I is then transferred Atg3 where it is conjugated to phosphatidylethanolamine (PE) to form LC3-II. The autophagosome then fuses with a lysosome to form the autolysosome. The autolysosome is where the encapsulated contents are degraded and subsequently breakdown products are released (e.g. amino acids) into the cytosol (Mizushima, 2007).

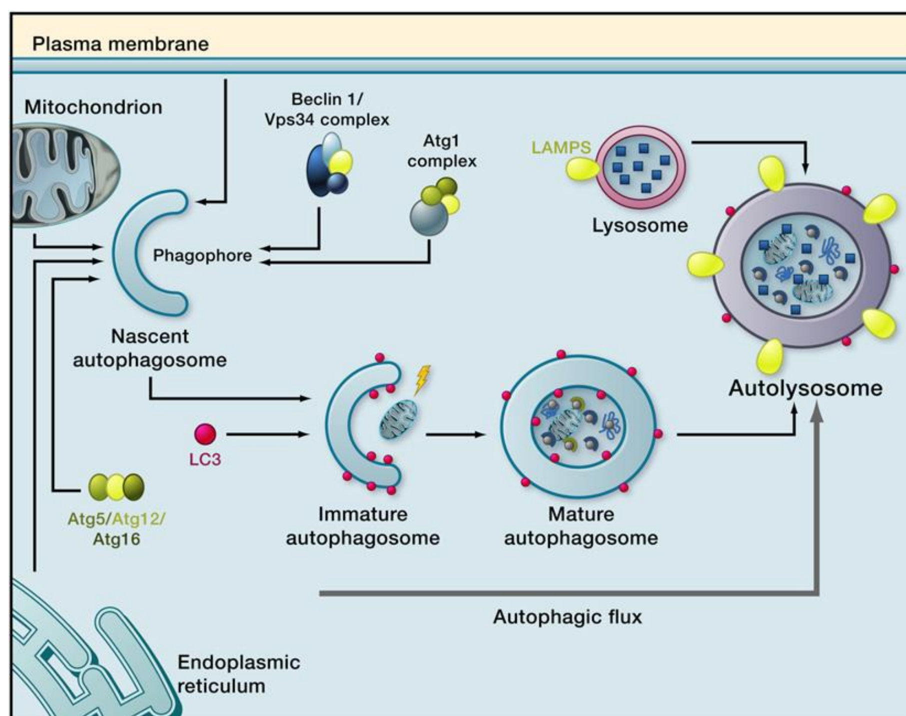



Figure 1-10. The autophagic clearance pathway taken from (Rubinsztein et al., 2011).
The main stages of autophagy are depicted here. Initiation of autophagy by Beclin-1 and Atg1/ULK-1 through formation of the phagophore. The immature autophagosome will engulf damaged proteins and organelles, and forms the mature autophagosome. This will then fuse with a lysosome to produce the autolysome where the contents are degraded and recycled.

### 1.11.1 Autophagy in ageing

Similarly, to mTORC1 signalling, autophagy has also been implicated in regulating ageing. In a genetic screen for short lived mutants in yeast, ten mutants were found that had defects in autophagy-related genes (Matecic et al., 2010), suggestive that downregulation of autophagy leads to a shorter lifespan. Other studies have investigated autophagy in long lived mutants of different species, for example, the daf-2 *C. elegans* model. This is a long lived mutant that has reduced insulin signalling. It was found that the increased lifespan induced by knockdown of daf-2 was abolished if autophagy-related genes were also knocked down (Meléndez et al., 2003), suggesting that upregulation of autophagy is required for mediating extension of lifespan. In a *Drosophila* model where lifespan was extended by feeding flies rapamycin, autophagy was also found to be increased. In addition, knocking down Atg5 (involved in autophagosome elongation) expression using RNA interference (RNAi) inhibited the extension of lifespan induced by rapamycin (Bjedov et al., 2010). A study conducted in *Drosophila* also found that mRNA levels of autophagy-related genes decreased in older flies compared to younger flies. This was correlated with an age-related decrease in Atg8 protein (Simonsen et al., 2008). Corroborating this, Atg5 was overexpressed in mice and enhanced autophagy was found together with increased lifespan

and motor function (Pyo et al., 2013). Loss of function mutations in autophagy-related genes such as Beclin-1 and Atg1 (both of which are required for initiation of the formation of the autophagosome) were found to reduce the lifespan of *C. elegans* (Tóth et al., 2008). Collectively these studies suggest that upregulation of autophagy can extend lifespan, highlighting autophagy as an important pathway in the regulation of ageing.

Autophagy's role in extension of lifespan is hypothesised to be through the removal of damaged proteins. This will be discussed in further detail below. However, autophagy is also involved in the removal of dysfunctional organelles, such as mitochondrion, from the cell. As discussed above in Section 1.8.1, mitochondria become dysfunctional with age producing increasing amounts of ROS. Autophagy has been demonstrated to degrade dysfunctional mitochondria. For example, mitochondria were isolated from skeletal mouse muscle where autophagy had been genetically knocked down and found to have impaired mitochondrial respiration and increased ROS (Wu et al., 2009). These studies collectively demonstrate that increased autophagy is associated with increased longevity. This effect is potentially mediated by its ability to remove dysfunctional organelles, such as mitochondrion and damaged proteins from the cell. The fact that many diseases, including AD are characterised by accumulations of misfolded and damaged proteins implicates the autophagy pathway in the pathogenesis of these diseases.

### 1.11.2 Autophagy in AD

Autophagy is a particularly important process in post mitotic cells, such as neurons, as cell turnover does not happen which would usually perturb the accumulation of intracellular debris. Therefore, a decrease in autophagic clearance in neurons could be potentially damaging. In AD brains, autophagy has been found to be downregulated. For example, decreased levels of Beclin-1 mRNA and protein levels have been found in the entorhinal cortex and dentate gyrus of AD patients (Pickford et al., 2008). As Beclin-1 is involved in the initiation of the formation of the autophagosome, this implies that the initiation of autophagy is inhibited in AD. In addition, studies in AD patients have reported that markers of autophagy co-localise with the plaques and NFTs found in the brain (Ma et al., 2010). This suggests that autophagy is upregulated in AD but is not able to aid in the clearance of these pathologies implying that autophagy is dysfunctional in AD.

Animal models have been utilised to further understand the relationship between autophagy and AD pathologies. Studies conducted in the 3xTg mouse model of AD found that after treatment with rapamycin the tau and Aβ pathologies that accumulate in this

model, were reduced. This was correlated with an induction of autophagy. This study also demonstrated specifically that tau and Aβ are degraded by lysosomes as indicated by immunohistochemistry showing localisation of Aβ and tau with Lamp2A, a lysosomal marker (Caccamo et al., 2010). A follow-up study from this group found that rapamycin needed to be administered before plaques and tangles are established to reduce pathology and related cognitive deficits in mice (Majumder et al., 2011). This suggests that autophagy is capable of removing smaller species of tau inhibiting the formation of large aggregates but is not able to degrade the larger aggregates once they are formed. In addition, methylene blue has been demonstrated to induce autophagy, both in vitro and in vivo (Congdon et al., 2012). Methylene blue was incubated with organotypic brain slices from P301L mice. This was found to reduce levels of aggregated insoluble tau, coinciding with increased levels of autophagic markers such as Beclin-1 and LC3-II which are involved in the formation of the autophagosome membrane. This study further showed that if autophagy was blocked by knocking down Beclin-1, methylene blue could no longer reduce levels of the insoluble fraction of tau (Congdon et al., 2012). These studies demonstrate that pharmacological agents can activate autophagy and reduce Aβ and tau proteins, preventing the formation of large aggregates.

Autophagy has also been genetically manipulated by the inhibition of specific components of the pathway. For example, a study conducted in *Drosophila* genetically knocked down cathepsin-D, a lysosomal aspartyl protease. This caused exacerbation of tau-mediated neurodegeneration and reduced lifespan (Khurana et al., 2010). Similarly, in a separate study in cells chloroquine was used to inhibit lysosomal hydrolases causing reduced tau clearance and subsequently causing tau to accumulate into insoluble aggregates (Hamano et al., 2008). This is evidence that autophagy can inhibit the accumulation of tau pathologies. Interestingly, it has also been found that tau can inhibit autophagy. Expression of tau in the *Drosophila* eye generated the formation of large autophagic intermediates which were not found in control flies. These autophagic intermediates were found to contain partially degraded tau (Bakhoum et al., 2014), suggesting that tau induces a gridlock in autophagic flux, inhibiting the autophagic process from completing. These studies demonstrate that autophagy is defective in AD and down-regulation or dysfunction of autophagy can result in the accumulation of tau pathology.

Autophagy has been shown to act downstream of mTORC1 signalling. Inhibition of mTORC1 signalling causes upregulation of autophagy and this has been demonstrated to increase lifespan, in a number of different species. This is thought to occur by autophagy-mediated removal of damaged proteins. Therefore, if mTORC1 pathways and autophagy

pathways become dysregulated with age this could lead to the accumulations of aggregated proteins seen in disease. To be able to investigate this hypothesis it will be important to assess how these pathways are altered in both ageing and tauopathy.

## 1.12 *Drosophila* as a model organism for studying ageing and tauopathy

To be able to investigate the roles of mTORC1 signalling and autophagy in ageing and tauopathy a model is required as it is not possible to manipulate these pathways in the human brain. *Drosophila* are a particularly good model for use in this study because they have been used extensively in both ageing and tauopathy research. Their relatively short lifespan and genetic tractability has made *Drosophila* particularly attractive for use in assessing regulators of ageing, including the TORC1 signalling and autophagy pathways. These pathways are also highly conserved between *Drosophila* and humans (Partridge et al., 2011, Chang & Neufeld, 2010). Not only this but approximately 70% of disease causing genes in humans are conserved in *Drosophila,* including those that cause AD (APP and presenilin) and tauopathies such as FTDP-17 (tau) (Reiter et al., 2001). In addition, genetic tools have been generated in *Drosophila* that have made it possible to express different ageing and tauopathy mutants in a temporal and spatial dependant manner.

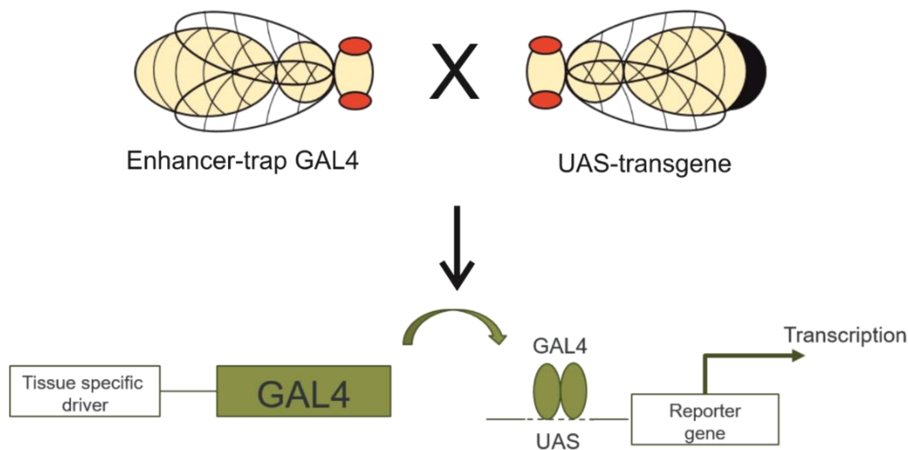
### 1.12.1 Temporal and spatial expression

The UAS/GAL4 system, pictured below in Figure 1-9, is an elegant expression system that allows for spatial expression of specific transgenes in *Drosophila*. This involves the crossing of two Fly lines that contain either a UAS or GAL4 element. GAL4 is a gene transcription regulator, that will bind to an upstream activating sequence (UAS) to activate transcription. The GAL4 lies upstream of a tissue specific promoter. Many different tissue specific GAL4 driver lines have been made that allow for the targeted expression of a specific protein of interest in any cell, subset of cells or whole tissues (Brand & Perrimon, 1993). For example, the Elav-GAL4 line has been created that drives expression of proteins pan-neuronally (Brand & Perrimon 1993).

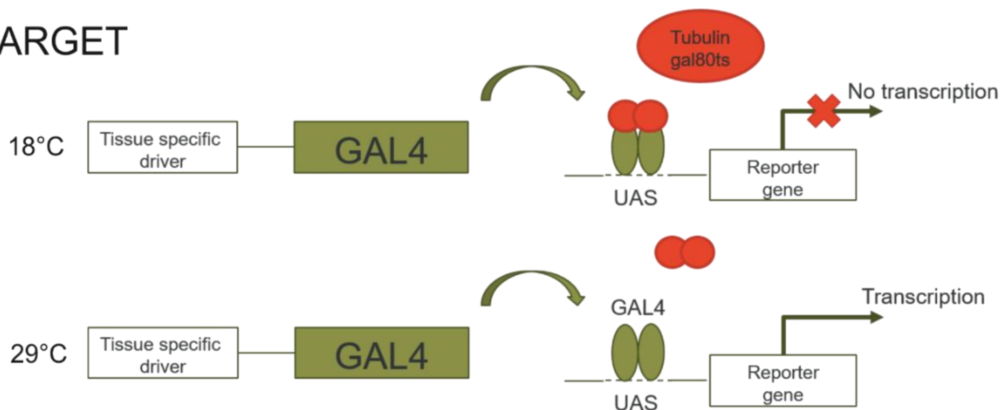
Adaptations of the UAS/GAL4 system have also been generated that allow for temporal expression of proteins. One of these adaptations, known as the TARGET expression system, uses a temperature-sensitive variant of a repressor of GAL4, namely GAL80$^{ts}$. At 29°C-30°C the Gal80$^{ts}$ is deactivated and allows for GAL4 expression of UAS-responder lines. At 18°C the Gal80$^{ts}$ is activated and represses GAL4, inhibiting the UAS-transgene

from being expressed (Suster et al., 2004). An alternative to the TARGET system is GeneSwitch which also allows for temporal and spatial expression of genes. Similarly, to TARGET, GeneSwitch is based on the UAS/GAL4 system. However, the GAL4 in GeneSwitch is fused to a progesterone receptor chimera which is hormone-inducible. This type of expression system requires the drug RU486 (also known as mifepristone) for GAL4 binding to UAS (Burcin et al., 1998). An advantage of using GeneSwitch over TARGET is that it is not reliant on a change in temperature, which can strongly affect flies' behaviour. Both the TARGET and GeneSwitch systems allow for expression of proteins both spatially and temporally. Importantly for this study, this allows for expression of transgenes only in adulthood and not during development. This is particularly useful for when studying pathways such as TORC1 signalling as this pathway has an important role in growth and development, as reviewed in (Laplante & Sabatini, 2012). Therefore, expression of manipulators of this pathway could well disrupt normal development. As this research is concerned specifically with ageing it will be important to determine that any potential effects of manipulating these pathways are not due to effects on development. This is made possible by the use of these expression systems.

UAS/GAL4




TARGET




Geneswitch

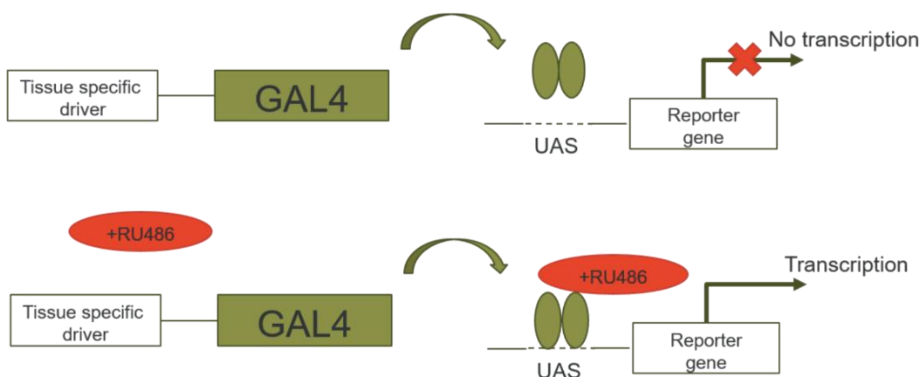



Figure 1-11. Gene expression systems in *Drosophila*.
The UAS/GAL4 system involves a tissue specific driver put upstream of the GAL4 element. When crossed with a fly containing a gene attached to a UAS then transcription occurs. The TARGET system is an adaptation of the GAL4/UAS system as this involves another element the GAL80$^{ts}$ which is activated at low temperatures (18°C) and binds to GAL4, inhibiting the binding to UAS. At 29°C the GAL80$^{ts}$ is inhibited and cannot bind to the GAL4, allowing for transcription. The GeneSwitch system works in a similar way but transcription occurs only in the presence of RU486 drug.

### 1.12.2 *Drosophila* as a model of ageing

Due to their genetic tractability *Drosophila* have been used extensively in exploring the pathways that underlie ageing. This means that a wide range of genetic mutants have been made available to study ageing. For example, as with mice (McCay et al., 1935) dietary restriction has been demonstrated to also extend the lifespan of flies (Chippindale et al., 1993, Chapman & Partridge, 1996). Using a number of different genetic mutants', pathways that underlie this phenomenon have been identified and explored in *Drosophila*. These pathways include, among others, insulin and TORC1 signalling. For example, the loss of the *Drosophila* insulin receptor substrate, chico, causes extension of lifespan (Clancy et al., 2001). This was found to be dependent on the concentration of dietary nutrition administered to the flies. The *chico* mutation was not able to induce extended lifespan in flies fed lower concentrations of diet (Clancy et al., 2002). This suggests that dietary restriction mediated extension of lifespan is dependent on the insulin signalling pathway.

*Drosophila* have also been used to demonstrate that the dTORC1 pathway is involved in extension of lifespan by dietary restriction. For example, overexpression of dTSC2, that inhibits dTORC1 signalling, was found to increase lifespan when ubiquitously expressed in all tissues of the fly. Again, this was found to be dependent on the nutritional content of the diet. The greater the concentrations of nutrients in the diet, the greater the increase in lifespan mediated by the mutant (Kapahi et al., 2004). This suggests that the dTORC1 signalling is also a pathway through which dietary restriction extends lifespan (Kapahi et al., 2004). This highlights the importance of this pathway in ageing and how *Drosophila* can be used to explore this.

Other studies have also demonstrated that an inhibition of the dTORC1 pathway causes an extension of lifespan in *Drosophila*. These studies have shed light on the mechanisms that underlie the effect of the dTORC1 pathway on ageing. For example, one study treated WT flies with rapamycin which induced extension of lifespan. This effect was correlated with decreased mRNA translation and increased autophagy (Bjedov et al., 2010). In addition, downregulation of autophagy has been independently demonstrated to decrease lifespan of *Drosophila* (Simonsen et al., 2008). This was carried out by expressing Atg8 (homolog of LC3) mutants in the nervous system of the flies. Interestingly, this effect was correlated with an increase in sensitivity to ROS (Simonsen et al., 2008).

Collectively these studies demonstrate how *Drosophila* have been used to understand age-related pathways and how these regulate ageing. These studies have generated not only

knowledge about the mechanisms that underpin ageing but also provided a range of genetic mutants that can be used to manipulate these pathways.

### 1.12.3 *Drosophila* as a model of tauopathy

In addition to studying ageing, *Drosophila* have also been used to further understand the role of tau in disease. *Drosophila* have provided various different insights into how tau becomes pathogenic and causes toxicity to neuronal cells. To do this, a number of different *Drosophila* models have been generated that express both WT and mutant forms of tau, which will be discussed further in Chapter 2. By utilising the UAS/GAL4 system, the expression of tau proteins has been targeted to specific neuronal populations. For example, tau expression has been demonstrated in all neurons using the Elav-GAL4 driver (Folwell et al., 2010). In addition, tau has been expressed in subsets of neurons including the motor neurons (D42-GAL4) (Mudher et al., 2004) and the mushroom body neurons (Kosmidis et al., 2010). In addition, a number of different assays have been used to assess the impact that tau expression has on these different populations of cells including behavioural readouts dependent on these neuronal populations. These include assays such as, lifespan (Folwell et al., 2010), larval locomotion (Mudher et al., 2004), adult locomotion (Roy & Jackson, 2014) and learning and memory (Mershin et al., 2004).

*Drosophila* models of tauopathy have also been used to examine aspects of tau pathogenesis such as in mediating destabilisation of microtubules (Cowan et al., 2010), defects in synapses (Chee et al., 2005) and axonal transport deficits (Mudher et al., 2004). Tau *Drosophila* models have also been used to demonstrate the effects that tau has on neurodegeration, both in the *Drosophila* eye (Jackson et al., 2002) and in the whole brain (Witmann et al., 2001). Using the 'rough eye' phenotype that is generated when toxic proteins are expressed in the eye has been particularly useful in identifying enhancer/suppressors of tau-mediated toxicity. For example, one such study that expressed a mutant form of tau (V337M) used the rough eye phenotype to investigate modifiers of tau toxicity (Shuman & Feany 2003). Collectively these studies demonstrate the rich history of using *Drosophila* to study tauopathies, providing a wealth of different models and assays that can be used in this study.

## 1.13 Research Aims

The overarching aim of this study is to understand how ageing can lead to a greater risk of AD. Specifically, this research will investigate how age related changes in cellular pathways can impact on tau pathology. The cellular pathways that will be investigated are TORC1 signalling and autophagy which have been implicated in both ageing and AD. Both *Drosophila* and human brain tissue will be used to study the connection between age-related pathways and tauopathy. This investigation will be broadly separated into four aims:

1. **To investigate age-related changes in tau-mediated phenotypes and pathology**

It is important to firstly understand how tau changes with age. To do this, two different isoforms of human tau, htau$^{0N3R}$ and htau$^{0N4R}$, will be expressed in the nervous system of *Drosophila* and assessed for changes in established ageing phenotypes, longevity and climbing. Any potential changes in these phenotypes will be correlated with changes in total tau levels, tau phosphorylation and tau solubility. This work will provide a clear understanding of how tau is affected during ageing.

2. **To characterise complex behaviours, relevant to AD, in tau-expressing *Drosophila***

It is possible to model behaviours in *Drosophila* that better correlate with AD symptoms than longevity and climbing phenotypes. These behaviours include circadian rhythms and learning and memory. These will be characterised in htau$^{0N3R}$ expressing flies by expressing htau$^{0N3R}$ in subsets of neurons pertaining only to circadian and memory functions in the *Drosophila* brain. This will provide an understanding of how expression of htau$^{0N3R}$ can directly alter these behaviours. This work could potentially demonstrate some of the mechanisms that underlie these symptoms in AD.

3. **To measure how the mTORC1 signalling and autophagy pathways change with age.**

It is currently unclear how mTORC1 and autophagic pathways change in the normal ageing brain. Human cortical tissue will be used for this, using patients of different age to measure how the activity of these pathways change with age. There is very little research conducted in this area, particularly in the human brain. This work is necessary to be able to understand fully, how these pathways are involved in the ageing process. Is it that age related changes in these pathways lead to the accumulations of proteins found in disease?

**4. To investigate how manipulations of the dTORC1 and autophagy pathways can affect tau mediated phenotypes and pathologies**

The final aim of this study is to understand whether manipulations of dTORC1 and autophagic pathways affect age-related tau phenotypes in $htau^{0N3R}$ expressing flies and whether this correlates with changes to tau protein. If these manipulations are able to reduce tau-mediated phenotypes this work will have implications for possible therapeutic interventions that impact tau.

# Chapter 2: Characterisation of ageing phenotypes in tau expressing *Drosophila.*

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## 2.1 Introduction

The overall aim of the research described in this Thesis is to understand how pathways implicated in ageing contribute to tau-mediated phenotypes and pathologies. To do this, it is important to firstly characterise potential age-related changes in tau-mediated phenotypes, which are associated with changes in tau pathology. *Drosophila melanogaster* lends its self as a particularly good model organism for this study as they have been used extensively in the past to study both ageing and tauopathy.

### 2.1.1 Tau mediated behavioural phenotypes

Different tau-mediated behavioural phenotypes have been investigated in various *Drosophila* models of tauopathy (Table 3). For example, several studies have found that expression of human tau reduces lifespan (Wittmann et al., 2001, Khurana et al., 2010, Folwell et al., 2010, Colodner & Feany, 2010). Deficits in locomotion have also been found with expression of human tau, both in larvae (Mudher et al., 2004, Sinadinos et al., 2012, Quraishe et al., 2013) and in adult flies (Folwell et al., 2010, Roy & Jackson, 2014). In addition to locomotion, expression of human tau has been found to cause defects in more complex behaviours, such as learning and memory (Mershin et al., 2004, Kosmidis et al., 2010). In fact, the mushroom bodies appear to be particularly vulnerable to the expression of human tau, with some isoforms of tau causing complete or partial ablation of the mushroom bodies (Kosmidis et al., 2010). Collectively these studies show that tau can induce behavioural phenotypes but often these behavioural phenotypes cannot be attributed to ageing in the studies conducted so far. Lifespan is clearly an age-related phenotype but testing locomotion, for example, in larvae can only assess tau-mediated defects at a single time point early on in the fly lifecycle. Often adult climbing has only been tested at a single time point or only tested in young to middle-aged flies. In addition, learning and memory assays, although tested in adult flies, are often not assessed for age-related changes. This demonstrates the need for longitudinal studies in *Drosophila* to be able to assess the contribution of age to tau-mediated behavioural phenotypes.

Table 3. Phenotypes produced in different *Drosophila* models of tauopathy.

| Isoform of tau expressed | Tau-mediated phenotypes and pathologies | Dysfunction/ Degeneration? | Age-related? | Reference |
|---|---|---|---|---|
| **0N3R** | Larval locomotor defects<br>Axonal transport dysfunction | Dysfunction | Larval model | (Mudher et al., 2004) |
| | Defects in NMJ morphology<br>Abnormal exo/endocytosis in NMJ<br>Abnormal transmission at NMJ<br>Defects in mitochondria | Dysfunction | Larval model | (Chee et al., 2005) |
| | Microtubule breakdown | Dysfunction | Larval model | (Cowan et al., 2010) |
| | No ablation of mushroom bodies, mushroom body function normal. | n/a | Adult driven expression but only one time point tested | (Kosmidis et al., 2010) |
| | Larval locomotor defects | Dysfunction | Larval model | (Sinadinos et al., 2012) |
| | Larval locomotor defects<br>Microtubule destabilisation<br>Axonal transport defects | Dysfunction | Larval model | (Quraishe et al., 2013) |
| **0N4R** | Reduced lifespan<br>Age-related neurodegeneration<br>Age-related loss of cholinergic neurons | Both | Age-related | (Wittmann et al., 2001) |
| | Rough eye phenotype<br>Vacuolisation in medulla | Degeneration | Developmental | (Jackson et al., 2002) |
| | Rough eye phenotype | Degeneration | Developmental | (Nishimura et al., 2004, Steinhilb et al., 2007, Iijima et al., 2010, Iijima-Ando et al., 2010) |
| | Rough eye phenotype<br>Increased filamentous actin | Degeneration | Developmental | (Fulga et al., 2007) |
| | Accumulation of high molecular weight and insoluble tau species | n/a | Age-related | (Blard et al., 2006) |
| | Deficits in associative olfactory learning and memory | Dysfunction | Only young adults tested | (Mershin et al., 2004) |
| | Age-related accumulation of insoluble, phosphorylated tau<br>Glial fibrillary tangle formation | Both | Age-related | (Colodner & Feany, 2010) |
| | Axonal transport dysfunction<br>Morphology defects in NMJ<br>Climbing phenotype<br>Reduced longevity | Both | Climbing and longevity phenotypes are age-related | (Folwell et al., 2010) |
| | Reduced lifespan<br>Increased apoptosis | Both | Age-related | (Khurana et al., 2010) |
| | Age-related decline in memory performance<br>Age-related vacuolisation<br>Age-related increase in tau phosphorylation | Both | Age-related | (Ali et al., 2012) |
| | Microtubule defects<br>Microtubule defects in NMJ | Dysfunction | Larval model | (Xiong et al., 2013) |
| **2N4R** | Rough eye phenotype | Degeneration | Developmental | (Chatterjee et al., 2009, Roy & Jackson, 2014) |
| | Ablation of mushroom bodies | Degeneration | Adult driven expression but only one time point tested | (Kosmidis et al., 2010) |
| | Reduced climbing phenotype<br>Microtubule/cytoskeleton disorganisation<br>Reduced axonal transport<br>Reduced synapsin in synaptic boutons | Both | Larvae and young adults used only. | (Roy & Jackson 2014) |

### 2.1.2 Tau-mediated neurodegeneration

The mechanisms that underlie how tau mediates behavioural phenotypes have also been investigated in *Drosophila*. One of these potential mechanisms is that tau can directly cause neurodegeneration. Neurodegeneration is one of the major hallmarks of AD and is thought to be a primary reason for the symptoms of the disease, as reviewed previously (Ballatore et al., 2007). Therefore, one line of research has investigated how tau can cause neurodegeneration, for which *Drosophila* models of tauopathy have been particular valuable. For example, human tau has been expressed in the *Drosophila* eye, with this found to cause a rough eye phenotype (Jackson et al., 2002, Nishamura et al., 2004, Fulga et al., 2007, Steinhilb et al., 2007, Chatterjee et al., 2009, Iijima et al., 2010, Iijima-Ando et al., 2010, Roy & Jackson 2014). Although these studies demonstrate that expression of human tau can be toxic to neuronal cells, this particular phenotype is generated using the GMR-GAL4 driver, which drives expression during development, and so is not caused by age-related changes in tau. However, others have expressed human tau pan-neuronally using the post-mitotic Elav-GAL4 driver and therefore not driven developmentally. These studies found age-related vacuolisation across the brain (Wittmann et al., 2001, Dias-Santagata et al., 2007) suggesting that tau-induced neurodegeneration is not only a consequence of developmental expression, but is also age dependent.

### 2.1.3 Using *Drosophila* to determine the mechanisms that underpin tauopathies – microtubule destabilisation

Another mechanism by which tau may be inducing behavioural phenotypes is by becoming dysfunctional. Tau normally binds to and stabilises microtubules, allowing efficient axonal transport to the synapse. It is hypothesised in AD that tau becomes hyperphosphorylated rendering it no longer able to bind to microtubules, causing the microtubules to become destabilised and to break down (Alonso et al., 1994). This has been demonstrated in *Drosophila* models of tauopathy (Cowan et al., 2010, Xiong et al., 2013, Quraishe et al., 2013, Roy & Jackson et al., 2014). As a consequence of microtubule destabilisation it has been found that expression of human tau causes disruption to axonal transport (Mudher et al., 2004, Folwell et al., 2010, Quraishe et al., 2013, Roy & Jackson 2014). It is thought that without the proper trafficking of organelles and proteins along the axon, synapses are likely to fail causing a break down in synaptic transmission and ultimately a loss of cell to cell communication. This has also been demonstrated in flies in the neuromuscular junction (NMJ) where defects have been seen after expression of human tau, such as reduction of

the overall size of NMJs, the appearance of satellite boutons and reduced synaptic transmission (Chee et al., 2005, Xiong et al., 2013, Roy & Jackson et al., 2014).

### 2.1.4 Using *Drosophila* to determine the mechanisms that underpin tauopathies – biochemical properties of tau

Alternatively, to dysfunction, tau could potentially mediate behavioural phenotypes and degeneration through a toxic-gain-of function. The tangles found in AD brains contain hyper phosphorylated, misfolded and aggregated tau, as reviewed previously (Goedert & Spillantini, 2006). Consequently, these particular biochemical properties have been assessed in *Drosophila* models of tauopathy. Many of the studies that have investigated tau phosphorylation have not investigated how phosphorylation of tau changes with age. However, those few studies that have investigated age-related changes in tau phosphorylation found an increase with age in phosphorylation at the Ser202/Thr205 (AT8), Ser262/Ser356 (12E8), Thr212/Ser214 (AT100) and Thr231 (AT180) sites (Wittmann et al., 2001, Colodner & Feany 2010). Although others have not assessed how phosphorylation of tau directly changes with age, they have demonstrated the important role that phosphorylation has in toxicity. For example, it has been observed that if certain tau kinases such as PAR-1 and GSK-3 are co-expressed with tau then the rough eye phenotype, induced by the expression of human tau, is enhanced (Nishamura et al., 2004, Chatterjee et al., 2009). Similarly, if specific serine sites on tau are mutated to alanines (rendering them unphosphorylatable), such as in the case with Ser262 and Ser356, then this abolishes the rough eye phenotype (Chatterjee et al., 2009). Other studies have shown that if GSK-3β is inhibited then tau induced locomotor, synaptic and axonal transport deficits are improved (Mudher et al., 2004). In addition, most studies in *Drosophila* have found that tau-induced dysfunction and degeneration occurs in the absence of large aggregates of tau such as the PHFs and NFTs found in AD brains. However, when expressing human tau in glial cells, it was found to be possible to induce age-related accumulation of structures that resemble NFTs (Colodner & Feany 2010). This same study also showed that tau becomes insoluble (Colodner and Feany 2010) and others have shown that tau also becomes increasingly misfolded with age (Wittmann et al., 2001). Collectively these studies suggest that the species of tau that mediate the majority of tau phenotypes in *Drosophila* are soluble, misfolded and phosphorylated forms of tau and are unlikely, in most cases to be large aggregates.

### 2.1.5 Aims

As described above, expression of human tau in *Drosophila* mediates behavioural phenotypes that are underpinned by changes to cytoskeletal structure and the degeneration of neurons. However, the majority of studies have only investigated these effects at single time points, usually either in larvae or young adult flies, (Table 1). Further to this, age-related phenotypes do not correlate well with the underlying changes in biochemical properties of tau such as phosphorylation and aggregation. Therefore, there is a lack of research into how tau phenotypes and pathologies change with age in *Drosophila*. There are also discrepancies between studies that appear to show different types/isoforms of tau producing different phenotypes. For example, different types of tau produce differential effects on the rough eye phenotype (Chen et al., 2007) and the ablation of the mushroom bodies (Kosmidis et al., 2010). Due to the discrepancies seen in these different models it was decided for this study that two, previously used, *Drosophila* lines would be assessed, one expressing 3R (htau$^{0N3R}$) and one expressing 4R human tau (htau$^{0N4R}$). Adult flies expressing either htau$^{0N3R}$ or htau$^{0N4R}$, pan-neuronally using the Elav-GAL4 driver, were assessed for both longevity and locomotor deficits as they aged. The biochemical properties of tau were then assessed for total levels, phosphorylation state and solubility changes in both young, 0-3 days' post eclosion (0 week old) and old (6 weeks old) flies. This longitudinal study will provide a comprehensive understanding of the effect of tau expression on age-related phenotypes and the pathology that underlies any potential age-related changes.

## 2.2 Methods

### 2.2.1 Fly stocks

Assays were conducted using male progeny from genetic crosses of female Elav$^{C155}$-GAL4 flies with either UAS-htau$^{0N3R}$, UAS-htau$^{0N4R}$, or Oregon R (WT controls) male flies. For full genotypes and details of fly stocks used, see Appendix E. Flies were raised on standard fly food (Bloomington recipe, see Appendix D) at 23°C, 12-hour light:dark cycle. w$^{1118}$ flies were used for backcrossing htau$^{0N3R}$ and Elav-GAL4 flies.

### 2.2.2 Longevity assay

Three cohorts of 10 male flies of each genotype were separated 0-3 days' post eclosion and then were transferred to new food twice a week and scored for any deaths three times a week. Survival analysis was carried out using GraphPad Prism software and a Kaplein-Meir curve was plotted. A Log-rank (Mantel-Cox) test was performed on the data.

### 2.2.3 Climbing assay (locomotion)

The climbing ability of adult male flies was tested weekly. Three cohorts of 10-15 flies were used for this assay. Flies were anesthetised with $CO_2$ and placed in 50mL measuring cylinders and left to recover and acclimatise for 15 mins. Assay was conducted by tapping flies to the bottom of the cylinder and recording the flies climbing up the sides of the measuring cylinder. The distance climbed by the flies was recorded at 10 s. The assay was repeated three times, with 1 minute rest between each trial and the mean was calculated. GraphPad Prism software was used for statistical analysis and a two-way analysis of variance (ANOVA) was performed on the climbing data with the Bonferroni correction used for multiple comparisons.

### 2.2.4 Tau solubility

A total of 10 fly heads were homogenised in 40 µl of TBS/sucrose buffer (50 mM Tris-HCl pH 7.4, 175 mM NaCl, 1 M sucrose, 5 mM EDTA). The samples were then spun for 2 min at 1000 *g* and the pellet discarded. The supernatant was spun at 186,000 *g* for 2 h at 4°C. The resulting supernatant was "S1" – the aqueous soluble fraction. The pellet was resuspended at room temperature in 5% SDS/TBS buffer (50 mM Tris-HCl pH 7.4, 175 mM NaCl, 5% SDS and protease inhibitor cocktail) and spun at 186,000 *g* for 2 h at 25°C. The resulting supernatant was "S2" – the SDS-soluble, aqueous-insoluble fraction. The

pellet was resuspended at room temperature in 5% SDS/TBS buffer (50 mM Tris-HCl pH 7.4, 175 mM NaCl, 5% SDS and protease inhibitor cocktail) and spun at 186,000 *g* for 2 h at 25°C as a wash spin; following which the supernatant was discarded. This pellet was then resuspended in 8 M urea, 8% SDS buffer (50 mM Tris-HCl pH 7.4, 175 mM NaCl, 8% SDS, 8 M urea and protease inhibitor) and agitated for 12-18 h at room temperature ("S3"). All samples were diluted in 2 x Laemmli buffer and boiled for 5 min. "S1" and "S2" were loaded equally whereas double the amount of "S3" was loaded compared to "S1" and "S2".

### 2.2.5 Western Blotting

Western blotting was performed to assess total tau levels, phosphorylation and tau solubility state. Adult fly heads were homogenised in buffer in a ratio of 1 head: 10μl buffer (150 mM NaCl, 50 mM MES, 1% Triton-X, protease inhibitor cocktail, 30 mM NaF pH7, 40 mM 2-glycerophosphate pH 7, 20 mM Na pyrophosphate pH7, 3.5 mM Na orthovanadate, 1% SDS, 10 μM staurosporine). Samples were centrifuged at 3000 *g* for 2 min and unhomogenised material was discarded. Then 2 x Laemmli buffer was added to the supernatants and samples were boiled at 95°C for 5 min. These samples and those prepared for the tau solubility assay were run on 10% SDS PAGE gels and transferred to nitrocellulose membrane. Total tau levels were detected using anti-human tau polyclonal antibody (1:15,000, Dako), and anti-kinesin (1:5000, Cytoskeleton) was used as a loading control. The conformation and phosphorylation antibodies used were pS262 (1:1000, Invitrogen), MC1 (1:200) and PHF1 (1:500), (both gifts from Peter Davies), AT8 (1:800), AT100 (1:1000), AT180 (1:100), (all from Source Biosciences) and Tau-1 (1:2000, Millipore). Levels of all phosphorylated and conformational variants of tau were compared to total tau levels. *Drosophila* tau levels were detected using anti-dtau (gift from Prof St. Johnstone, University of Cambridge, UK) used at 1:2000 and compared to levels of kinesin. Signal was detected using fluorescent secondary antibodies against rabbit (IRDye, LI-COR) or mouse (Alexa-Fluor, Invitrogen), both used at 1: 20,000. A LiCor scanner with Odyssey software was used to detect the signal and ImageJ software was used to measure band densities. GraphPad Prism was used for statistical analysis and unpaired, two-tailed t tests were performed on the data.

### 2.2.6 Backcrossing (relevant to Figure 2-6)

Htau$^{0N3R}$ and Elav-GAL4 flies were backcrossed to w$^{1118}$ flies for seven generations. At each generation the mating females were snap frozen for DNA extraction. Genomic PCR was then carried out to check that htau$^{0N3R}$ gene was present in each generation.

### 2.2.7 Genomic PCR

Flies were homogenised in lysis buffer (10 mM Tris pH 8, 10 mM EDTA, 100 mM NaCl and 0.1% SDS and 2% proteinase K), then incubated at 55°C for one hour prior to homogenisation. The samples were spun at 13,000 rpm for 10 min to pellet undigested material. The supernatant was removed and mixed with equal volumes of isopropanol. The samples were then spun at 10,000 rpm for 5 min to pellet the DNA. The DNA pellet was then washed twice in 70% ethanol by centrifuging for 5 min at 10,000 rpm each time. After drying, the samples were resuspended in distilled water. A Nanodrop was used to ensure that each sample contained sufficient quantities of non-contaminated DNA.

### 2.2.8 PCR amplification

A total of 12.5 µl of redtaq and 9.5 µl distilled water was used per sample with 1 µl each of the forward and reverse primers and 1 µl of the DNA. The primer sequences used for detection of htau$^{0N3R}$ were forward 5'-GGAGCCCAAGAAGGTGGC-3' and reverse 5'-TGCTCAGGTCAACTGGTTTG-3'. The programme for detecting htau$^{0N3R}$ included an initial denaturing step of 30 s at 94°C and then 30 cycles of denaturation for 30 s at 94°C, annealing for 60 s at 60.6°C and extension for 60 s at 68°C. There was a final extension of 5 min at 68°C after which the samples were kept at 4°C. 2% agarose gels were used to run PCR products. Agarose gels were made up in TBE buffer with 0.01% Gel Red. 12 µl of PCR product was added to each lane of the gel. DNA bands were visualised using a Gel Doc XR+ Gel Documentation System (BIO-RAD).

### 2.2.9 Statistical analysis

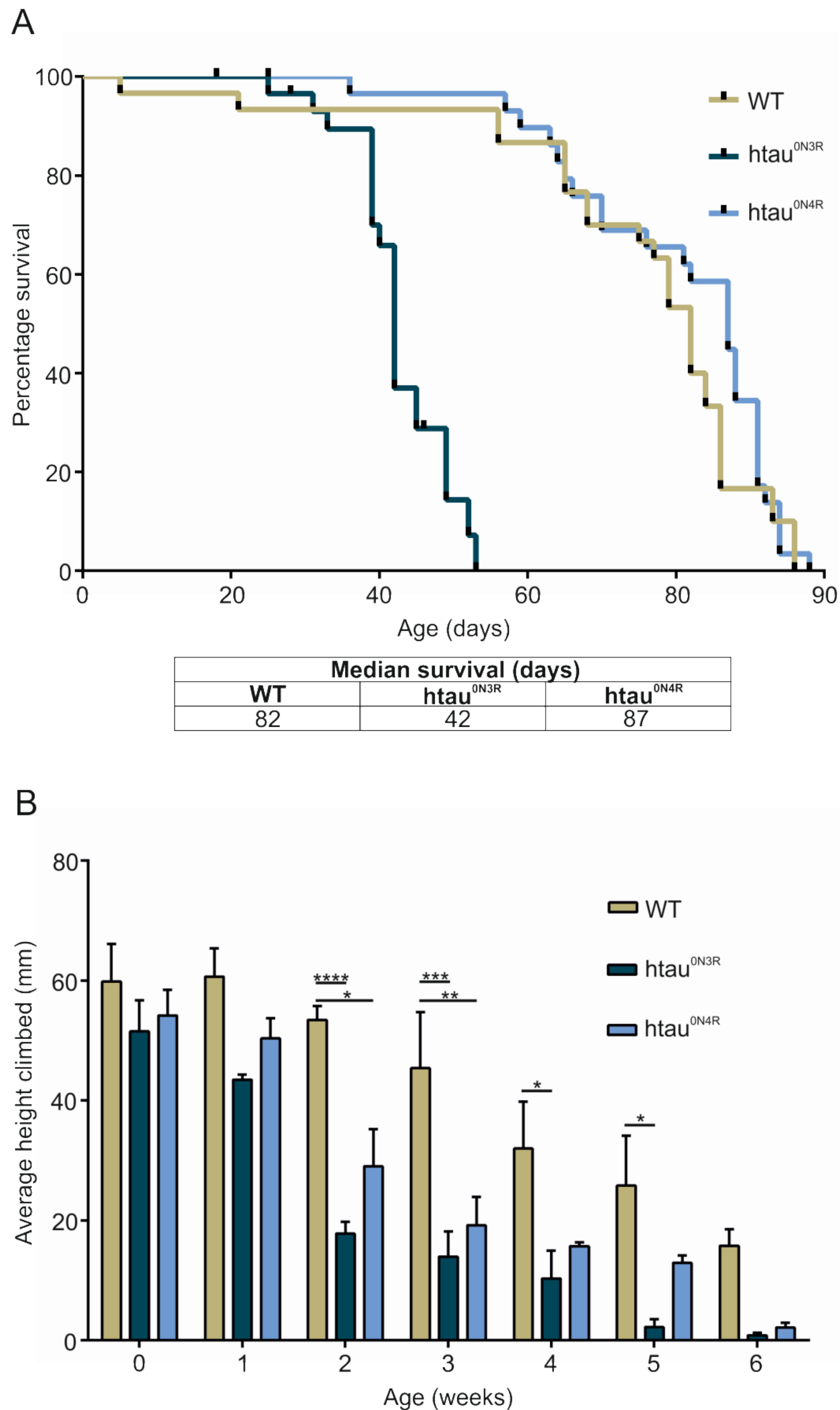
All values are presented as the mean ± standard error of the mean. To compare differences between groups, statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). The statistical test used for each assay is indicated separately. $p<0.05$ was considered to indicate a statistically significant difference.

## 2.3 Results

### 2.3.1 Htau$^{0N3R}$ produces greater deficits in age-related phenotypes compared with htau$^{0N4R}$

The primary aim of the studies described in this Chapter was to characterise tau phenotypes that are relevant to ageing. Age-related phenotypes, such as lifespan (Wittmann et al., 2001) and climbing (Folwell et al., 2010) have been used previously to determine how tau expression can affect ageing. Therefore, longevity and climbing phenotypes were assessed using two different human tau expressing *Drosophila* lines, one expressing 0N3R human tau (htau$^{0N3R}$) and another expressing 0N4R human tau (htau$^{0N4R}$). (Figure 2-1a) shows that htau$^{0N3R}$ flies die significantly quicker than both htau$^{0N4R}$ and WT control flies (Mantel-Cox test, p=0.0001). WT and htau$^{0N4R}$ flies do not have significantly different lifespans. WT and htau$^{0N4R}$ have similar median lifespan ages of 82 and 87 days respectively, whereas htau$^{0N3R}$ has a median lifespan of 42 days. This shows that htau expression shortens lifespan and that htau$^{0N3R}$ flies have a significantly shorter lifespan compared with htau$^{0N4R}$ flies.

Measuring the climbing ability of flies has been used previously to assess how locomotor function declines with age (Folwell et al., 2010, Roy & Jackson 2014). The climbing ability of the different isoform expressing lines was tested once a week for a total of 6 weeks due to the vast majority of flies not being able climb after this point (Figure 2-1b). A 2-way ANOVA was conducted on the three different groups (WT, htau$^{0N3R}$ and htau$^{0N4R}$) and the difference was found to be significant (p=0.0002). Multiple comparisons following the Bonferroni correction revealed that htau$^{0N3R}$ flies were significantly worse than WT flies at weeks 2, 3, 4 and 5. Whereas htau$^{0N4R}$ flies were found to be significantly worse than WT at weeks 2 and 3 only. Htau$^{0N3R}$ and htau$^{0N4R}$ flies did not display significant differences in climbing ability at any time point, but there was a trend for htau$^{0N4R}$ flies to be better than htau$^{0N3R}$ flies. Collectively these data show that both htau expressing lines exhibit age-related declines in climbing ability. However, htau$^{0N3R}$ flies have more severe age-related behavioural deficits compared to htau$^{0N4R}$ flies.




| Median survival (days) | | |
|---|---|---|
| WT | htau$^{0N3R}$ | htau$^{0N4R}$ |
| 82 | 42 | 87 |

Figure 2-1. Different isoform expressing flies have different ageing phenotypes.
(A) Htau$^{0N3R}$ flies have a significantly shorter lifespan than both WT flies (p=0.0001) and htau$^{0N4R}$ (p=0.0001) (n=30). The lifespan of htau$^{0N4R}$ flies is not significantly different to that of WT flies. Mantel-Cox test was used to analyse the data. (B) Climbing ability of htau$^{0N3R}$, htau$^{0N4R}$ and WT flies is significantly different between the 3 groups (two-way ANOVA; p=0.0002) (n=30). Error bars are plotted as ± S.E.M.

### 2.3.2 Htau$^{0N3R}$ and htau$^{0N4R}$ flies express different levels of human tau

Previously, increased tau expression has been shown to cause a worsening of tau induced phenotypes (Ubhi et al., 2007, Chen et al., 2007) and so the next step was to ensure that the differences seen in behavioural phenotypes between htau$^{0N3R}$ and htau$^{0N4R}$ flies were not due to differences in tau expression. Human tau expression was probed both in young (0 week, 0-3 day old) and old flies (6-week old) in both lines, shown in (Figure 2-1a). It was observed that htau$^{0N3R}$ flies have higher expression levels of human tau compared with htau$^{0N4R}$ at 0 weeks (t test, p=0.0001) and at 6 weeks (p=0.0066). It was also found that tau expression in htau$^{0N3R}$ flies was significantly higher in 6 week old flies, compared with expression in htau$^{0N3R}$ at 0 weeks (p=0.0045). There was also found to be a significant increase in human tau expression (p=0.0251) when comparing htau$^{0N4R}$ 6 wks to htau$^{0N4R}$ 0 wks (Figure 2b). Overall, the htau$^{0N3R}$ flies exhibited higher tau expression levels compared with htau$^{0N4R}$ flies, indicating a possible underlying reason for the differences in behavioural phenotypes. In addition, these data show that both tau isoforms accumulate with age.

Given the finding that tau accumulates with age, the next step was to understand whether this only occurred with the human tau or also with *Drosophila* tau (dtau) in WT flies. It was also important to understand whether human tau expression alters the expression of dtau. Dtau expression was therefore probed in WT, htau$^{0N3R}$ and htau$^{0N4R}$ flies , 0 and 6 weeks old (Figure 2-2c). Dtau expression was observed to increase with age in both WT (p=0.0193) and htau$^{0N4R}$ flies (p=0.0419), but not in htau$^{0N3R}$ flies. There was also found to be more dtau at 0 weeks in WT flies compared with htau$^{0N4R}$ flies (p=0.0022) and significantly more dtau was found at 6 weeks compared with htau$^{0N3R}$ flies (p=0.0309). These data show that in WT flies endogenous dtau also accumulates with age, similar to human tau. Furthermore, human tau expression appears to alter the levels of dtau.

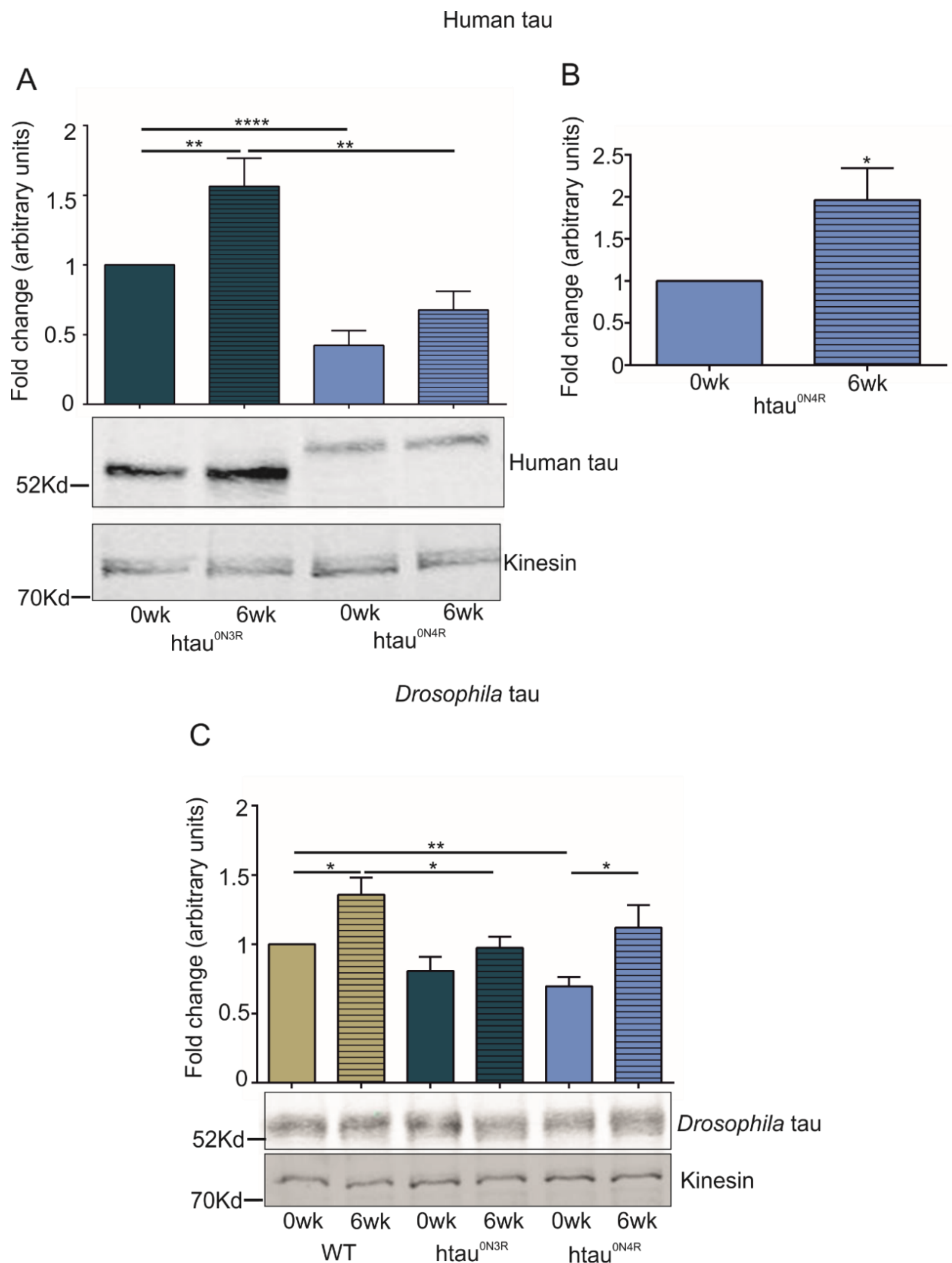



Figure 2-2. Expression of both human tau and *Drosophila* tau differs for htau$^{0N3R}$ and htau$^{0N4R}$ expressing flies.
(A) Expression of human tau in heads of young flies (0 wks old) and old flies (6 wks old) in both htau$^{0N3R}$ and htau$^{0N4R}$ flies (n=7-14). Htau$^{0N3R}$ total human expression was found to be significantly higher than htau$^{0N4R}$ at 0 wks (p=0.0001) and at 6 wks (p=0.0066). There is significantly more tau in htau$^{0N3R}$ 6 wk flies compared with htau$^{0N3R}$ 0 wk flies (p=0.0045). (B) Fold change of htau$^{0N4R}$ expression (6 wk) compared to htau$^{0N4R}$ (0 wk) (p=0.0251). (C) Expression of dtau in heads of young (0 wk) and old (6wk) WT, htau$^{0N3R}$ and htau$^{0N4R}$ flies. Dtau accumulates with age, when comparing 0 and 6 wk old files in both WT (p=0.0193) and htau$^{0N4R}$ (p=0.0419), but not in htau$^{0N3R}$ flies. Unpaired, two-tailed t tests were used for statistical analysis. Error bars are plotted as ± S.E.M.

### 2.3.3 Isoform specific differences in behavioural phenotypes not due to differences in expression

Due to previous findings that increased amount of tau correlates with phenotype severity (Ubhi et al., 2007) it may well be the case that the differences in the phenotypes (Figure 2-1) are due to differences in protein level (Figure 2-2). Therefore, an alternative htau$^{0N4R}$ line was used that when assessed for human tau expression level via western blot (Figure 2-3a), showed similar human tau expression levels to htau$^{0N3R}$ expressing flies. This alternative line is referred to as htau$^{0N4Rhigh}$ from here after. Htau$^{0N3R}$ and htau$^{0N4Rhigh}$ expressing flies were assessed for longevity and climbing, as performed previously for the low expressing htau$^{0N4R}$ line in Figure 2-1. In this case, htau$^{0N3R}$ flies still had significantly decreased lifespan compared with htau$^{0N4Rhigh}$ flies (p=0.0001), with median lifespan ages of 46 days for htau$^{0N3R}$ and 64 days for htau$^{0N4Rhigh}$ flies (Figure 2-3b). Assessment of climbing ability (Figure 2-3c) showed that htau$^{0N3R}$ flies performed significantly worse than htau$^{0N4Rhigh}$ flies (ANOVA, p=0.0001). Analysis using the Bonferroni correction revealed that htau$^{0N3R}$ was significantly worse that htau$^{0N4Rhigh}$ flies at weeks two and four. This suggests that the differences in phenotypes between htau$^{0N3R}$ and htau$^{0N4R}$ flies are not caused solely by differing levels of human tau expression.

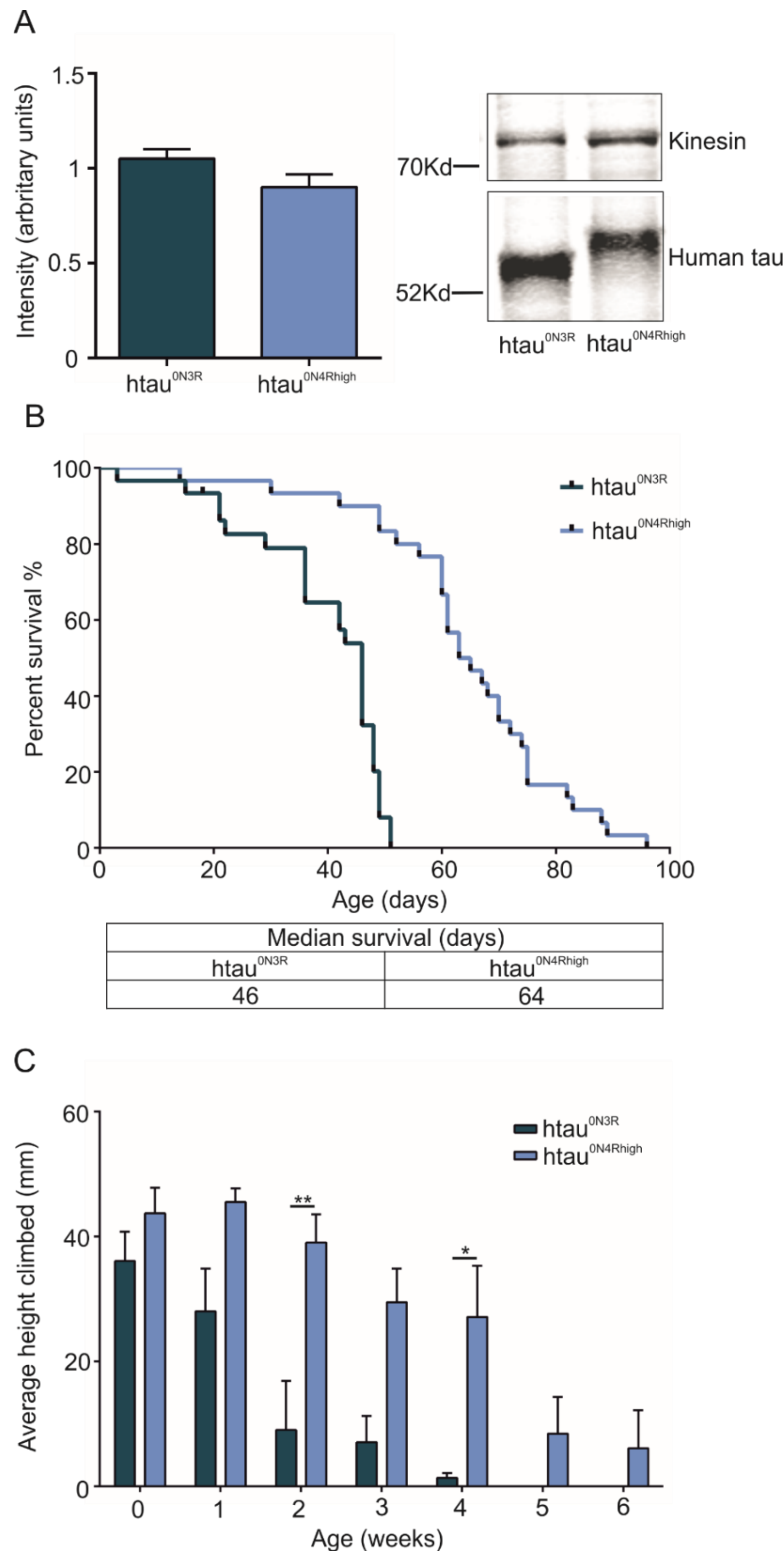



Figure 2-3. Comparison of htau$^{0N3R}$ and htau$^{0N4R}$ lines that have equal tau expression.
(A) A representative western blot with quantification of homogenates of heads from newly eclosed flies (0-3 days old) showing no significant difference in the expression of human tau when comparing htau$^{0N3R}$ and htau$^{0N4Rhigh}$. (B) Comparison of longevity of htau$^{0N3R}$ and htau$^{0N4Rhigh}$ expressing flies (n=30), htau$^{0N4Rhigh}$ expressing flies live significantly longer than htau$^{0N3R}$ flies (p=0.0001). (C) Climbing ability of htau$^{0N3R}$ and htau$^{0N4Rhigh}$ flies was found to be significantly different (2- way ANOVA; p=0.0001) (n=30). Analysis using the Bonferroni correction revealed that htau$^{0N3R}$ was significantly worse than htau$^{0N4Rhigh}$ flies at weeks 2 and 4. Error bars are plotted as ± S.E.M.

### 2.3.4 Htau$^{0N3R}$ and htau$^{0N4R}$ expressing flies have different phosphorylation profiles

The phosphorylation of tau is thought to be important in tau toxicity so it was crucial to assess any potential differences in the phosphorylation state of the tau expressed in the htau$^{0N3R}$ and htau$^{0N4R}$ lines (Figure 2-4). Five disease-associated phosphorylation epitopes were assessed using PHF-1 (Ser396/Ser404), AT8 (Ser202/Thr205), AT100 (Thr212/Ser214), AT180 (Thr231) and pSer262 antibodies. Tau-1, an antibody which recognises tau when it is not phosphorylated at Ser199/Ser202/ Thr205 sites was also used and MC1 was used to assess conformational changes in tau protein. The phosphorylated level of tau (Figure 2-4) was measured as a ratio of phosphorylated tau to total human tau expression. No differences were observed with age or with isoform with the PHF-1, AT8 and AT180 antibodies. pSer262 was increased with age in htau$^{0N3R}$ flies (p=0.0089). There was a significant reduction in signal observed with the AT100 antibody, with age, in htau$^{0N3R}$ flies (p=0.0001), with an increase seen in Tau-1 expression in htau$^{0N3R}$ flies (p=0.0258). There were no age-related changes in phosphorylation at any site assessed in the htau$^{0N4R}$ flies. These data show that there are differences in the phosphorylation profiles, with age, between the different isoforms, offering a possible explanation for the differences in age-related phenotypes.

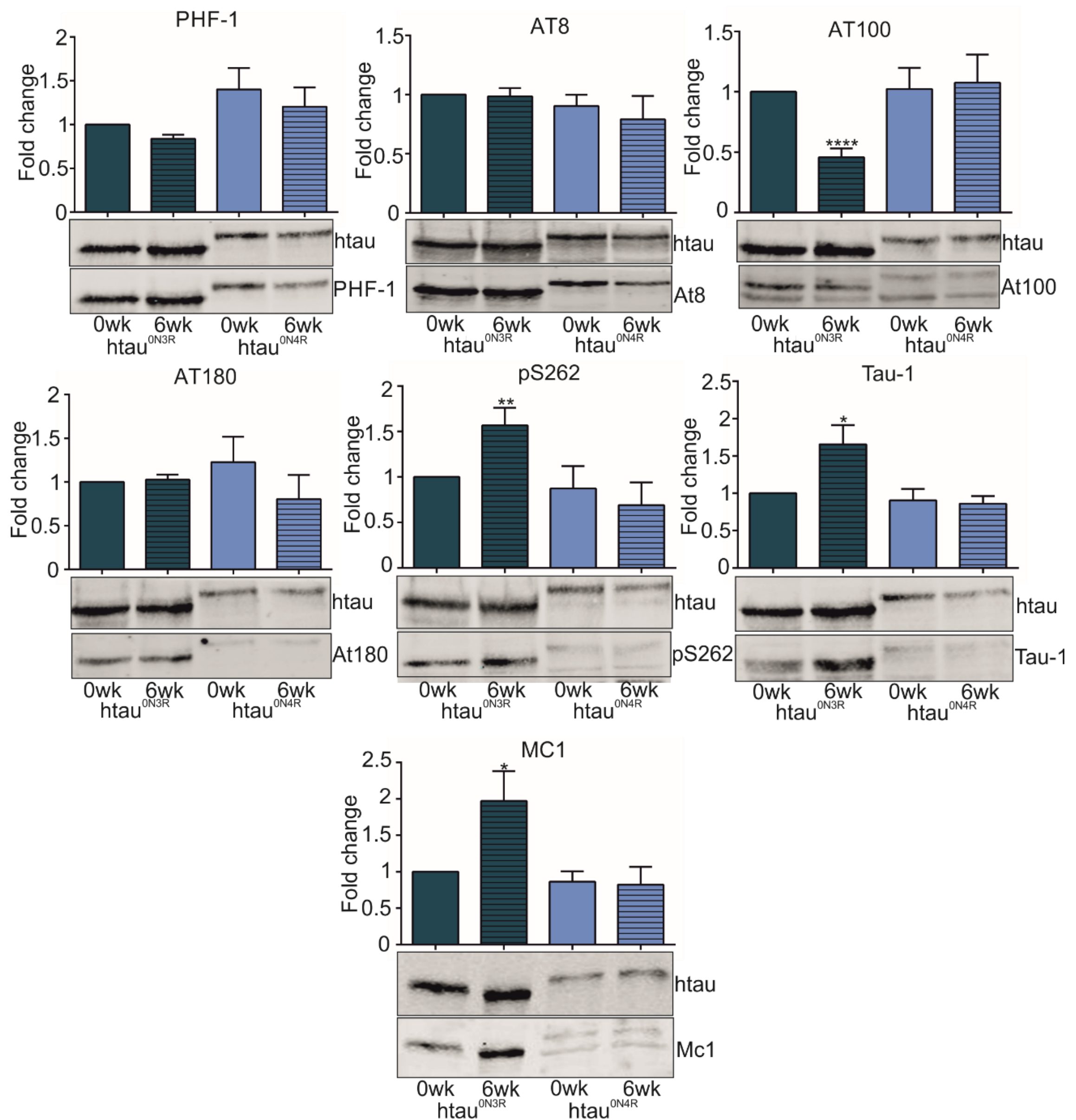



Figure 2-4. Western blots assessing phosphorylation state in htau$^{0N3R}$ and htau$^{0N4R}$ young (0 wk) and old (6 wk) flies.

The quantification shown represents the amount of phosphorylated tau relative to total tau levels and to htau$^{0N3R}$ 0 wk samples (n=5-12). No significant difference with age at PHF-1, AT8, and AT180 sites in either htau$^{0N3R}$ or htau$^{0N4R}$ expressing flies. htau$^{0N3R}$ flies show significant increase, with age, in phosphorylation at the pS262 site (p=0.0089). Also, in htau$^{0N3R}$ flies, there is a decrease, with age in the phosphorylation at the AT100 site (p=0.0001). Dephosphorylation at Ser199/202/Thr 205 site (Tau-1) is increased in htau$^{0N3R}$ flies. Tau reactive for MC1 increases with age in htau$^{0N3R}$ flies (p=0.0258) Unpaired, two-tailed t tests were used for statistical analysis. Error bars are plotted as ± S.E.M.

### 2.3.5 Tau solubility is similar between htau$^{0N3R}$ and isoforms htau$^{0N4R}$

In AD brains, it has been shown that tau forms insoluble aggregates including PHFs and NFTs, so it was important to understand the solubility state of the human tau expressed in htau$^{0N3R}$ and htau$^{0N4R}$ flies. Htau$^{0N3R}$ and htau$^{0N4R}$ heads were homogenised and separated into three fractions using a series of centrifugation steps. The three fractions contained the soluble fraction "S1", SDS soluble fraction "S2" and SDS insoluble fraction "S3". (Figure 2-5) shows that the majority of tau in both lines was present in the S1 fraction with less in the S2 fraction and very low amounts in the S3 fraction. It was also observed that the amount of insoluble tau found in the "S3" fraction did not increase with age or with the isoform being expressed. Although there appeared to be a trend for a decrease in the amount of insoluble tau in htau$^{0N3R}$ flies and a trend for an increase in htau$^{0N4R}$ flies, with age. These data suggest that the age-related phenotypes in longevity and climbing are not due to large insoluble aggregates of tau and are induced by smaller soluble species of human tau.

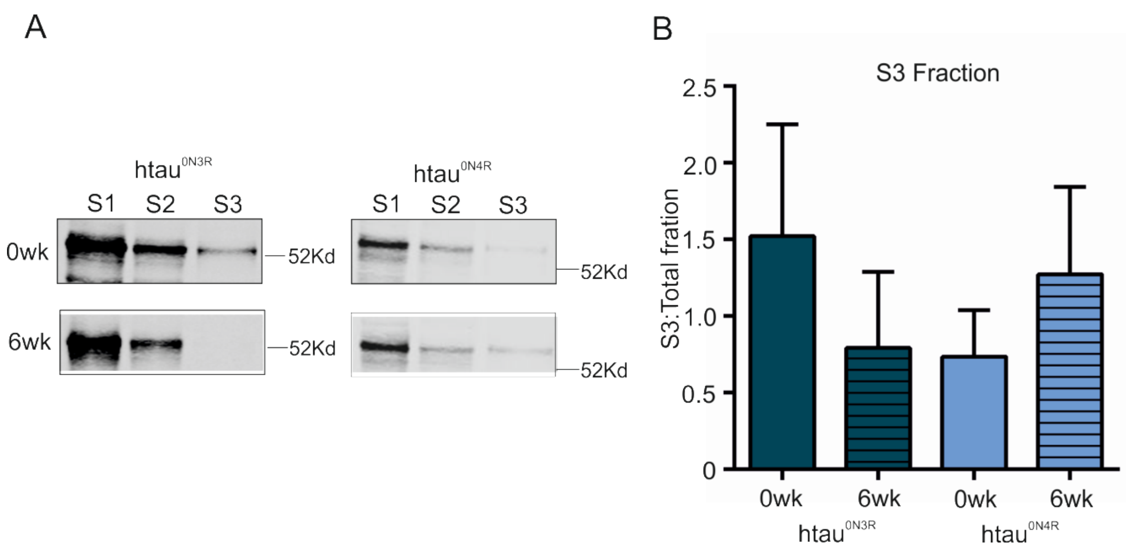
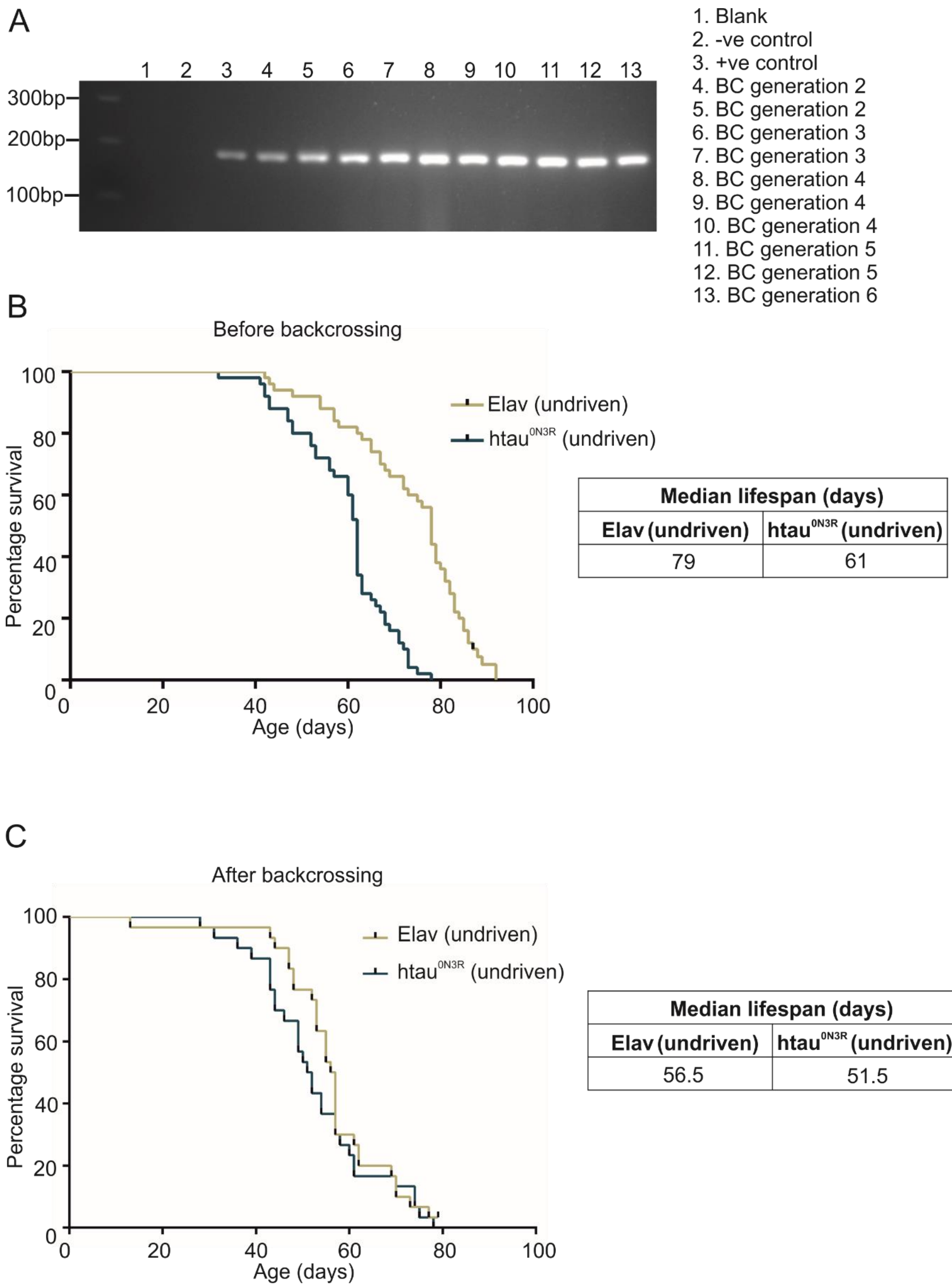



Figure 2-5. Differences in tau solubility between htau$^{0N3R}$ and htau$^{0N4R}$.
(A) Western blots of adult heads from young (0 wk) and old (6 wk) flies in the soluble (S1), SDS-soluble (S2) SDS-soluble and SDS-insoluble (S3) fractions. (B) Quantification of S3 fraction compared with the sum total of all fractions (n=4). There is no significant difference in the amount of insoluble tau between htau$^{0N3R}$ and htau$^{0N4R}$ lines or with age in either line. Unpaired, two-tailed t tests were used to test for significance. Error bars are plotted as ± S.E.M.

### 2.3.6 Backcrossing alleviates genetic background differences but maintains tau expression

It was decided that subsequent experiments would be carried out using only $htau^{0N3R}$ flies due to $htau^{0N4R}$ not having strong age-related phenotypes. It was possible that some of the differences seen in phenotypes between different tau expressing lines could be due to genetic background differences, hence the process of backcrossing was explored. The process of backcrossing to the same genetic background can be used to ensure that the different phenotypes exerted by different lines are only due to the genetic manipulation and not due to differences in genetic background. To do this, the $htau^{0N3R}$ flies were crossed with $w^{1118}$ flies, a white-eyed control line widely used for backcrossing, for seven generations. (Figure 2-6a) shows data from a genomic PCR performed on flies from each generation to ensure that the tau gene expression was not lost with each back-crossed generation. (Figure 2-6b and c) illustrate the reasons for backcrossing lines. When assessing the lifespan of the Elav-GAL4 (driver line) uncrossed and $htau^{0N3R}$ parent lines before backcrossing (Figure 2-6b), Elav flies lived significantly longer than $htau^{0N3R}$ flies ($p=0.0001$). After backcrossing (Figure 2-6c), Elav-GAL4 and $htau^{0N3R}$ flies do not have significantly different lifespans. This demonstrates that the process of backcrossing alleviates the genetic background effects that contribute to longevity phenotypes.




| Median lifespan (days) | |
|---|---|
| Elav (undriven) | htau$^{0N3R}$ (undriven) |
| 79 | 61 |

| Median lifespan (days) | |
|---|---|
| Elav (undriven) | htau$^{0N3R}$ (undriven) |
| 56.5 | 51.5 |

Figure 2-6. Backcrossing alleviates differences in genetic backgrounds, allowing comparisons of different lines.
(A) Genomic PCR demonstrated that with every backcrossed generation, tau expression was retained. (B) Longevity of parent non-driven Elav and htau$^{0N3R}$ flies before backcrossing. Elav flies live significantly longer than htau$^{0N3R}$ flies (n=30) (p=0.0001). (C) After backcrossing Elav and htau$^{0N3R}$ flies do not have significantly different lifespans (n=30). Longevity analysed by Mantel-Cox test.

## 2.4 Discussion

### 2.4.1 Summary

Age-related changes in tau-mediated phenotypes and pathologies were demonstrated here, by the pan-neuronal expression of two different human tau isoforms (htau$^{0N3R}$ and htau$^{0N4R}$). It was shown that these lines caused different ageing phenotypes with htau$^{0N3R}$ having a more severe effect on lifespan and climbing compared with htau$^{0N4R}$. It was found that, although there were differences in levels of human tau expression, this was not found to be the only contributing factor to the differences in age-related phenotypes. The different htau-expressing flies also showed different tau phosphorylation profiles, with age. The different lines, however did not show any differences in the solubility of the human tau protein being expressed, suggesting that the species of tau responsible for age-related tau phenotypes in *Drosophila* are small soluble phosphorylated forms.

### 2.4.2 Different isoforms of tau exert different phenotypes

Although there has been little research to directly compare htau$^{0N3R}$ and htau$^{0N4R}$ expression in *Drosophila* previously, it has been found that htau$^{0N3R}$ and htau$^{0N4R}$ isoforms exert different phenotypes when expressed in the mushroom bodies. Htau$^{0N4R}$ induces loss of the mushroom body structure whereas htau$^{0N3R}$ does not cause the same toxicity (Kosmidis et al., 2010). This could be construed as a contradiction to the results shown here, however it is possible that the different isoforms exert differential levels of toxicity depending on the tissue in which they are expressed. When comparing learning and memory in the same lines used in this study, htau$^{0N4R}$ flies performed worse than htau$^{0N3R}$ flies in a learning and memory assay, see Appendix A (Sealey et al., unpublished), most likely due to htau$^{0N4R}$ being more toxic in the mushroom bodies (Kosmidis et al., 2010). In a different study, although not comparing between 3R and 4R isoforms the differences in the expression of dtau and htau$^{2N4R}$ in the eye were compared. Here they found that htau$^{2N4R}$ had a worse rough eye phenotype compared with the expression of dtau. This study also investigated potential modifiers of tau; and interestingly they found that, although some modifiers altered the rough eye phenotype of both dtau and htau$^{2N4R}$, other modifiers only affected one or the other type of tau (Chen et al., 2007), suggesting that different types of tau may be functionally different and interact with different proteins.

Many of the studies that have investigated the expression of human tau proteins in *Drosophila,* expressed human tau in the eye to produce a rough eye phenotype, as in the study discussed above (Chen et al., 2007). These studies have been conducted using either wild-type or mutant 4R tau which cause toxicity in the eye (Jackson et al., 2002, Fulga et al., 2007, Steinhilb et al., 2007, Iijima et al., 2010, Iijima-Ando et al., 2010), but no studies have directly compared $htau^{0N3R}$ and $htau^{0N4R}$ expression in the eye. Previous data from the Mudher laboratory demonstrated that when $htau^{0N3R}$ and $htau^{0N4R}$ were expressed in the eye, $htau^{0N4R}$ induced greater toxicity than $htau^{0N3R}$, suggesting that the expression of different tau proteins in different tissues can induce a variety of phenotypes, see Appendix A (Sealey et al., unpublished). The reasons that underpin this may be due to structural differences between 3R and 4R tau isoforms. It has been demonstrated that 4R isoforms bind to microtubules more strongly than 3R isoforms (Butner & Kirschner, 1991) (Goode et al., 2000), with this due to the 31-amino acid insertion containing the second microtubule binding domain being absent in 3R isoforms. Goode et al., (2000) also observed that the way in which 3-repeat and 4-repeat isoforms bind to microtubules differs, as depicted in Figure 2-7. Although tau is thought to have very little secondary/tertiary structure in solution, when tau binds to microtubules the secondary structure is induced depending on the specific interactions of tau with the microtubules. As there are specific differences between the ways 3R and 4R isoforms bind to microtubules, the secondary structures formed are quite different (Goode et al., 2000). The distinct structures formed by the different isoforms may well alter how other proteins interact with tau when tau is bound to microtubules. It may also mean that other proteins may not be able bind to the microtubules as efficiently causing differences in transport of cargo up and down the axon.

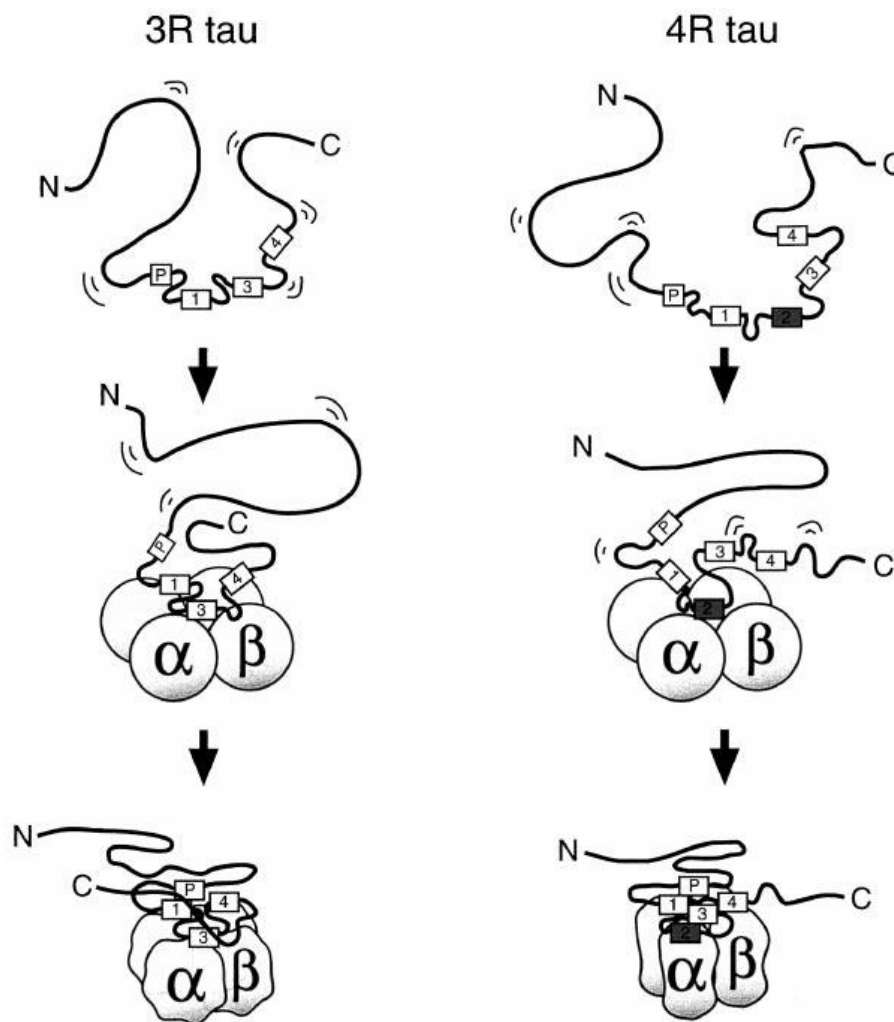



Figure 2-7. The binding of 3R and 4R isoforms to tau differs taken from (Goode et al., 2000).
Tau, in solution, has little secondary structure but upon binding to microtubules becomes more ordered, forming a secondary structure that depends on the interactions that the specific isoform has with the microtubules. As the 3R and 4R isoforms bind differently to microtubules the subsequent secondary structures formed also differ.

### 2.4.3 Tau accumulates with age

It has been shown here that tau accumulates with age in the brains of *Drosophila,* observed in both the isoforms investigated as well as for the native dtau. Much of the research that has been conducted in *Drosophila* using expression of human tau has not investigated how the tau protein levels change with age. Wittmann et al. (2001) reported that no change in tau protein levels were observed, with age, when human tau was expressed. However, in that study, flies were only aged for ten days (Wittmann et al., 2001) and this is too early to assess any real age-related changes in tau protein levels as flies can live up to 3 months. Studies conducted in brains taken from an inducible rTg4510 mouse model that expresses P301L mutant human tau, have shown that total tau levels increase, with age, from 1 month to 4.5 months (Berger et al., 2007). Similarly, in a mouse model that over-expresses a mutant form of 4R tau (R406W mutation) very little human tau was observed when using

immunohistochemistry on the whole brain at 3 months. However, by 6 months, staining was detected in the hippocampus and amygdala. By 8 months, human tau was also found in the neocortex and caudate putamen as well as the hippocampus. By 14 months, the accumulation of human tau was seen in the whole neocortex and subcortical regions (Ikeda et al., 2005). Collectively, this demonstrates that tau does accumulate with age although it is not clear why this occurs.

Increased tau expression has been reported in human AD brains. However, much of this work has been conducted using CSF (Sunderland et al., 2003, Green et al., 1999), meaning that it is difficult to decipher whether this is due to increased tau in the brain or whether more tau is being released into the CSF. There have, however, been other studies that have directly investigated tau levels in brain homogenates and observed that the amount of total tau is at least 4 fold increased in AD brains compared with control brains (Khatoon et al., 1992). Genetic studies have found that different haplotypes of tau drive higher tau expression correlating with an increased risk of AD (Iqbal et al., 2005). What is not clear is why tau expression increases in AD. Two possible hypotheses for the increase in tau in AD are that either there is more tau being produced through translation. Alternatively, less tau is being cleared. Most studies do not report an increase in the amount of tau mRNA found in AD brains (Mah et al., 1992). However, some studies have reported increased tau mRNA in selected regions of the brain, for example, in the hippocampus (Barton et al., 1990) There is more evidence for the accumulation of tau being due to reduced clearance. Two clearance mechanisms have been identified as potentially becoming dysfunctional in AD; the UPS and autophagy. Ubiquitin has been found to accumulate in the tangles in AD brains (Perry et al., 1987), suggesting that although tau protein is being targeted by ubiquitination for degradation by the proteasome, the UPS is not able to clear away the tau. In support of this, deficits in proteasome activity have been found in the hippocampus of AD brains (Keller & Markesbery, 2000). Similarly, components of autophagy, such as Beclin-1, have been found to be decreased in the entorhinal cortex and dentate gyrus of AD brains (Pickford et al., 2008). Other studies in human AD brains have found that markers of autophagy co-localise with neurofibrillary tangles (Ma et al., 2010). This suggests that autophagy is activated by the accumulation of tau but is not able to clear the tau found in tangles. This could be because tau becomes gridlocked, getting stuck and not being cleared away, as shown in (Bakhoum et al., 2007). Collectively these studies provide evidence that total tau levels are increased in AD brains, consistent with what has been shown here in *Drosophila*, and that this could well be due to increased synthesis or reduced clearance of tau.

### 2.4.4 Differences in phenotypes are not due to expression level

Studies have shown that tau can induce toxicity in a dosage-dependent manner. For example, when two copies of dtau are expressed, this increases the rough eye phenotype compared with the expression of a single copy of dtau (Chen et al., 2007). Similar dose-dependent effects on both axonal transport and larval locomotion were observed when two copies of dtau were expressed. (Ubhi et al., 2004). In the current study, $htau^{0N3R}$ was observed to be expressed at higher levels compared with $htau^{0N4R}$, and it is possible that this directly caused the reduced lifespan and greater climbing defects. Although it is not possible to rule out a contribution of the higher expression of $htau^{0N3R}$, it is certainly not the only factor involved. When assessing a different $htau^{0N4R}$ line ($htau^{0N4Rhigh}$) which exhibited similar levels of human tau expression to $htau^{0N3R}$, lifespan and climbing phenotypes were still greatly different to each other, with $htau^{0N3R}$ flies consistently performing worse than $htau^{0N4Rhigh}$ flies. In addition, as discussed above, the lower-expressing $htau^{0N4R}$ line was used for studies on learning and memory and eye toxicity (see Appendix A Figure 6-1 and Figure 6-2). Although it showed lower tau expression, the line exhibited more potent phenotypes in these tissues than the $htau^{0N3R}$ line (Sealey et al., unpublished). Overall, this suggests that the level of tau expressed is less important than the tau isoform and the tissue it is expressed in.

### 2.4.5 Specific phosphorylation residues are important for phenotypes

The phosphorylation state of tau, which was shown to differ between isoforms, could also play a role in the differences seen between the different transgenic lines. Increased phosphorylation at Ser262 was observed in $htau^{0N3R}$ flies but not in $htau^{0N4R}$ flies. Ser262 has been reported to be a site that is phosphorylated early in NFT development (pre-tangle) (Augustinack et al., 2002). This site has also been found to be one of the early phosphorylation sites that can trigger the downstream phosphorylation of other sites on tau (Nishamura et al., 2004). In addition, if the Ser262 is mutated to an alanine, tau toxicity is reduced (Nisamura et al., 2004). In that study, a form of mutant 4R htau (R4O6W) was used which causes moderate eye degeneration. On mutating Ser262 and Ser356 to alanines ($htau^{R406WS2A}$) the rough-eye phenotype caused by the expression of $htau^{R406W}$ was found to be reduced. Conversely, co-expression of PAR-1 kinase, which directly phosphorylates tau at Ser262 exacerbated the rough-eye phenotype caused by expression of $htau^{R406W}$ (Nishimura et al., 2004, Chatterjee et al., 2009). In addition, the $htau^{R460WS2A}$ line showed less toxicity in the mushroom bodies (Kosmidis et al., 2010). Blocking phosphorylation at Ser262 reduced ablation of the mushroom bodies by the expression of $htau^{R406W}$ and

consequently improved memory deficits back to control levels (Kosmidis et al., 2010). There has also been research conducted to further understand the importance of Ser262. Fischer et al., (2009) mutated the Ser262 to glutamate, in vitro, which mimics phosphorylation, and found that this resulted in tau being unable to bind to microtubules (Fischer et al., 2009). This suggests that the phosphorylation of Ser262 impairs binding of tau to microtubules, resulting in microtubule destabilisation. Taken together, these studies show the importance of the Ser262 site and suggests how increased phosphorylation of this site, with age, could contribute to the worsened ageing phenotypes seen in the htau$^{0N3R}$ flies.

A decrease of AT100 immunoreactivity, with age, was also observed in the htau$^{0N3R}$ flies. This has also been reported in the rTg4510 mouse (Dickey et al., 2009). These researchers showed that, although they did see an initial increase in AT100 positive tau in the brains of 3 and 5.5 month old mice, at 9 months there was sharp decline in AT100 immunoreactivity. They suggested that this may be caused by conformational changes or other post-translational modifications that lead to reduced access of the AT100 antibody to this site (Dickey et al., 2009). This may well be the case, as it was shown in this work that there was an age-related increase in MC1 staining, a conformation specific antibody that only binds to tau when the N-terminal of tau has folded back on its self and interacts with the microtubule-binding domain (Weaver et al., 2000). Age-related increases in MC1 were found and coincided with age-related decreases in the htau$^{0N3R}$ flies, but not in the htau$^{0N4R}$ flies. Other studies have also reported age-related changes in MC1 tau immunoreactivity both in flies (Wittmann et al., 2001) and in mice (Dickey et al., 2009). It was also shown in this current study that there was an increase in Tau-1 immunoreactivity, which recognises non-phosphorylated tau. This has also been reported in a different fly model of tauopathy, where it was shown that the phosphorylation of tau at Ser262 stabilised de phosphorylated tau unbound to microtubules (Ando et al., 2016). Collectively, these data suggest that increased phosphorylation at Ser262 could be driving the age-related phenotypes in htau$^{0N3R}$ flies by reducing binding to microtubules, triggering conformational changes and stabilising non-phosphorylated tau.

### 2.4.6 Phenotypes seen in the absence of insoluble tau

There is much debate in the field about which species of tau exerts toxic effects. It has been demonstrated here that expression of human tau in *Drosophila* can produce age-related phenotypes without the production of large insoluble aggregates, such as NFTs. Whilst from the work here it is not possible to state the exact species of tau that causes the observed phenotypes, it has been demonstrated that these species are soluble or SDS-

soluble forms of tau. This echoes what others have reported in other fly models that overexpress human tau. Wittmann et al., used electron microscopy to show that expression of 4R tau, though causing neurodegeneration, showed no evidence of NFTs or large filamentous structures of tau. (Wittmann et al., 2001). In fact, very few studies have reported finding filamentous tau in the brains of tau-expressing flies, suggesting that the phenotypes, at least in flies, are caused by smaller, soluble forms of tau. There have been similar findings in mouse models expressing 4R tau with the P301L mutation, both in the rTg4510 and JNPL3 mouse models. rTg4510 mice show memory deficits at 3.5 months although no noticeable NFT pathology is found in the mouse brains at this time. These researchers did, however, see higher molecular weight species of tau, migrating on western blots at 140 kDa/170 kDa, which they termed "multimers" (Berger et al., 2007). This study therefore again demonstrated that NFTs are not necessary for tau-induced phenotypes, but that smaller aggregates, such as oligomers, are the toxic species. Future work should include determining the exact species of tau that is present in the fly models used here and how these species evolve over time.

### 2.4.7 Genetic background is important when studying ageing phenotypes

One can suggest that the differences seen in the phenotypes of the flies expressing different isoforms (Figure 2-1) may be due, at least in part, to the genetic background of the flies. Lifespan can be very different depending on the genetic background of the flies (Partridge & Gems, 2007). This is clearly shown in (Figure 2-6b) when assessing the different parent lines, Elav-GAL4 and htau$^{0N3R}$, which have significantly different lifespans. After backcrossing, however, these lines no longer showed significantly different lifespans. By backcrossing lines, it is possible to more accurately measure the contribution of a specific perturbation on lifespan, such as the expression of human tau. It is not only lifespan that may well be affected by genetic background; other studies have also shown that genetic background also significantly affects climbing phenotypes (Gargano et al., 2005). In future work, it will be important to decipher the contribution of genetic background to the differences in the phenotypes seen in the htau$^{0N3R}$ and htau$^{0N4R}$ flies by backcrossing both lines to the same genetic background. Additional controls could also be used, to strengthen the data, that better match the genetic background of the experimental flies used here, for example crossing w1118 with either isoform would produce control flies that contain one copy of the htau but with a genetic background that closely resembles the experimental line.

### 2.4.8 Conclusions and future directions

It has been demonstrated here that different isoforms of tau cause differences in age-related phenotypes, which are underpinned by changes in the biochemical properties of tau. This may well have implications for human diseases as different isoforms are found to underlie different tauopathies. For example, both 3R and 4R isoforms are found in the tau pathology in AD, Down's Syndrome, ALS and certain forms of FTDP-17, whereas only 4R isoforms are found in CBD, PSP and different forms of FTDP-17. Conversely, in PiD the tau pathology is made up of only 3R isoforms, reviewed in (Buée et al., 2000). The reason why different isoforms of tau underlie different diseases is likely due to their different biochemical properties. Differences in properties such as phosphorylation, conformation and aggregation could well underlie the selectivity of these isoforms in causing distinct effects in different tissues.

In this thesis, now that age-related changes in tau-mediated phenotypes and pathologies in *Drosophila* have been characterised, it is now possible to assess the contributions that age-related pathways have on tau-mediated phenotypes and pathologies. It would be interesting to investigate whether other, more complex behaviours are also vulnerable to human tau expression, therefore this will be explored in the next Chapter. Only the $htau^{0N3R}$ line will be used in subsequent experiments due to the stronger age-related behavioural phenotypes of this isoform.

# Chapter 3: Characterisation of AD relevant complex behaviours in tau expressing *Drosophila*

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## 3.1 Introduction

The previous chapter demonstrated how lifespan and locomotive behaviours can be used to measure the effect that expression of human tau has on ageing phenotypes. However, these behaviours do not closely represent the complexity of the symptoms experienced by AD patients. The primary symptom of AD is a deficit in memory, beginning with short term memory and progressing in later stages of the disease to deficits in long term memory. In addition, early on in disease, AD patients have disruptions in circadian rhythms and, as the disease progresses, patients can experience other symptoms such as depression, anxiety, language or communication problems and decreased locomotor abilities. Although it is not possible to model all of these symptoms, some of the complex behaviours that are core to these symptoms can be modelled in *Drosophila*, such as learning and memory and circadian rhythms. This Chapter will assess the impact that $htau^{0N3R}$ has on these behaviours.

### 3.1.1 Circadian rhythms

Circadian rhythms are defined as any biological process that has an endogenous daily oscillation. Circadian rhythms are highly conserved across most organisms including *Drosophila* and humans. *Drosophila,* similarly to humans have a ~ 24-hour cycle that is synchronised by external environmental cues such as light and temperature and a central internal body clock (Panda et al., 2002). In humans a central clock is contained within the hypothalamic suprachiasmatic nuclei (SCN). The SCN is made up of around 20, 000 neurons and is broadly separated into two main compartments, the dorsal shell and the ventral core. The dorsal shell contains neurons positive for arginine vasopressin (AVP) and the ventral core neurons are positive for vasoactive intestinal polypeptide (VIP). AVP and VIP are neuropeptides that are transmitted across the SCN network allowing for synchronisation of the clock neurons. The network that governs circadian activity in *Drosophila* is less complex than the mammalian SCN, containing around 150 neurons. The central clock, in *Drosophila,* is governed only by around 18 neurons, these neurons contain the neuropeptide (pigment dispersing factor) Pdf, which acts like AVP and VIP in the mammalian clock, to synchronise the *Drosophila* central clock. The molecular clock that governs circadian rhythms in *Drosophila* and humans is highly conserved with minor differences, with transcription-translation negative feedback loops being at the core of the molecular clock, as shown in (Figure 3-1). In the SCN in humans, the transcription factor CLOCK dimerises with BMAL1, and this heterodimer (CLOCK: BMAL1) then translocates to the nucleus and activates transcription. CLOCK: BMAL1 activates the transcription of PERIOD (PERs) and CRYPTOCHROME (CRYs) via E-box elements.

PERs: CRYs then hetrodimerise in the cytoplasm and subsequently translocate to the nucleus leading to the inhibition of CLOCK: BMAL1-mediated transcription, thereby inhibiting the transcription of PERs and CRYs. This mechanism is very similar in *Drosophila*, although some components are different, see Figure 3-1. In *Drosophila* CYCLE (CYC), an orthologue of BMAL1, performs a similar function and dimmerises with CLOCK. Also, in *Drosophila*, TIMELESS (TIM) performs the role that CRYs does in the mammalian clock (Gertsner & Yin 2010).

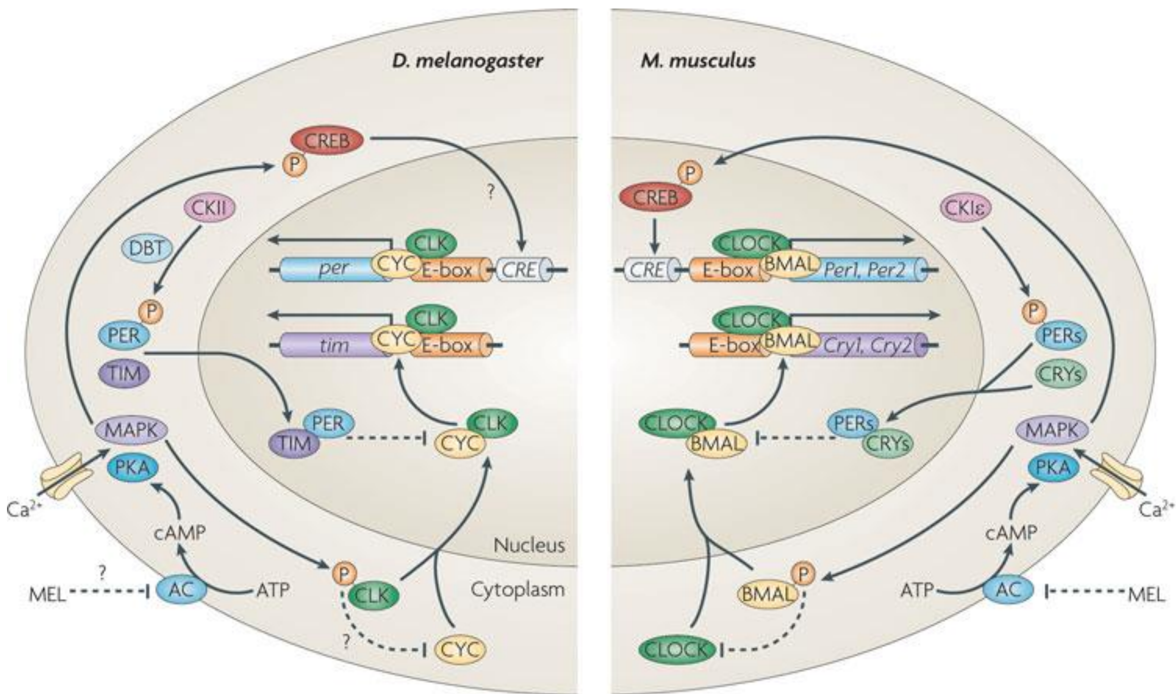



Figure 3-1. Comparison of the *Drosophila* and mammalian central clock oscillator, taken from (Gerstner & Yin, 2010).
In both mammals and *Drosophila,* a transcription-translation negative feedback loop controls the central molecular clock. Transcription factor CLOCK dimerises with BMAL in mammals and CYC in *Drosophila*, and heterodimers (CLOCK: BMAL) translocate to nucleus and activates the transcription of PERs (in both mammals and *Drosophila)* and CRYs (in mammals)/TIM (in *Drosophila*).

### 3.1.2 Measuring circadian rhythms in *Drosophila*

In both humans and *Drosophila,* the circadian oscillations are able to entrain to environmental cues such as light and temperature but they are also self-perpetuating. This means that it is possible to assess the rhythms of the central clock without environmental cues by putting animals onto constant darkness and temperature. This is termed “free running conditions” and can be used to measure the behavioural output of the central clock. In order to do this, the locomotor activity of flies can be measured using specifically designed equipment that can measure activity constantly for days or even weeks at a time.

To do this, individual flies are placed in small glass tubes and inserted into devices that pass an infrared beam down the middle of the tube. The breaking of this beam by the flies' movement can then be recorded. In using this experimental setup, it is possible to see that flies, similar to humans, operate a 12:12 activity cycle, being active during the day and resting/ sleeping during the night. Flies also have anticipatory peaks of activity at both dawn and dusk, with a relatively low level of activity in between these two peaks of activity, see Figure 3-2 (Grima et al., 2004).

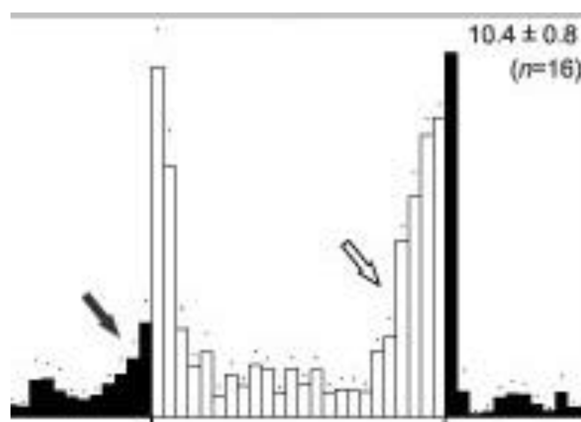



Figure 3-2. Daily activity profile of wild type *Drosophila*, adapted from (Grima et al., 2004).
Conducted in a light:dark 12:12 cycle, with the dark bars indicating the flies' activity in the dark part of the cycle and the light bars indicating activity during the light part of the cycle. The arrows on the profile indicate the beginning of both the morning anticipatory peak (black arrow) and the evening anticipatory peak (white arrow).

Monitoring locomotor activity in this way has been instrumental in being able to understand the mechanisms that underlie circadian rhythms. These systems are ideal for testing different mutant flies and assessing their contribution to circadian rhythms. For example, flies with a null mutation in the clock gene *per* (produces PERIOD protein; $per^0$) were found to have arrhythmic behaviour, meaning that the flies did not have the characteristic 12:12 cycles of activity and rest (Konopka & Benzer, 1971). Studies such as these have paved the way in identifying and characterising the now established molecular oscillations of the highly conserved central clock.

Due the relative simplicity of being able to measure the locomotor behaviour readout of the central molecular clock, flies lend themselves as an ideal model to identify the underlying mechanisms of disease associated disruptions in circadian rhythms. AD is a particular disease in which disruptions in circadian rhythms have been shown to be an early symptom of the disease. AD patients experience disruptions in their sleep/wake cycles with patients being less active during the day and more active nocturnally (Hatfield et al., 2004, Prinz et al., 1982). AD patients also experience what is known as "sundowning", where AD symptoms are exacerbated at specific times of day, usually late afternoon or early evening

(Volicer et al., 2001). To attempt to understand what underlies these circadian disturbances in AD patients, animal models of AD have been used to assess changes in circadian rhythms on expression of disease-associated proteins, Aβ and tau. Recently, studies conducted in flies that overexpress Aβ peptides have been assessed for changes in circadian disturbances with age. These studies found that Aβ expression resulted in the loss of circadian behavioural rhythms which became progressively worse with age (Chen et al., 2014, Long et al., 2014). In a different approach, it has been demonstrated that disruption to the regulation of the molecular clock enhances neurodegeneration and tau mediated phenotypes in *Drosophila*. Price et al., (1998) utilised a kinase-dead mutant of double time $Dbt^{K/R}$, a key regulator of the molecular clock, in *Drosophila*, that mediates Per degradation (Price et al., 1998). Co-expression of $Dbt^{K/R}$ with $htau^{0N4R}$ was found to enhance $htau^{0N4R}$ mediated neurodegeneration in the eye and $htau^{0N4R}$ mediated deficits in longevity and climbing phenotypes (Means et al., 2015). Although this demonstrates that circadian rhythms can affect tau-mediated phenotypes, it has not yet been demonstrated whether tau is able to disrupt circadian behaviours, as has been shown with Aβ. Therefore, this is an important avenue of research left to fully understand the mechanisms that underlie circadian rhythm disturbances in AD.

### 3.1.3 Learning and memory in *Drosophila*

Although disruption of circadian rhythms is an early symptom of AD, the main symptom that is most prominent in the disease is loss of memory, and it is this that is the central focus of disease-modifying therapies. The mechanisms that underlie the different forms of learning and memory in humans are highly complex and involve various regions of the brain. These memory-associated regions of the brain, such as the hippocampus, are also found to be particularly susceptible to tau and Aβ pathology in AD. This suggests that tau and Aβ have important roles in the mechanisms that underlie the memory deficits in AD. This has also been demonstrated in various mouse models that express both tau and Aβ proteins (Oddo et al., 2003, Schindowski et al., 2006).

To further elucidate how tau affects learning and memory, researchers have utilised *Drosophila* models. The majority of these studies have used olfactory classical conditioning assays where flies associate an odour with a negative stimulus (e.g. electric shock), or with a positive stimulus (e.g. food reward). After a period of training, if the flies have learnt the association, they will avoid the odour associated with the negative stimulus and favour the odour that is associated with a positive reward (Beck et al., 2000). Genetic and pharmacological manipulations have been conducted in conjunction with these assays.

These studies found that the region of the *Drosophila* brain responsible for this type of learning is the mushroom bodies (MB) (de Belle & Heisenberg, 1994, McGuire et al., 2001). The mushroom bodies, depicted in (Figure 3-3) are made up of 2500 neurons contained within each of the two brain hemispheres. Using the classical olfactory assay, it was found that expression of htau$^{0N4R}$ caused a 25-30% deficit in learning ability compared to control flies. htau$^{0N4R}$ expression also caused deficits in memory at both 90 and 180 minutes' post training (Mershin et al., 2004). A different study found that 4R-isoforms of tau, driven throughout the CNS, selectively caused ablation of the mushroom bodies (Kosmidis et al., 2010). Few studies have investigated the impact that htau$^{0N3R}$ has on learning and memory and so it is important to understand this.

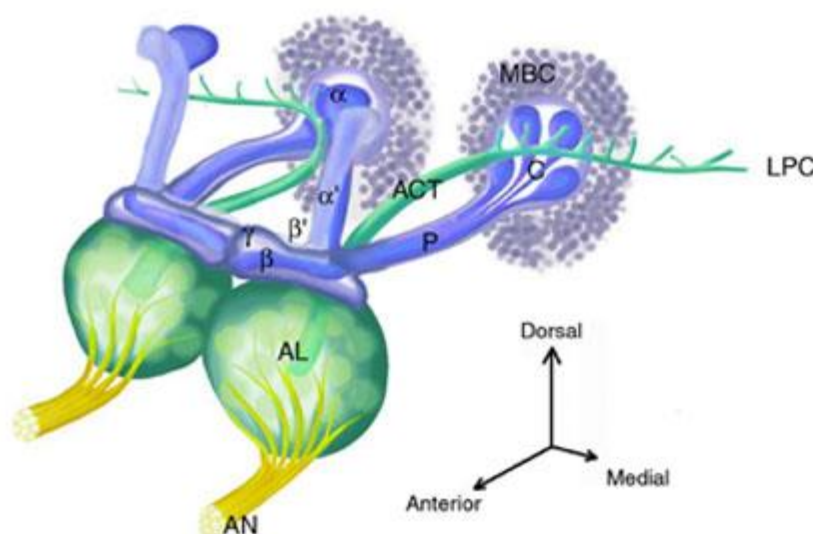



Figure 3-3. Anatomical organisation of the *Drosophila* mushroom bodies (MB).
The mushroom bodies (in blue) are made up of parallel bundles of fibres, namely the Kenyon cells. Odour information travels along the antennal nerve (AN) to the antennal lobe (AL). This information is then relayed to the calyx (C) of the mushroom body (MBC). The antennocerebral tract (ACT) connects individual glomeruli in the antennal lobe to the calyx. There are three distinct classes of neurons that make up the MBs: α/β, α/β' and γ, which make up the different MB lobes. (Heisenberg et al., 2003). Image, courtesy of Ron Davies laboratory.

An alternative assay for learning and memory that has been used previously involves aversive phototaxic suppression (APS). These assays involve an aversive stimulus being associated with light. Flies are instinctively phototaxic (Hirsch & Boudreau, 1958); when given a choice, flies will always move towards a lit area and away from a darkened area. Therefore, it is possible to take advantage of this instinctive behaviour for use in a learning assay. The APS assay consists of two chambers, one light and another dark (Figure 3-4). The light chamber is teamed with an aversive stimulus, for example quinine, which is a bitter-tasting chemical. After a period of training, flies with intact learning capabilities will be able to associate the light with this aversive stimulus and will avoid the light, remaining

in the dark chamber. The APS assay has been used previously to investigate how expression of human tau in the mushroom bodies of flies effects learning and memory. Seugnet et al., (2009) found that expression of 4R human tau in the mushroom bodies causes deficits in short term memory (Seugnet et al., 2009). Although APS assays are currently used less in the field than the olfactory assays discussed above, they do have the advantage of being simpler, making them more cost effective to run. In the past these assays have only been used to assess single flies, meaning that it is very time consuming to assess ample numbers of flies. Therefore, in this study, it was important to investigate whether it is possible to test populations of flies in this assay.

### 3.1.4 Aims

The aims of this Chapter will be to explore both circadian rhythms and learning and memory, as AD-relevant complex behaviours, in htau$^{0N3R}$ expressing flies. To do this, htau$^{0N3R}$ will be expressed in the Pdf neurons (central clock neurons) to understand whether htau$^{0N3R}$ can directly induce deficits in core circadian clock function. In addition, htau$^{0N3R}$ will be expressed in the mushroom bodies and using an APS assay will elucidate the contribution that htau$^{0N3R}$ makes to learning and memory. These behaviours will also be assessed in flies expressing htau$^{0N3R}$ for changes that may occur with age.

## 3.2 Methods

### 3.2.1 Fly Stocks

The circadian rhythm experiments were conducted using unmated females and male progeny from genetic crosses of Pdf-GAL4 (Renn et al., 1999) or Pdf-GAL4; tubulin-GAL80$^{ts}$ (McGuire et al., 2003) drivers crossed with UAS-htau$^{0N3R}$. w$^{1118}$ flies were crossed with UAS-htau$^{0N3R}$ to be used as undriven controls. Additional controls were Pdf-GAL4 and Pdf-GAL4; tubulin-GAL80$^{ts}$ crossed with w$^{1118}$ as a control for the driver background. Pdf-GAL4 driven experiments were performed at 23°C. For the temperature sensitive experiments flies were reared at 17°C and then moved after eclosion to 29°C to induce expression in adulthood.

The learning and memory assays were carried out using OreR flies (wild type flies). Mushroom body (MB) GeneSwitch flies were crossed with UAS-htau$^{0N3R}$ For full genotypes and details of fly stocks used, see Appendix E. MB GeneSwitch will drive expression in the mushroom bodies if the drug RU486 is present in the food. RU486 was only added to the food in adulthood and not during development, allowing for adulthood expression.

The flies in all experiments were kept on "SYA" diet containing in 1 litre (100g yeast, 50g sucrose, 50g cornmeal, 10g Agar, with propionic acid and nipagin).

### 3.2.2 RU486 treatment

After eclosion, 0-3 day old flies were moved to either food containing RU486 or to control diet that did not contain RU486. A 100 mM stock solution of RU486 was made up in 100% ethanol. Diet containing RU486 was then made up to a final concentration of 200 μM.

### 3.2.3 Circadian locomotor assays

Individual flies were lightly anaesthetised and put into glass tubes containing SYA diet. These glass tubes were placed in monitors that make up the *Drosophila* Activity Monitoring System (DAM System, TriKinetics). Monitors were then placed in light controlled boxes and were entrained for at least 3 days of 12:12 light: dark cycle at the relevant temperature for the experiment and then moved to constant darkness. The flies' locomotion, in free running conditions, by recording infra-red beam breaks in 5 minute bins. The flies'

locomotion was monitored until the flies died, seen as a disappearance of any spikes on the actograms and recorded as a measure of longevity. Median actograms were produced per week of recording in free running conditions, any flies that died within the week of testing were discounted from the results. Period lengths and relative rhythmic power were calculated for individual flies using ClockLab software (ActiMetrics, Willmette, IL, USA). Period length was determined by chi-square periodgram analysis for 7-day intervals. Relative rhythmic power was determined by calculating the ratio of the peak amplitude divided by the significance threshold. Flies were deemed strongly rhythmic if the relative rhythmic power ratio was found to be higher than 1.5 and deemed weakly rhythmic if 1.5 or less. GraphPad Prism was used for statistical analysis and to plot average values for period lengths and rhythmic power (±S.E.M). Average activity profiles for when flies were in 12:12 light dark conditions were also generated using ClockLab Software.

### 3.2.4 Learning and memory assay

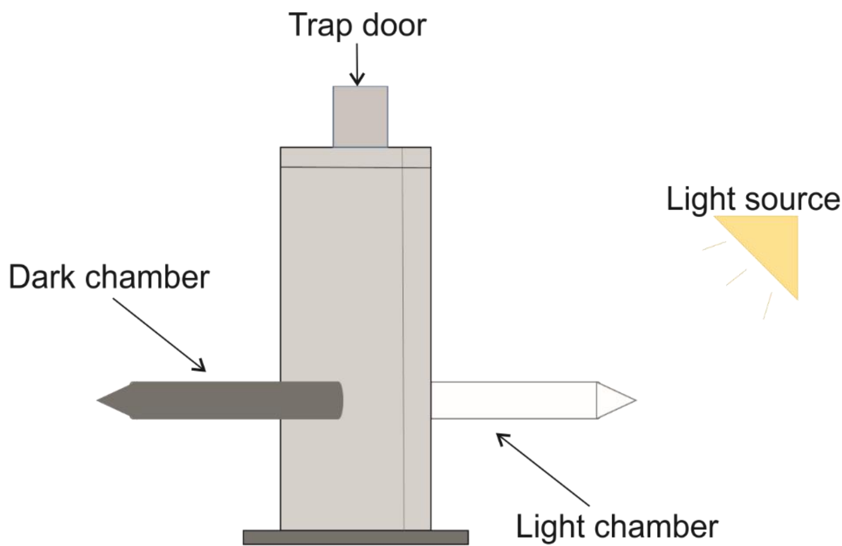
The APS assay was adapted from assays used previously (Le Bourg & Buecher, 2002) see (Figure 3-4) for set up. The day before the assay, flies were anaesthetised and cohorts of 10-15 flies were separated and put into 15mL falcon tubes containing SYA diet. The assay was then performed on the next day between 2-4 hours after the lights were switched on. The assay was conducted in the dark with exception of a red light to aid the experimenter. A goose necked light source was used to illuminate the light chamber of the apparatus. Each cohort was tested by firstly tapping the flies directly into the dark chamber (wrapped in foil to stop light penetrating the tube) and fastened into the side of the apparatus that is away from the light source. Flies were left to acclimatise for 1 minute in the dark before commencing the phototaxic phase of testing. As the assay is dependent on a phototaxic response, the phototaxic phase was used as a screening process to remove cohorts of flies that were deemed not to be phototaxic. The phototaxic phase was made up of 4 trials and performed using an agar coated light chamber with no aversive stimulus. Each trial involved tapping flies down to the bottom of the dark chamber, switching on the goose necked lamp, releasing the trap door that separates the two chambers and counting the number of flies that enter the light chamber within 15 seconds. This process was repeated an additional 3 times. There needed to be at least 50% of flies on average across the four trials, of the phototaxic phase, entering the light chamber in 15 seconds for the cohort to be deemed as being phototaxic. Cohorts deemed not to be phototaxic were not then used for the learning phase of the assay. The learning phase of the assay was made up of 4 blocks of 4 trials (16 trials in total) and each trial was conducted in the same way as the

phototaxic trials with the addition of light chambers containing quinine in the agar coating for when the aversive stimulus was being used. The 16 trials were performed immediately one after the other. At the end of the learning phase flies were left in the light before the 4 trials that made up the test phase of the assay. Different amounts of time were left between the learning and test phases of the assay depending on the memory time point assessed (1-30 minutes). Results of the assay were analysed by calculating the mean number of flies entering the light chamber for each phase of the assay. This was then converted to a performance index by finding the proportion of flies that were avoiding the light chamber during the test phase of the assay compared to the proportion of flies originally deemed to be phototaxic. To compare experimental conditions Mann-Whitney statistical tests were conducted on the data, using GraphPad Prism. These assays were performed in conjunction with a masters student, Jemma Knight. I designed , set-up and developed the assay. Jemma tested the flies, used here, and collected the data. I then analysed and interpreted the data with Jemma.

### 3.2.5 Statistical analysis

All values are presented as the mean ± standard error of the mean. To compare differences between groups, statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). 2-way ANOVAs with Bonferroni multiple comparisons were used for testing differences with age. Unpaired Student's t tests were performed on pairwise comparisons. $p<0.05$ was considered to indicate a statistically significant difference.

A

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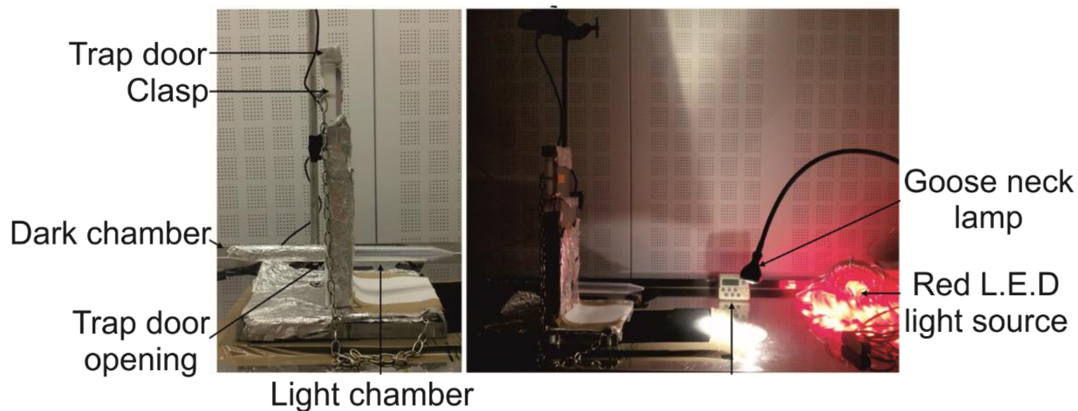


C

| Block | Phototaxic Block | Training Block 1 | Training Block 2 | Training Block 3 | Training Block 4 | Test Block |
|---|---|---|---|---|---|---|
| Trial no. | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 |
| Condition | Light | Light and Quinine | | | | Light |

Figure 3-4. APS assay experimental setup and study design.
(A) Schematic of the APS apparatus, showing the central partition with a trap door separating the dark and light chambers. The light source is angled towards the light chamber. (B, left) Photograph of the actual setup. (B, right) Experimental setup in the dark, during testing. Red light used while the testing is being carried out. (C) Study design comprising of 4 phototaxic trials where the light chamber does not contain quinine. The phototaxic block is followed by 16 trials of training, this is the learning phase. The light chamber in the training phase contains the aversive stimulus, quinine. A set period of time then separates the training phase and the 4 trials of the testing block. This period of time depends on the memory time point to be assessed. In the test block the chamber is swapped for an empty chamber that does not contain quinine.

## 3.3 Results

### 3.3.1 Circadian rhythms decline with age

As discussed above, disruptions to circadian rhythms are an early symptom of AD (Hatfield et al., 2004, Prinz et al., 1982). Others have also found that disruptions in circadian rhythms are found in normal ageing as well (Weitzman et al., 1982), therefore it is important to understand the relative contributions of both age and tau to disruptions in circadian rhythms. The first step was to analyse how circadian rhythms change with age. To investigate this, control flies (non-expressing htau$^{0N3R}$) were put into constant darkness and monitored for locomotive changes, with age, until the flies died. By qualitatively analysing weekly median actograms it was shown that free running rhythms started to breakdown by week 4 in male flies (Figure 3-5a). By week 6, all flies were arrhythmic shown in (Figure 3-5a) quantified in (Figure 3-5b). All female flies were shown to become arrhythmic by week 5, shown in (Figure 3-6a) and quantified in (Figure 3-6b). Comparing between genders, females appeared to be, overall, less rhythmic than males shown by a decrease in the amount of strongly rhythmic flies across all time points (Figure 3-5b and Figure 3-6b). These data show that during normal ageing flies' circadian rhythms decline with age and that female flies, in general, have weaker circadian rhythms than male flies.

### 3.3.2 Tau-expressing flies have disruptions to circadian rhythms at early time points

As it has been shown here that normal flies' rhythms decline with age, the next step was to understand whether tau expression in the Pdf neurons could alter this age-related decline in rhythms. To do this, htau$^{0N3R}$ was expressed in Pdf neurons using the Pdf-GAL4 driver. Flies were entrained for at least 3 full days in light: dark, 12:12 hour cycle and then put into constant darkness and monitored for their locomotion until they died. Again, rhythmic power was used as a quantitative readout of rhythmicity and flies were scored for being strongly rhythmic, weakly rhythmic and arrhythmic. Similar to controls, the median actograms showed rhythms until week 4 in male htau$^{0N3R}$ expressing male and female flies (Figure 3-5c,d) and (Figure 3-6c,d) but there were less strongly rhythmic flies at early time points, compared to controls. It was found that in younger ages (weeks 1 & 2) there were less strongly rhythmic flies with htau$^{0N3R}$ expression in both male and females (Figure 3-5d and Figure 3-6d). However, there were less htau$^{0N3R}$ arrhythmic flies when comparing older ages (weeks 5&6) with controls. This suggests that htau$^{0N3R}$ does not exacerbate age-related decline in rhythms but does notably disrupt the rhythms of young flies.

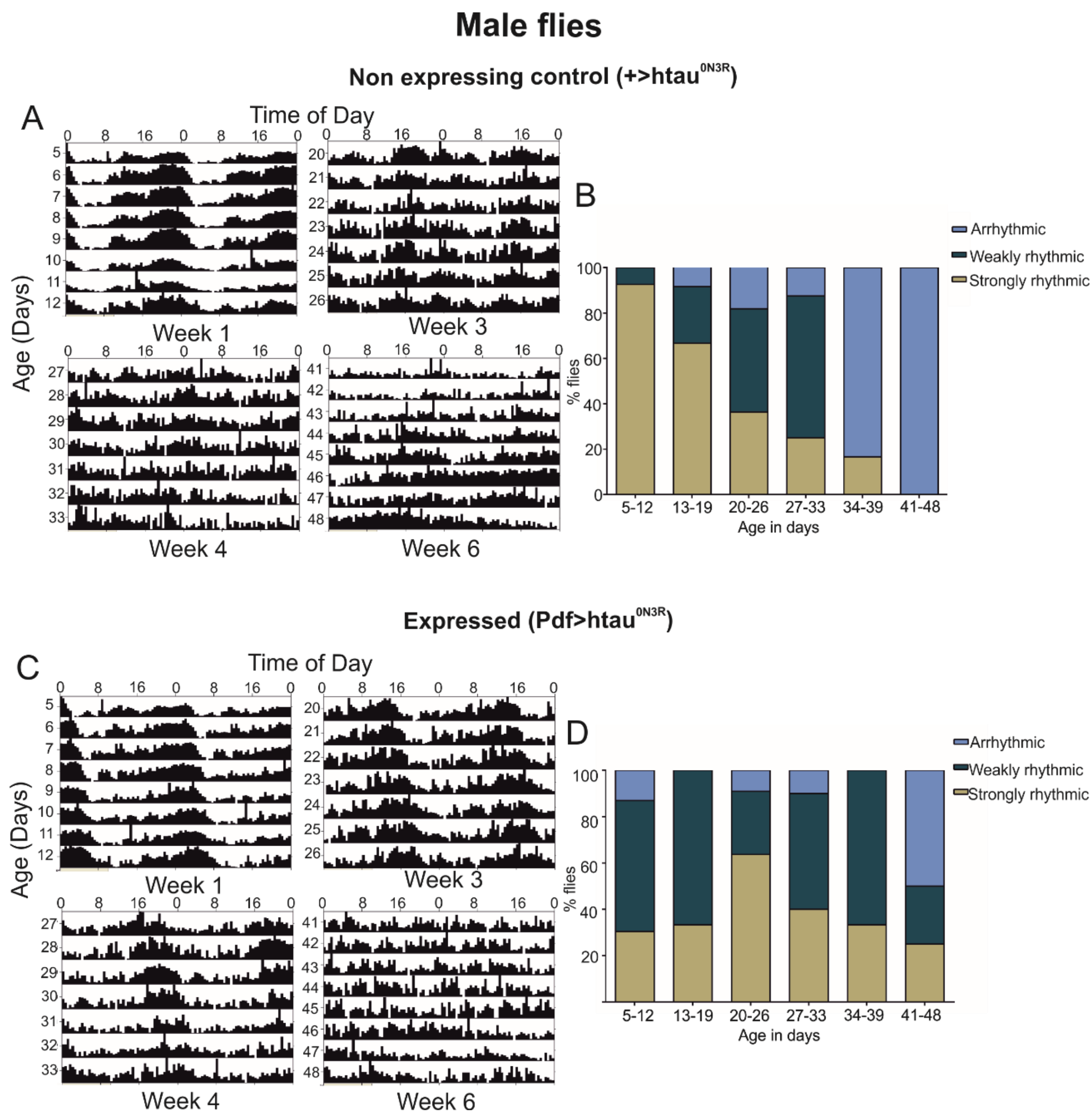


Figure 3-5. Assessment of rhythmicity with age of male control and htau$^{0N3R}$ expressing flies.
Week 1 corresponds to (5-12d) flies, week 2 (13-19d), week 3 (20-26d), week 4 (27-33d), week 5 (34-39d), week 6 (41-48d). (A) Median actograms showing the locomotor activity of male control (htau$^{0N3R}$ crossed with w$^{1118}$ flies) flies (starting n=27) over 6 weeks. (B) Quantification of rhythmicity in male control (non-expressing) flies. (C) Median actograms showing locomotor activity of male htau$^{0N3R}$ expressing flies using Pdf-GAL4 driver (starting n=23) over 6 weeks. (D) Quantification of rhythmicity Pdf expressing htau$^{0N3R}$ male flies.

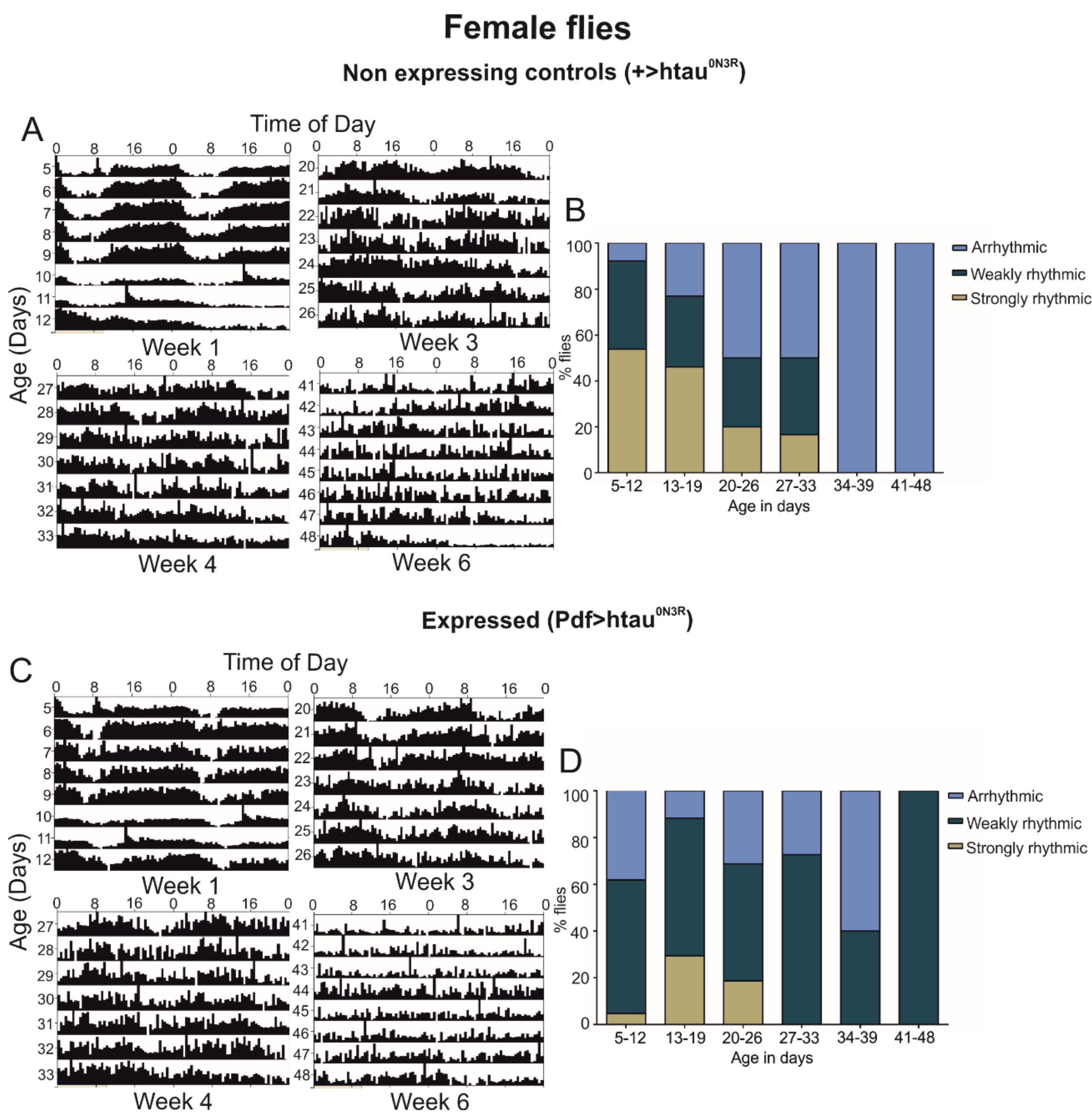


Figure 3-6. Assessment of rhythmicity with age of female control and htau$^{0N3R}$ expressing flies. Week 1 corresponds to flies 5-12-day old (d) flies, week 2 (13-19d), week 3 (20-26d), week 4 (27-33d), week 5 (34-39d), week 6 (41-48d). (A) Median actograms of the free running locomotor activity of female control (htau$^{0N3R}$ crossed with w$^{1118}$) flies (starting n=26) over 6 weeks, (B) Quantification of rhythmicity for female non expressing controls. (B) Median actograms showing locomotor activity of female htau$^{0N3R}$ expressing flies using Pdf-GAL4 driver (starting n=21) over 6 weeks, (D) Quantification of rhythmicity for male Pdf expressing htau$^{0N3R}$ flies.

### 3.3.3 Expression of htau$^{0N3R}$ in Pdf neurons increases period length

Another way in which expression of htau$^{0N3R}$ could affect circadian rhythms, other than changing the distribution of rhythmicity, is by altering the period length. Period length is defined as the duration of a full circadian cycle ie. the reciprocal of its frequency (Refinetti et al., 2007). Period length was determined using ClockLab for each individual fly for weeks 1-4. Weeks 5 and 6 were not analysed as there were not enough rhythmic flies left alive to be able to generate a period length for these older time points. A 2-way ANOVA revealed that expression of htau$^{0N3R}$ in Pdf neurons significantly extended period length in both males (p=0.0001) (Figure 3-7a) and in females (p=0.0282) (Figure 3-7b). Multiple comparisons revealed that period length was significantly increased in males at each time point (Figure 3-7a) compared with controls. It was also found that the period length was significantly increased, with age, comparing weeks 1 and 4 in males (Figure 3-7a). In females, period length was found to be significantly increased at weeks 2, 3 and 4 (Figure 3-7b). These data demonstrate that expression of htau$^{0N3R}$ in Pdf neurons can increase period length, slowing the internal clock of flies. This extension of period length is increased, with age, in male flies but not in female flies.

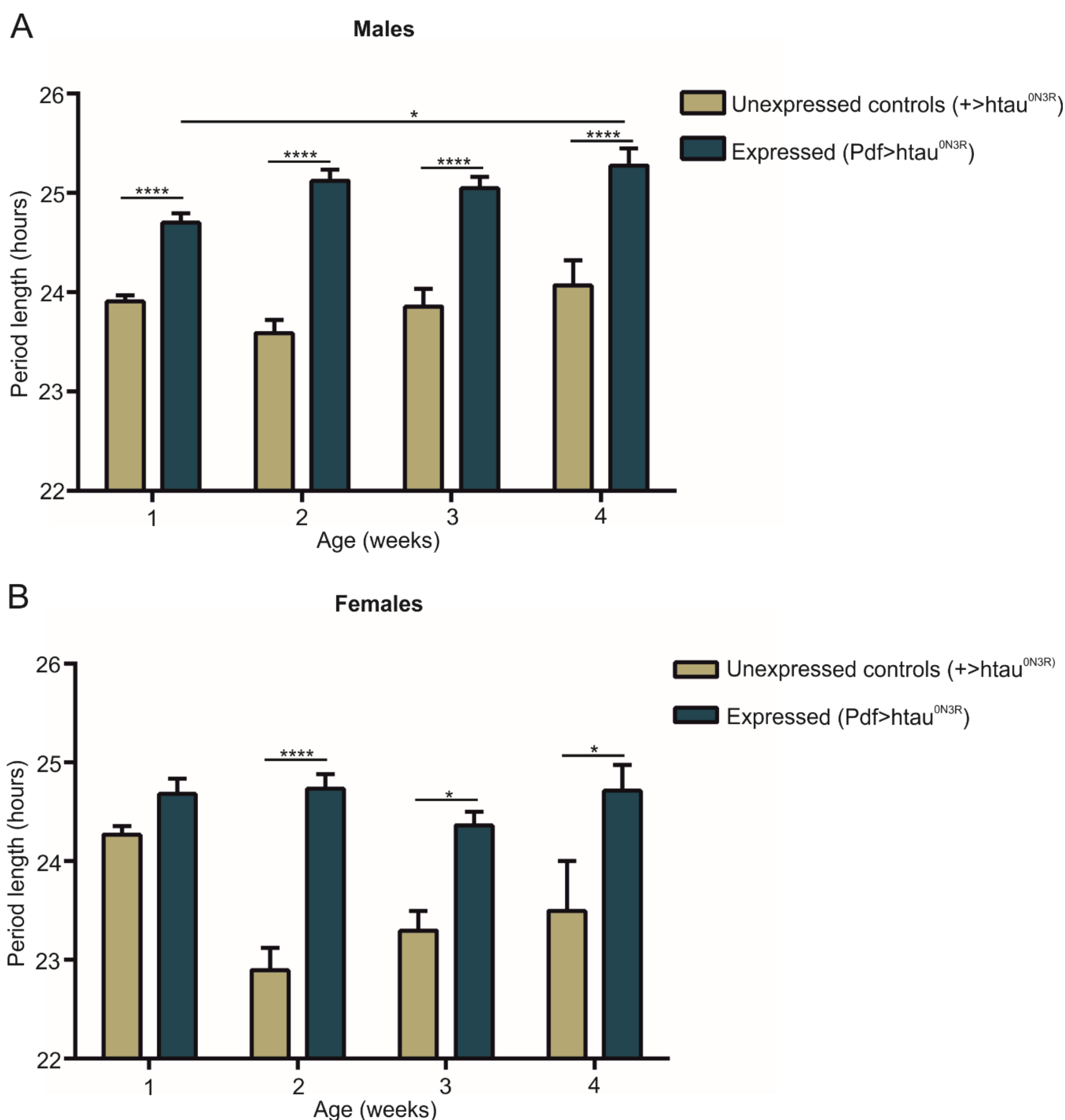


Figure 3-7. Assessment of period length with age in both male and female flies.
Week 1 (5-12d) flies, week 2 (13-19d), week 3 (20-26d), week 4 (27-33d). Period length was determined for rhythmic flies. (A) Male htau$^{0N3R}$ flies (n=20) were found to be significantly different to control flies (n=27) (2-way ANOVA, p=0.0001). Age was also found to be a significant factor (p=0.0282). (B) Female htau$^{0N3R}$ flies (n=13) were found to be significantly different to control flies (n=24) (2-way ANOVA, p=0.0001), age was also found to be a significant factor (p=0.0001). Error bars are plotted as ± S.E.M.

### 3.3.4 Expression of htau$^{0N3R}$ in Pdf neurons decreases rhythmical power

It has been shown here that htau$^{0N3R}$ alters the proportions of strongly, weakly rhythmic and arrhythmic flies (Figure 3-5 and Figure 3-6) but to understand how htau$^{0N3R}$ expression in Pdf neurons affects the strength of rhythms, rhythmic power needs to be quantified. Relative rhythmic power was computed for individual flies that were classified as either strongly or weakly rhythmic for weeks 1-4 of flies being in free running conditions (Figure 3-8). A 2-way ANOVA was conducted on the data. It was found that htau$^{0N3R}$ flies were significantly different to control flies (p=0.0017) (Figure 3-8a). Multiple comparisons revealed that for male flies at week 1, controls had a higher rhythmic power than htau$^{0N3R}$ expressing flies. There was also a trend for male control flies to have a stronger rhythmic power than htau$^{0N3R}$ flies at week 2 but this was not found to be significant. At weeks 3 and 4 rhythmic power was found to be similar between male control and htau$^{0N3R}$ expressing flies due to the significant decrease in rhythmic power with age in the control flies. Multiple comparisons revealed a significant decrease in rhythmic power at weeks 3 and 4 in male flies compared to week 1 in male flies. In female flies (Figure 3-8b) htau$^{0N3R}$ expressing flies were found to be significantly different to control flies (2-way ANOVA p= 0.0239). Multiple comparisons did not reveal that female htau$^{0N3R}$ expressing flies were different to controls at any single time point. There was, however, a trend for female htau$^{0N3R}$ flies to have a lower rhythmic power compared to control flies, at each time point. There was also a trend for female control flies to have a decrease in rhythmic power, with age, but not for htau$^{0N3R}$ expressing females.

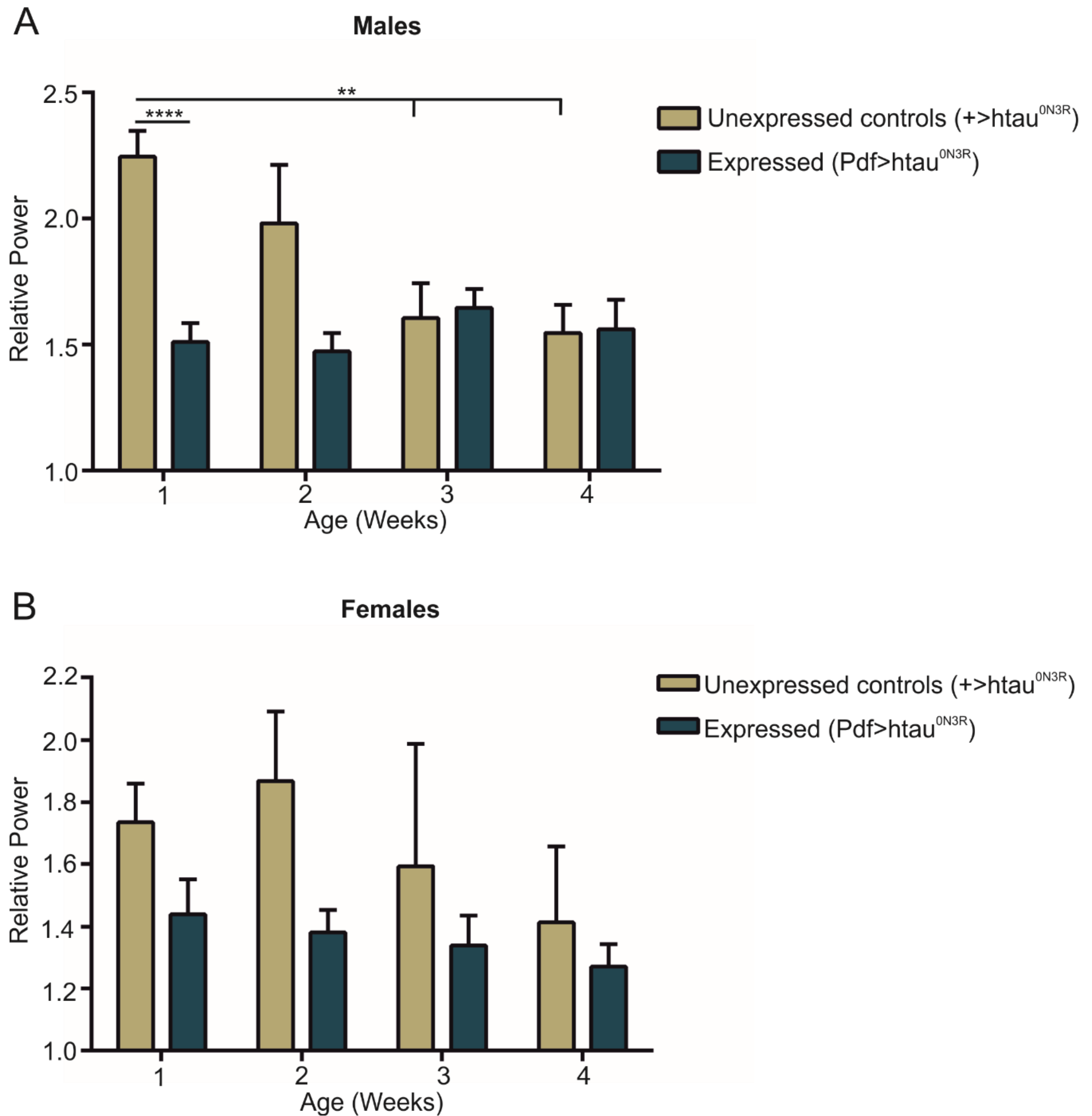


Figure 3-8. Assessment of relative rhythmic power, with age, in both male and female flies. Relative power was determined for weakly and strongly rhythmic flies. Week 1 (5-12d), week 2 (13-19d), week 3 (20-26d), week 4 (27-33d). (A) Male $htau^{0N3R}$ flies (n=20) were found to be significantly different to control male flies (n=27) (2-way ANOVA, p=0.0017). Relative power in female flies (B) $htau^{0N3R}$ flies (n=13) was found to be significantly different to control female flies (n=24) (2-way ANOVA, p=0.0239).

### 3.3.5 htau$^{0N3R}$ expressing flies have disrupted sleep/wake rhythms

An early and prominent symptom of AD is disruptions to sleep/wake rhythms, with patients becoming more active during the night. It is currently not clear what causes this disruption, therefore, it was important to understand the role that tau might play in this particular symptom of AD. To investigate this, 3-5 day old htau$^{0N3R}$ expressing flies were assessed for activity for 3 days of light: dark cycles. Average activity profiles were generated using ClockLab software for both control (non-expressing) and htau$^{0N3R}$ male and female flies. Average activity profiles, shown in (Figure 3-9), were generated for male control flies (a) and htau$^{0N3R}$ flies (b), as well as female controls (c) and htau$^{0N3R}$ flies (d). Quantification of the percentage of total activity that occurred nocturnally is presented in (Figure 3-9e). Control flies were found to have characteristic daily activity profiles with morning and evening peaks with a period of low activity in between and low activity at night. Htau$^{0N3R}$ expression significantly increased nocturnal activity both in males ($p=0.0157$) and in females ($p=0.0002$), compared with controls. This result demonstrates that expression of htau$^{0N3R}$ in Pdf neurons increases nocturnal activity, particularly in female flies. These data demonstrate that expression of human tau causes disruptions in sleep/wake circadian rhythms, offering a possible mechanism for the disruptions in sleep/wake cycles that develop in patients with AD.

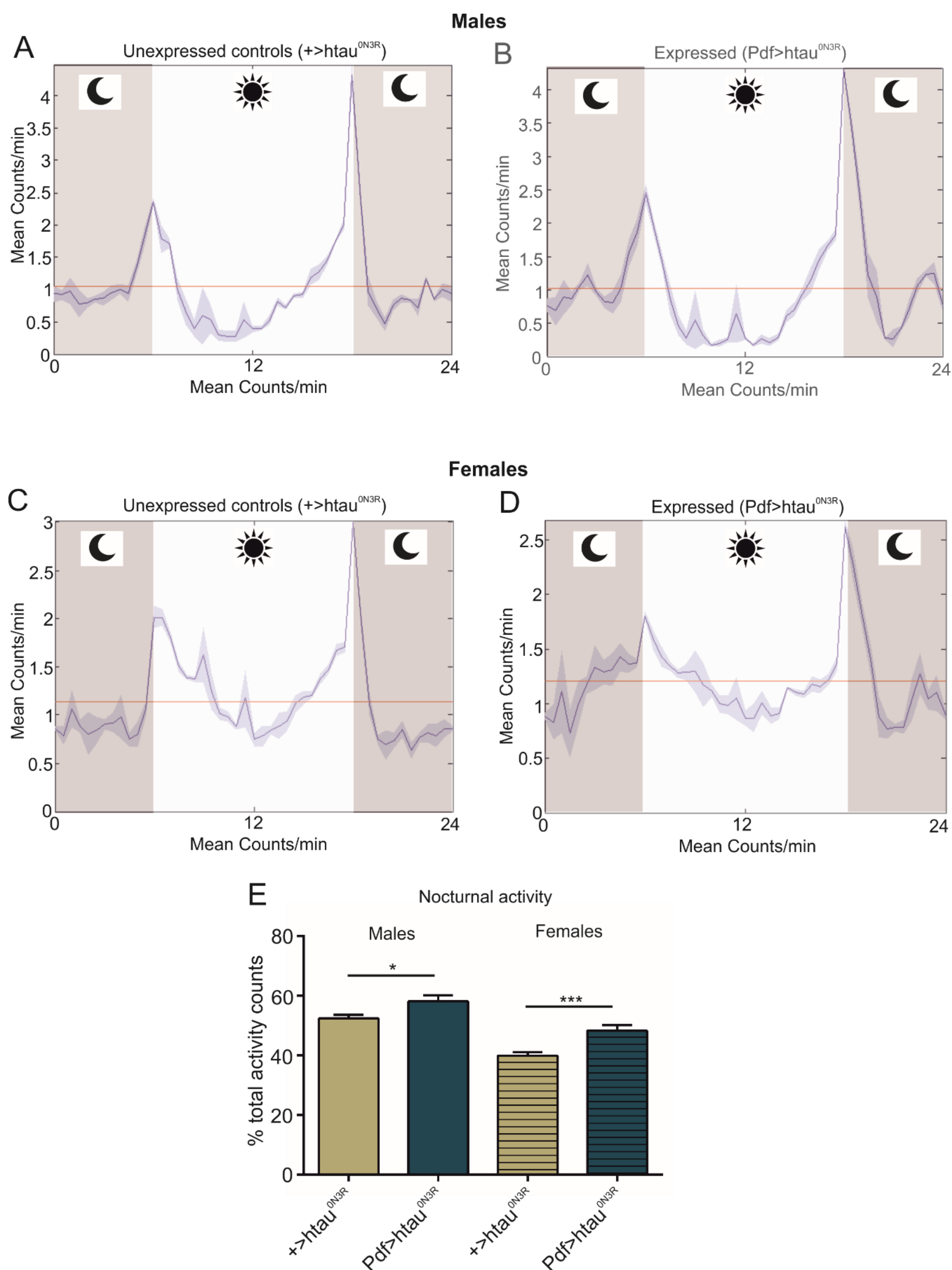


Figure 3-9. Htau$^{0N3R}$ expression increases nocturnal activity of young (newly eclosed flies).
Average activity profiles for 3 days of 12:12 light: dark cycle of 0-3day old flies. presented in A-D. (A) Unexpressed males (n=27), (B) Pdf expressed males (n=22), (C) unexpressed females (n=38), (D) Pdf expressed females (n=21). Nocturnal activity is shaded grey. (E) Average nocturnal activity accounts as a % of total activity accounts. Pdf expressed htau$^{0N3R}$ males have significantly increased nocturnal activity compared to controls (p=0.0157). Pdf expressed htau$^{0N3R}$ females have significantly increased nocturnal activity compared to controls (p=0.0002). Unpaired two-tailed t-tests were performed on the data.

### 3.3.6 htau$^{0N3R}$ induced extension of period length is independent of developmental defects

It has been shown here that htau$^{0N3R}$ expression using the Pdf-GAL4 driver causes an extension of period length. This was demonstrated in week 1 of flies being in constant darkness (Figure 3-7). As Pdf-GAL4 drives expression during development, from late embryonic stage onwards, it is possible that htau$^{0N3R}$ mediated period extension could be a developmentally driven phenotype. To understand the contribution of expression of htau$^{0N3R}$ during development circadian locomotor behaviour was assessed in flies where htau$^{0N3R}$ was expressed only in adulthood. This was done using the TARGET expression system, using a driver line that contains both Pdf-GAL4 and Tubulin-GAL80$^{ts}$. The GAL80$^{ts}$ inhibits expression of tau at low temperatures (17°C), by binding to the GAL4 element (McGuire et al., 2003). Whereas at higher temperatures, GAL80$^{ts}$ is no longer able to bind to GAL4 and allows expression of htau$^{0N3R}$. Therefore, if flies are kept at 17°C during development and moved to 29°C during adulthood, then it is possible to express htau$^{0N3R}$ only in adulthood. As an additional control where expression is not induced during adulthood, flies were also assessed at 17°C where expression is not induced. Flies have a much shorter lifespan at 29°C so they could only be analysed for the first week of being in constant darkness.

It was found that rhythmicity was not affected in htau$^{0N3R}$ flies of either gender at 17°C (Figure 3-10a). This was to be expected as expression of htau$^{0N3R}$ is not induced at this temperature using the TARGET expression system. Rhythmicity was also similar between htau$^{0N3R}$ and control flies at the 29°C, when expression is induced (Figure 3-10b). Period length was also found to be similar between control and htau$^{0N3R}$ flies at 17°C in either gender (Figure 3-10c). At 29°C period length was found to be significantly extended in male flies when htau$^{0N3R}$ expression was switched in adulthood ($p=0.0002$) (Figure 3-10d). No difference in period length was found in female flies at 29°C. Relative power was not found to be different in either male or female flies at either temperature (Figure 3-10e and Figure 3-10f). It was noted that an increase in temperature correlated with increased relative power in male flies but decreased relative power in female flies, regardless of genotype. Collectively, these data show that although some aspects of the circadian phenotype in htau$^{0N3R}$ flies may be in part due to developmental expression, the extended period length, at least in males, can be induced with short term adulthood expression of htau$^{0N3R}$. Other aspects of circadian behaviour may also be affected by adulthood expression of htau$^{0N3R}$ at older ages, but it was not possible to assess this here due to high levels of mortality at 29°C.

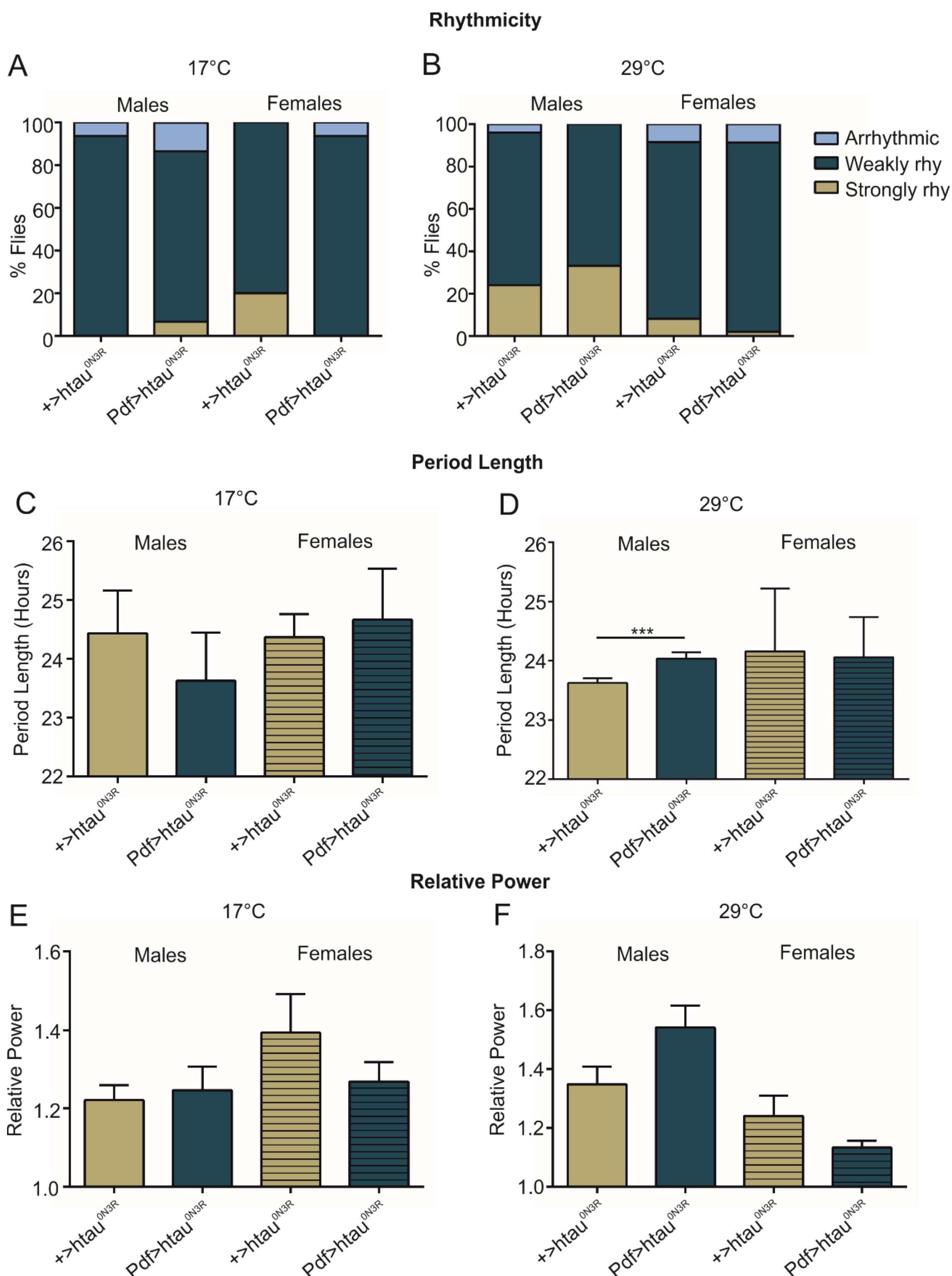


Figure 3-10. Period length is still extended if htau$^{0N3R}$ is expressed only in adulthood.
Pdf-GAL; Tubulin GAL80$^{ts}$ driver was used to express htau$^{0N3R}$ in Pdf neurons in adulthood by keeping flies at 17°C during development and moving flies to 29°C during adulthood. Results presented here are on 3-10 day old flies (n=11-43). (A) No difference in rhythmicity of htau$^{0N3R}$ expressing flies compared to control flies at 17°C. (B) No difference in rhythmicity at 29°C.(C) No differences in period length at 17°C with either gender or genotype. (D) At 29°C there is a significant increase in period length with expression of htau$^{0N3R}$ in males (p=0.0002) but not in females. There are no differences in relative power for either gender or genotype at either (E) 17°C or (F) 29°C.

### 3.3.7 Development of a high throughput APS assay to measure learning and memory in *Drosophila*

Although disruption of circadian rhythms is an early deficit reported in AD, the primary symptom of AD is a decline in memory. Expression of human tau has been found to disrupt learning and memory in both flies (Mershin et al., 2004, Kosmidis et al., 2010) and in mice (Tatebayashi et al., 2002, Schindowski et al., 2006). Therefore, it was important to understand the effects of $htau^{0N3R}$ used here, in learning and memory. To do this, an adaption of an APS assay was developed that has high throughput capabilities. This assay, shown in (Figure 3-4) trains flies to associate an aversive stimulus (quinine) with light. Flies that learn this association, during the testing phase, will avoid the light chamber by remaining in the dark chamber.

This part of the work was done in conjunction with a master's student, Jemma Knight. The first step in developing this assay was to investigate quinine as the aversive stimulus and to assess whether cohorts of wild type flies can learn to avoid the light chamber due to the presence of quinine. Cohorts containing 10-15 wild type flies were put through the assay and exposed to different concentrations of quinine. A range of doses of quinine has previously been used for these assays, therefore, concentrations were used that encompassed these variations (Seugnet et al., 2009, Ali et al., 2011). 0.025M, 0.05M and 0.1M quinine concentrations were added to the agar that was used to coat the inside of the light chambers. Plain agar with no addition of quinine was used for control light chambers. It was found that wild type flies responded to quinine shown by increased avoidance of the light chamber in a dose responsive manner (Figure 3-11). The higher the performance index (PI), the more flies that were associating the light chamber with quinine and therefore remained in the dark chamber during the test phase of the assay. The PI of flies exposed to the 0.025M dose of quinine was not found to differ from flies exposed to the control chamber (no quinine). Both 0.05M ($p=0.0198$) and 0.1M ($p=0.0006$) quinine concentrations were found to significantly increase PI. These data demonstrated that quinine does act as an aversive stimulus and that to maximise response of flies' behaviour, higher concentrations of 0.5M and 0.1M should be used. Therefore, it was decided that all subsequent experiments were to be performed using the 0.1M dose of quinine.

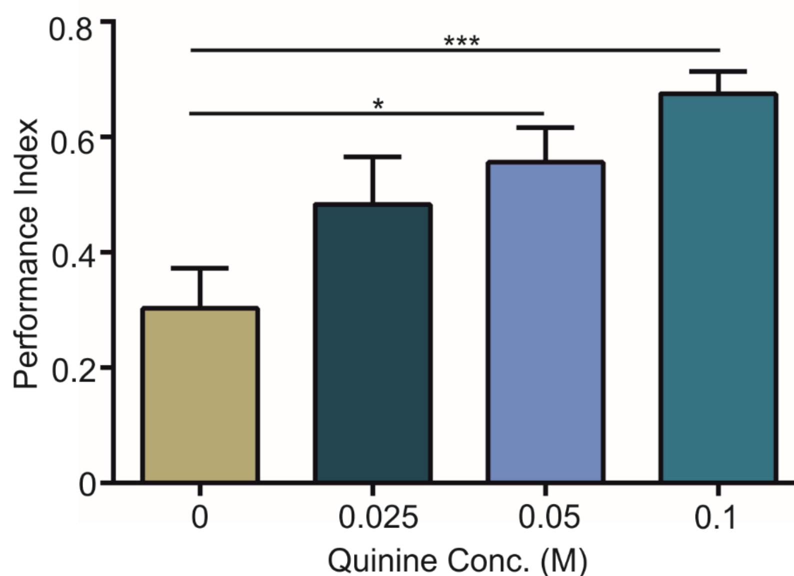


Figure 3-11. Effect of increasing dose of quinine in learning and memory assays on wild type flies. Performance index is a measurement of the flies' ability to associate the light chamber of the assay with quinine, measured by the fly's avoidance of the light chamber. n= 8-11 cohorts of 15 flies. 0.05M quinine was found to significantly improve performance of wild type flies (Mann-Whitney p= 0.0198) compared to there being no quinine present in the light chamber. 0.1M quinine was also found to significantly improve performance compared to there being no quinine (p=0.0006). Data produced in conjunction with Jemma Knight.

Quinine concentrations were assessed using a test/memory phase that was 1 minute after the training phases had been completed. The next step was to understand whether this time interval between training and testing could be increased. As short term memory is an important aspect of AD, memory was primarily investigated at time points associated with the short term memory timeframe in flies. Short term memory in *Drosophila* has been found to encompass time points of up to 1 hour in length (Mcguire et al., 2005), therefore 1, 5, 10, 15 and 30-minute time points were primarily assessed, as shown in Figure 3-12. It was found that the performance index of WT flies significantly decreased the longer the interval was between the learning and test phase. Using a Mann-Whitney statistical test it was found that even at 5 minutes there was a significant decrease in performance (p=0.0253), although this result was due to an outlier cohort that without it the 5-minute time point would not have been significantly different to the 1-minute time point. The 10-minute time point did not affect the performance of the flies compared to the 1-minute time point. However, by 15 minutes there was a significant decrease in performance compared to 1 minute (p=0.0017) and also when comparing the 30-minute time point with the 1-minute time point (p=0.0001). These data demonstrate that the association of light with the bitter tasting quinine diminishes relatively quickly with time after the training phase. Therefore, this assay is useful in testing short term memory up to between 10-15 minutes from training.

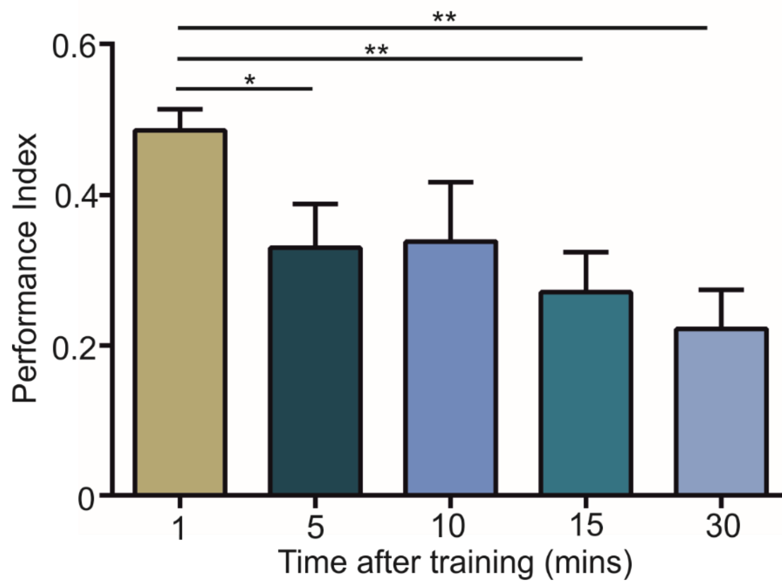


Figure 3-12. Assessing the time that memory of quinine aversive stimulus is retained for in WT flies.
Performance index is a measurement of the flies' ability to associate the light chamber of the assay with quinine, measured by the flies' avoidance of the light chamber. Cohorts of flies were tested containing 15 flies each. After 5 minutes (n=11 cohorts) there is a significant decrease in performance compared to the 1-minute time point (n=41 cohorts) (p=0.0253) but at the 10-minute time point (n=10 cohorts), the performance index is not significantly decreased compared to the 1-minute time point. Performance continues to decline at 15 minutes (n=10 cohorts) (p=0.0017) and at 30 minutes (n=10 cohorts) (p=0.0001). Data produced in conjunction with Jemma Knight.

### 3.3.8 Expression of htau$^{0N3R}$ induces impaired memory in high throughput APS assay

This assay was developed so that it would be possible to investigate how the expression of htau$^{0N3R}$ affects short term memory, therefore this was the next step. The MB GeneSwitch driver was used to express htau$^{0N3R}$ in the mushroom bodies for 1-week post eclosion. Expression of htau$^{0N3R}$ caused a significant reduction in PI (Figure 3-13) (p=0.0006 n=90). This demonstrates that htau$^{0N3R}$ expression in the mushroom bodies, in adulthood, causes deficits in short term memory.

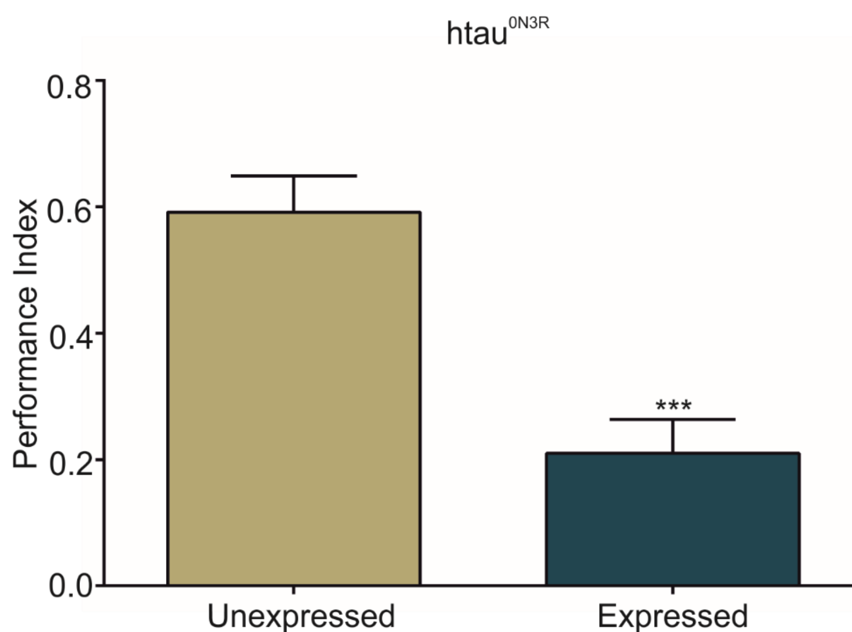


Figure 3-13. Adult expression of htau$^{0N3R}$ in the MBs induces short term memory deficits.
The MB GeneSwitch driver was used to express htau$^{0N3R}$ only in the mushroom bodies during adulthood. Female progeny was either kept on food with RU486 (expressed) or without RU486 (unexpressed) for 1 week prior to testing. 6 cohorts of 15 flies for both conditions were used. Significance was assessed using a Mann-Whitney test (p=0.0006).

## 3.4 Discussion

### 3.4.1 Summary

The data presented here show that it is possible to model AD relevant behaviours, namely circadian rhythms and learning and memory, in *Drosophila*. It was shown here that normal flies become less rhythmic, with age, and that tau expression in the Pdf neurons disrupts this normal decline by causing changes to the proportions of rhythmic flies, particularly in early ages. Tau expression was also found to increase period length in both male and female flies if expressed during development and throughout life. This extension of period length was still found in male flies when tau was expressed only in adulthood suggesting this phenomenon is not entirely developmental in nature. It was also found that tau causes disruption to sleep/wake cycles, with tau expressing flies being more active during the night than control flies. In addition, learning and memory was also assessed in *Drosophila*. A high throughput APS short term memory assay was developed that is based on teaming quinine as an aversive stimulus with phototaxic behaviour. Flies were trained to learn and remember an association between light and quinine and to avoid the light chamber of the assay. This assay was found to be effective in measuring short term memory, the primary symptom of AD, and also revealed that expression of htau$^{0N3R}$ in the mushroom bodies caused a deficit in short term memory of the flies.

### 3.4.2 Circadian rhythms: Ageing causes disruption to circadian rhythms

Here it was shown that in control flies, not expressing htau$^{0N3R}$, circadian rhythms declined with age. Flies became increasingly arrhythmic and had decreased rhythmic power, with age. This has been shown before in various species from invertebrates to humans. In *Drosophila*, Luo & Sehgal (2012) found with age, both decreased rhythmicity in free running conditions and an increase in period length (Luo & Sehgal, 2012). The reported extension in period length with age differs from what was found here, where no change in period length was seen in control flies. This may be due to the way in which the study was conducted. Luo et al., (2012) were able to assess much older ages as they did not monitor the rhythms constantly throughout the flies' life, instead analysing flies for a few weeks at three different ages. This meant that the flies were aged outside of the monitoring system and therefore lived to much older ages (60-70d) than were tested here (41-48d). This means that it is possible to see an increased period length in flies with age if older flies can be examined. Others have also found increased arrhythmia with age in *Drosophila* and interestingly, in addition, fragmented sleep (Koh et al., 2006). Sleep was not assessed here but this would be

an interesting future investigation as in humans it is well documented that sleep/wake cycles become disrupted with age in people. Older people exhibit increased periods of time sleeping during the day and increased wakefulness during the night (Bliwise, 1993). In addition, other disruptions in circadian rhythms have been found in aged people exemplified by a decrease in the amplitude of core body temperature rhythms and a decrease in period length, with age (Weitzman et al., 1982). Others have also found reduced amplitude in human circadian melatonin rhythms with age (Kloeden et al., 1993). Additionally, disruptions in circadian rhythms with age have also been found in rodents. A study in rats reported a shortening of the free running period in circadian output behaviours such as water drinking and body temperature (Witting et al., 1994). Collectively these studies demonstrate the highly conserved nature of declining circadian rhythms with age. What is currently unknown, is which mechanisms underlie these age-related changes.

Age-related changes in the SCN have been suggested to underlie age-related declines in rhythmicity. Altered functionality of the SCN with age has been demonstrated by decreased electrophysiological output and decreased levels of the neuropeptides, vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP) as reviewed in (Froy, 2011). In addition, in a study where the SCN was transplanted from young rats into old rats, circadian rhythms in corticotrophin-releasing hormone (CRH) and locomotor activity were restored (Li & Satinoff, 1998). An age-related decrease has also been found in clock gene expression in various species including zebrafish (Zhdanova et al., 2008), rats (Asai et al., 2001), hamsters (Kolker et al., 2003) and in mice (Wyse & Coogan, 2010). These studies show that an age-related decrease in clock gene expression may well underlie changes in clock neurons in the SCN and therefore in circadian rhythm outputs. What causes this remains elusive.

Another interesting avenue of research is how age-related changes in the clock may well underlie the ageing process. For example, mice lacking the clock protein BMAL1 have a reduced lifespan and have increased age-related pathologies such as osteoporosis, cataracts and reduced muscle mass (Kondratov et al., 2006). Similarly in flies, a null mutation in the clock gene *Per* gives rise to increased accumulation of reactive oxidative species, and reduced lifespan (Krishnan et al., 2009). It has also been found that loss of BMAL1 leads to increased neurodegeneration in mice (Musiek et al., 2015). These studies demonstrate that not only can disruptions to the circadian clock cause accelerated ageing but may well have implications for neurodegenerative diseases as well.

### 3.4.3 Circadian rhythms: tau expression causes increased period length

It has been shown here that tau expression in the central clock neurons causes an increase in period length. This is the first study to investigate that the expression of tau alters circadian rhythms in flies. However, others have assessed circadian rhythms in the triple transgenic mouse model (3x Tg) of AD, which has both plaque and tangle pathology. They found that the transgenic mice had decreased nocturnal activity and increased daytime activity compared to control mice and that this preceded plaque and tangle pathology (Sterniczuk et al., 2010). This echoes what was found here as mice are usually nocturnal animals. However, as the mouse model exhibits both Aβ and tau pathology it is not possible to understand which of these pathologies is contributing to the disruptions in circadian rhythms. To date, no other group has investigated directly how tau expression in clock neurons affects circadian rhythms, in *Drosophila*, but others have investigated other disease related proteins such as Aβ. Expression of Aβ with the arctic mutation, causes flies to become less rhythmic and have decreased rhythmic power in free running conditions (Chen et al., 2014). Chen et al., (2014) also found that Aβ induced toxicity in the clock neurons, and suggested that this may well underlie the Aβ induced deficits on circadian behaviours (Chen et al., 2014). Others in our group have investigated the morphology of the Pdf neurons after the expression of tau and found axonal swellings. This is thought to be an early stage of degeneration of axons. Future work should include assessing the impact of tau on Pdf neurons with age and whether tau can also cause degeneration of the Pdf neurons, as previously shown with Aβ (Long et al., 2014).

Tau or indeed Aβ toxicity in clock neurons may well underlie the defects in circadian rhythms seen in AD patients. AD patients have fragmented sleep/wake cycles where they exhibit decreased daytime activity and disrupted night time sleep, similar to what is found in normal ageing but with exacerbated symptoms (Bonanni et al., 2005). It is shown here that tau expression in the *Drosophila* central clock neurons is enough to cause disruptions in the sleep/wake cycles demonstrating a mechanism that could underlie the disruptions in sleep/wake cycles in AD patients. In addition, AD patients experience a phenomenon known as sun downing which is an exacerbation of other AD symptoms at particular times of the day, typically late afternoon and evening (Martin et al., 2000). These disruptions to circadian rhythms in patients are seen early in the pathogenesis and have even been investigated as a predictive or diagnostic tool to track AD pathology. For example, a study conducted in over 1000 older women found that disruptions in circadian activity rhythms were associated with the development of MCI and dementia (Tranah et al., 2011). These studies open the question of whether AD pathology perpetuates disruptions in circadian

rhythms or vice versa that disruptions in circadian rhythms can propagate AD pathology and related symptoms. Here it has been demonstrated that tau overexpression can disrupt circadian rhythms in a *Drosophila* model but further research is required to find out whether this is an early event in AD.

As there have been very few studies that express tau in the clock neurons, it is not currently known what mechanisms may underlie tau-induced disruption of circadian rhythms. It is possible that expression of the highly phosphorylated htau$^{0N3R}$ causes destabilisation of microtubules in the Pdf neurons. This would have a knock-on impact on axonal transport, causing disruptions to Pdf signalling. Pdf is a circadian output signal and synchronises the central clock neurons (Park et al., 2000). Therefore, disruptions in the release of Pdf from central clock neurons could well disrupt circadian behavioural rhythms. Future work is needed to confirm that disruption of microtubules in the clock neurons is what underlies the tau induced disruptions in behavioural rhythms. To do this, one could over express other agents that are involved in stabilising microtubules. One such protein, known as Short stop (Shot) crosslinks actin and microtubules. Mutations in Shot cause microtubule destabilisation similar to hyperphosphorylated tau (Sanchez-Soriano et al., 2009) and therefore it would be interesting to investigate whether these mutations also increase period length. Additionally, in support of this theory, analysis of AD brains have shown alterations to the cytoskeleton of SCN neurons (Swaab et al., 1985). These studies demonstrate the potential role of microtubule destabilisation in tau-mediated disruption of circadian rhythms. This also highlights the potential therapeutic benefit of microtubule stabilising agents for treatment in early stages of AD.

A different possible mechanism that may underpin tau induced disruption to circadian rhythms involves GSK-3β, a key kinase that phosphorylates tau. GSK-3β (sgg in flies) performs many different roles in addition to phosphorylating tau, including phosphorylating TIM and regulating period length in the molecular circadian clock. A study conducted in *Drosophila* found that overexpression of sgg causes a shortened period length in *Drosophila*, whereas reduction of sgg function lengthens period (Martinek et al., 2001). This was found to be due to advanced entry of PER into the nucleus, occurring 2-3 hours earlier than controls when sgg is overexpressed. It is possible that htau$^{0N3R}$ expression decreases the amount of sgg available to phosphorylate TIM, resulting in delayed nuclear translocation of PER/TIM. Other members of our laboratory have demonstrated that htau$^{0N3R}$ expression causes a delayed nuclear translocation of PER. Phosphorylation of TIM promotes nuclear translocation of PER/TIM complexes and therefore decreased phosphorylation of TIM would result in decreased or delayed nuclear translocation of

PER/TIM increasing period length (Martinek et al., 2001). Further evidence of the involvement of GSK-3 includes using lithium (an inhibitor of GSK-3) which has been used in range of species and shown to increase period length in hamsters (LeSauter & Silver, 1993), rats (Kripke & Wyborney, 1980) and primates (Welsh & Moore-Ede, 1990). This suggests that increasing GSK-3 activity could in theory rescue the lengthened period seen with overexpression of htau$^{0N3R}$. However, this has been shown to increase phosphorylation and therefore toxicity of htau$^{0N3R}$. This means that attempts to rescue the circadian disruptions in tau using GSK-3β activation could cause potentially toxic effects in other parts of the brain and should be explored cautiously. Regardless of this, it is important to fully understand the link between tau, GSK-3β and the circadian molecular clock.

### 3.4.4 Learning and memory: High throughput APS assay developed to assess short term memory

An APS assay was developed here that involves flies remembering an association of light with an aversive stimulus, in this case quinine. It was demonstrated here that avoidance of the light chamber was dependent on the quinine concentration. This type of assay that has utilised quinine as an aversive stimulus was first demonstrated in (Le Bourg & Buecher, 2002). Since then, another study established that memory mutants are deficit in this assay and this was due to mutants not being able to learn an association between light and quinine (Seugnet et al., 2009). The assay developed here was based on a previous design (Ali et al., 2011) with the addition of testing cohorts instead of individual flies. This important addition allows for many more flies to be tested in a shorter time frame, increasing the capabilities of the assay. These are important characteristics when designing an assay that is able to screen a number of different mutations. Classical olfactory conditioning assays have often been used to measure large cohorts of between 50-100 flies, therefore providing scope for further increasing the number of flies used in this assay.

This assay was demonstrated here to be a measure of short term memory, as performance was found to significantly decrease within 15 minutes. Others have also shown that this assay can be used as a measure of short term memory (Seugnet et al., 2009). However, it has been reported that this assay may be used to measure longer term memory (Ali et al., 2011). These investigators tested memory after 6 hours post training, but we could not reproduce this here. Assessment of memory at 3 hours post training showed that flies could not remember the association between light and quinine (data not shown). It is possible that minor differences in the set-ups underlie these discrepancies, such as the

mode of administering the quinine and also the strength of the light source. It is also possible that if the amount of training trials was increased, this could well elicit a longer term memory in the flies (Malik & Hodge, 2014). These aspects of the assay could be explored in future work to improve the sensitivity of the assay.

### 3.4.5 Learning and memory: htau$^{0N3R}$ expression in mushroom bodies induces deficits in short term memory

It was demonstrated here that expression of htau$^{0N3R}$ in adult mushroom bodies caused learning deficits with expression of htau$^{0N3R}$. Others have also shown that expression of htau in flies can cause deficits in learning and memory. This has been demonstrated both when expressed pan neuronally (Kosmidis et al., 2010, Ali et al., 2011) and also when localised only to the mushroom bodies (Mershin et al., 2004, Seugnet et al., 2009). All but Kosmidis et al., (2010) utilised 4 repeat isoforms of human tau. Kosmidis et al., (2010) found that pan neuronal expression of htau$^{0N3R}$ did not cause deficits in olfactory learning. This is the contrary to what we found here, where expression of htau$^{0N3R}$ did cause deficits. This could be because of different neurons being activated in the two assays. The APS assay is based on taste and therefore has inputs from the gustatory system. It is known that within the gustatory system there are specific cells that recognise bitter tastes, such as quinine, namely the Gr66a cells (Wang et al., 2004). When activated, these cells elicit responses in specific regions of the mushroom bodies (Harris et al., 2015). It is possible that the APS assay and classic olfactory assays involve different neurons in the mushroom bodies. It was highlighted in Chapter 2 that different isoforms of tau could well have different effects in different cells and tissues. The fact that htau$^{0N3R}$ expression causes deficits in APS learning and memory and not in olfactory learning highlights the importance of understanding exactly which neurons in the brain are susceptible to certain isoforms of tau and what underlies this.

Studies conducted in both humans and mice have also demonstrated that tau pathology correlates with cognitive deficits. Various studies conducted in AD patients demonstrated that severity of dementia is positively correlated to the number of neurofibrillary tangles in the brain (Arriagada et al., 1992, Bancher et al., 1993, Guillozet et al., 2003). Memory has also been found to decline with increasing Braak staging (Braak & Braak, 1995, Grober et al., 1999). Various mouse models of AD have been created that display memory deficits (Oddo et al., 2003). However, many of these models involve both Aβ and tau pathology and so it is difficult to tease out the relative contribution that tau has on learning and memory. Recent years have seen the emergence of pure tau pathology mouse models. A

mouse model that expresses a P301S mutant form of 4R-tau developed NFTs together with spatial memory deficits (Schindowski et al., 2006). Others have also found that if transgenic tau (P301L) expression is suppressed, then tau-mediated spatial memory deficits are improved (Santacruz et al., 2005). This particular study found that, although levels of tau expression were suppressed, NFTs still accumulated. This suggests that the tau species involved in inducing learning and memory deficits are not NFTs. It was shown in Chapter 2 that, even in aged flies, htau$^{0N3R}$ does not form large insoluble aggregates. Therefore, it is likely that the htau$^{0N3R}$- mediated deficits in learning and memory, seen here, are caused by small soluble species of htau$^{0N3R}$, particularly given the finding that these deficits are induced within 1 week of expression. It will be important to investigate whether the expression of htau$^{0N3R}$ is causing neurodegeneration or toxicity in the mushroom bodies. However, this is not believed to be likely because others have shown that htau$^{0N3R}$ does not cause overt neurodegeneration in the mushroom bodies (Kosmidis et al., 2010). This therefore suggests that the learning and memory deficit seen here is more likely to do with dysfunction of tau as opposed to tau-mediated degeneration.

### 3.4.6 Conclusions and future directions

Two different behavioural assays have been used here to measure htau$^{0N3R}$ deficits. These assays are highly relevant to this study as they closely model, aspects of AD. It is also possible to measure these different behaviours with age, although this was not done for learning and memory, due to time constraints. It has been demonstrated here that htau$^{0N3R}$ expression causes deficits in both of these behaviours in *Drosophila*. This study, together with others, demonstrates that tau expression is directly related to the symptoms of AD. This highlights the importance of discovering therapeutics for AD that target pathogenic tau. Now that htau$^{0N3R}$ deficits have been characterised here, it will possible to use these assays in subsequent experiments. It will be possible to assess how manipulations to ageing pathways can affect these phenotypes.

# Chapter 4: Investigation of how age-related changes in TORC1 signalling and autophagy pathways impact on tauopathy

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## 4.1 Introduction

The aim of this chapter is to understand how cellular pathways implicated in ageing firstly, change with age and secondly, how they impact on tauopathy. There are various pathways that have been implicated in both ageing and AD but the work presented here has focused on the roles of certain pathways that are involved in protein turnover, specifically mTORC1 signalling and autophagy. Here, studies conducted in both humans and transgenic *Drosophila* models will be used to explore these two pathways both in relation to ageing and tauopathy, to further understand how age-related changes in these pathways contribute to tauopathy.

### 4.1.1 The role of mTORC1 signalling in ageing and AD

mTORC1 signalling is a key regulator of many processes involved in cell growth including, among others, protein synthesis, metabolism and autophagy. Due to mTORC1's role in these vital and diverse processes, it is not surprising to find this pathway has been implicated in a range of diseases, such as cancer, metabolic diseases, and also neurodegenerative diseases such as AD (Laplante & Sabatini, 2012). The mTORC1 pathway has also been found to be important in regulating the ageing process. For example, pharmacological inhibition of the TORC1 pathway, using the drug rapamycin, leads to an increase in lifespan of various different species including *Drosophila* (Bjedov et al., 2010), *S. cerevisiae* (Powers et al., 2006) *C. elegans* (Robida-Stubbs et al., 2012), and mice (Harrison et al., 2009). Similarly, when the mTORC1 pathway has been genetically down-regulated an increase in longevity is found in *Drosophila* (Kapahi et al., 2004), *S. cerevisiae* (Bonawitz et al., 2007), *C. elegans* (Hansen et al., 2007) and in mice (Selman et al., 2009). Collectively these studies demonstrate that inhibition of the TORC1 pathway increases longevity and that this is highly conserved between species, implicating the TORC1 pathway as an important regulator of ageing.

mTORC1 signalling has also been implicated in AD. Li et al., (2005) found increased activity of the pathway in AD brains, demonstrated by increased phosphorylation of components of the mTORC1 pathway (mTOR and 4E-BP). A separate study also found that another downstream component of the mTORC1 pathway, pS6K, co-localises with phospho tau (AT8) in tangles and dystrophic neurites in AD brains (An et al., 2003). In animal models of AD, rapamycin has been found to reduce tau-mediated phenotypes, such as the rough eye phenotype in flies (Berger et al., 2006). Rapamycin has also been shown to reduce Aβ-mediated deficits in learning and memory in a mutant APP mouse model of AD

(Spilman et al., 2010). Similarly, in the 3xTg mouse model of AD, treatment with rapamycin was shown to significantly improve learning and memory and reduce both tau and Aβ pathology (Caccamo et al., 2010). These studies demonstrate that mTORC1 signalling is closely associated with AD and that inhibition of the mTORC1 pathway appears to ameliorate both tau and-Aβ mediated phenotypes and pathologies.

### 4.1.2 Autophagy in ageing and AD

Autophagy is inhibited by the mTORC1 pathway and is intrinsically linked to the effects of mTORC1 pathway on both ageing and AD. In yeast, a genetic screen for short lived mutants identified ten mutants that all had defects in autophagy-related genes (Matecic et al., 2010). In *Drosophila*, it was found that rapamycin-mediated an extension of lifespan and this was correlated with an increase in autophagy in the fly gut (Bjedov et al., 2010). Loss-of-function mutations in autophagy-related genes, such as Beclin-1 and Atg1, (both involved in the initiation of autophagy) were found to reduce the lifespan of *C. elegans* (Toth et al., 2008). Additionally, caloric restriction, which has been shown to consistently reduce lifespan, requires autophagy to mediate its effects on lifespan in *C. elegans* (Jia et al., 2007). These studies demonstrate the importance of the autophagic pathway in the regulation of lifespan.

Autophagic clearance has been implicated in AD, with markers for autophagy co-localising with the plaques and tangles found in AD brains (Ma et al., 2010). Additionally, induction of autophagy using rapamycin, in the 3xTg mouse model of AD, improved learning and memory deficits and ameliorated tau and Aβ pathology. This was correlated with increased autophagy in these animals (Caccamo et al., 2010, Majumder et al., 2011). In a separate study, organotypic brain slices from P301L mutant tau mice were incubated with methylene blue which induces autophagy and reduced tau levels (Congdon et al., 2012). In a fly model of tauopathy (htau$^{R406W}$), removal of the lysosomal aspartyl protease (cathepsin D), exacerbated tau-mediated neurodegeneration and correlated with a reduction in lifespan (Khurana et al., 2010). These studies demonstrate that autophagy is associated with both ageing and AD. It is not clear whether age-related changes in both mTORC1 signalling and autophagy contribute to the build-up of toxic aggregates of tau and Aβ, and consequently increase the risk of AD.

### 4.1.3 Aims

Although it is clear that both mTORC1 signalling and autophagy are intrinsically linked to both ageing and AD, it is not known how age-related changes in these pathways impact on tauopathy. Therefore, the aims of this Chapter are to firstly understand how mTORC1 signalling and autophagy change in normal ageing in the human brain. This is currently a neglected area of research, particularly in the ageing human brain. To be able to investigate this, human cortical brain tissue resected from living patients of different ages will be used. These patients are undergoing neurosurgery for a number of different inflictions including epilepsy and glioma. The tissue is resected from the cortical region of the brain outside the focal area and is classed as non-pathological. This type of tissue is useful for this study, as to be able to measure the activity of the mTORC1 pathway, the phosphorylation of various mTORC1 pathway components need to be probed. It is known that phosphate groups are rapidly removed from proteins during *post-mortem* delays (Matsuo et al., 1994) and therefore data from *post-mortem* brain may well not be representative of the situation in the living brain. In addition to assessing how the mTORC1 and autophagy pathways change with age, this same resected tissue will be used to correlate potential changes in these pathways with total and phosphorylated tau levels.

Although using resected human cortical tissue is useful for measuring how pathways change with age, it is not possible to manipulate these pathways in this tissue and investigate resultant changes in tau protein. Therefore, transgenic *Drosophila* have been generated that express $htau^{0N3R}$ (as used in Chapters 2 and 3) together with genetic manipulations of the dTORC1 pathway and autophagy pathways, shown in (Figure 4-1). The dTORC1 pathway will be manipulated by co-expression of the GTPase $Rheb_{RNAi}$ to knock down Rheb expression. Overexpression of Rheb has been shown to activate the dTORC1 pathway (Stocker et al., 2003) and to increase tau-mediated neurodegeneration before in a *Drosophila* model of tauopathy (Khurana et al, 2006), therefore knocking down Rheb expression is expected to reduce tau-mediated phenotypes. Separately to this, the autophagic initiator Atg1 will be co-expressed with $htau^{0N3R}$ to upregulate the autophagy pathway. Atg1 is the *Drosophila* homolog of ULK-1 and is a regulatory kinase that controls the initiation of autophagy (Scott et al., 2007). To ensure that these genetic manipulations do not affect the development of the flies, the GeneSwitch expression system will be used. This system, shown in (Figure 4-6a), only induces gene expression if RU486 is administered to the flies. The RU486 will only be added to the fly diet post eclosion and not during development, ensuring any resultant phenotypes are not confounded by developmental effects. Genetic manipulations of the dTORC1 and autophagy pathways will be expressed

in this way, with co-expression of htau$^{0N3R}$, and resultant tau-mediated phenotypes and pathologies will be measured. These studies aim to understand the relative contribution that these manipulations have on age-related tau-mediated phenotypes and pathologies. These studies, together with the data from the human cortical tissue will provide a better understanding of how age-related changes of these two pathways potentially contribute to tau pathology.

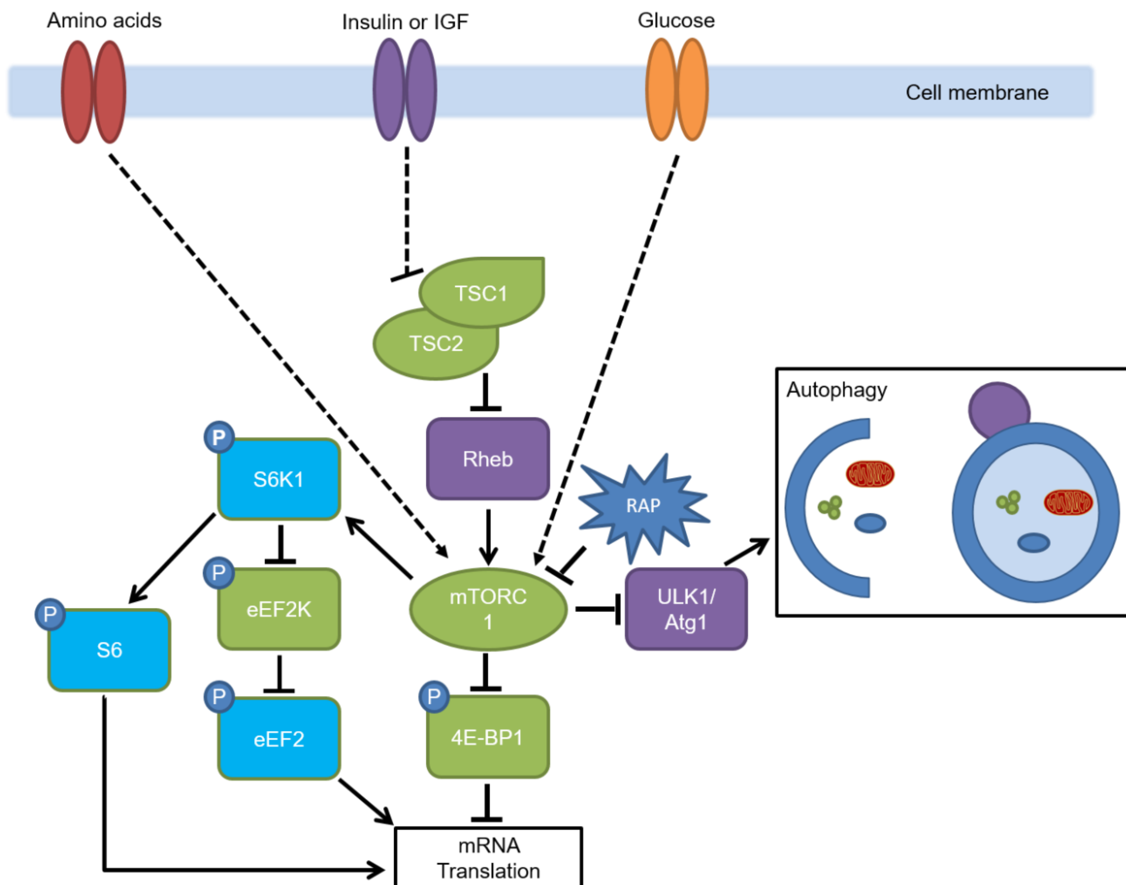



Figure 4-1. Probing the TOR and autophagy pathways in humans (mTOR) and *Drosophila* (dTOR). This diagram highlights the downstream components of the TORC1 signalling pathway that will be assessed in human brain tissue and *Drosophila* including pS6K, pS6 and peEF2 (light blue boxes). Also highlighted are where the pathway has been maniuplated using transgenic *Drosophila* lines. Flies will express both htau and Rheb (upstream of TORC1 complex) and Atg1, where TOR signalling is known to inhibit the autophagic pathway (shown in purple boxes).

## 4.2 Methods

### 4.2.1 Human tissue processing

The use of human tissue was carried out in collaboration with the laboratory of Dr. Mariana Vargas-Caballero. REC Reference: 12/NW/0 794, HTA LN: 12009. Study reference number: SRB002/14. All procedures on human tissue were performed in accordance with the Human Tissue Act 2004 with approval from the Faculty of Medicine Ethics Committee of the University of Southampton and the Southampton Research Biorepository. The cortical tissue used in this study was resected from living patients undergoing neurosurgery for the removal of deeper structures following written informed consent. All patient data was anonymised. The patients were being treated for medial temporal epilepsy, dysembryoplastic neuroepithelial tumour, malformation (cavernous or arteriovenous), or glioma, see (Table 4) for further details. This surplus tissue that would normally be discarded during such procedures was taken from outside the epileptic focus or tumour. Following resection in theatre, the cortical tissue was put into artificial cerebrospinal fluid (ACSF) immediately after resection. ACSF contained 110 mM choline chloride, 26 mM $NaHCO_3$, 10 mM D-glucose, 11.6 mM sodium ascorbate, 7 mM $MgCl_2$, 3.1 mM sodium pyruvate, 2.5 mM KCl, 1.25 mM $NaH_2PO_4$ and 0.5 $CaCl_2$. The tissue was then snap frozen on dry ice within 10 min of removal from the patient. The tissue sample was then homogenised as a 10% tissue solution in homogenisation buffer that contained 10 mM Tris Base, 150 mM NaCl, 0.05% Tween-20, 1 mM Na orthovanadate, 10 mM NaF, 10 μM Staurosporine,1x protease inhibitor cocktail and 1 mM Tyrophostin. Samples were briefly spun at 3000 *g* for 2 min and the pellet was discarded. A protein assay was then conducted on the samples and protein concentration was normalised across samples. The samples were then boiled in 2 x SDS sample buffer at 95°C for 5 mins.

Table 4 Patient information for human samples used.

| Patient no. | Age of patient | Gender | Diagnosis | Resected tissue | Used to probe |
|---|---|---|---|---|---|
| **19** | 19 | M | Arteriovenous Malformation | Left frontal lobe | S6, S6K, p62, Total tau, PHF-1 |
| **10** | 28 | M | Hippocampal Sclerosis/Atrophy in the left hippocampus | Left anterior temporal cortex | S6, S6K, p62, Total tau, PHF-1 |
| **18** | 30 | M | Cavernous Malformation | Right temporal lobe | S6, S6K, p62, Total tau, PHF-1 |
| **14** | 32 | M | Hippocampal Sclerosis | Left temporal lobe | S6, S6K, PHF-1 |
| **16** | 36 | F | Hippocampal Sclerosis | Right anterior temporal lobe | PHF-1 |
| **11** | 42 | F | Glioma | Right anterior temporal lobe | S6, S6K, p62 |
| **9** | 45 | M | Arteriovenous Malformation | | S6, Total tau, PHF-1 |
| **21** | 49 | M | Hippocampal Sclerosis | Right temporal lobe | p62, Total tau, PHF-1 |
| **4** | 52 | M | Epilepsy/Right Amygdalohippocampectomy | Right anterior temporal lobe | S6K, p62 |
| **12** | 55 | M | Glioma | Left parietal lobe | S6, S6K, p62, Total tau, PHF-1 |
| **5** | 60 | F | Epilepsy/Left Amygdalohippocampectomy | Left anterior temporal lobe | p62, Total tau, PHF-1 |
| **17** | 62 | F | Hippocampal Sclerosis | Right anterior temporal lobe | S6K, p62, Total tau, PHF-1 |
| **13** | 69 | F | Right Glioblastoma (GBM) | Right frontal lobe | S6, S6K, Total tau, PHF-1 |
| **20** | 70 | F | Arteriovenous Malformation | Left posterior temporal lobe | S6, p62, Total tau, PHF-1 |
| **8** | 71 | M | Right Glioblastoma (GBM) | Right temporal lobe | S6, Total tau, PHF-1 |

### 4.2.2 Fly stocks

The following fly stocks were used for this study: Elav-GeneSwitch, Elav-GAL4, UAS-htau$^{0N3R}$, UAS-Atg1, UAS-Atg$_{RNAi}$, UAS-Rheb and UAS-Rheb$_{RNAi}$ (Bloomington Stock Centre) and MB-GeneSwitch (a kind gift from Ron Davis lab). Full details of all fly stocks used here can be found in Appendix E. Double transgenic flies were made by a series of crosses using a double balancer line (Bloomington Stock Centre #3703), details of which can be found in the Appendix C (Figure 6-4). Flies were raised and maintained at 25°C on SYA diet. Only female flies were used for these studies.

### 4.2.3 RU486 treatment

After eclosion, 0-3 day old flies were moved to either food containing RU486 or to control diet that did not contain RU486. A 100 mM stock solution of RU486 was made up in 100% ethanol. Diet containing RU486 was then made up containing final concentrations of 100 µM and 200 µM RU486 for dose response experiments. The high dose of 200 µM RU486 was then used for all subsequent experiments.

### 4.2.4 Climbing assays

Climbing assays were performed as previously described in 2.2.3, with some minor differences. Five cohorts were used here, instead of three, each containing 10 flies in each cohort. The climbing ability of these flies was then tested on a weekly basis for four weeks. Flies were transferred to the measuring cylinders without use of anaesthetisation. Distance climbed was recorded at 10 seconds after tapping down. The assay was repeated three times per day of testing. A 2-way ANOVA was performed on the data with Bonferroni multiple comparisons using GraphPad Prism.

### 4.2.5 Longevity assays

Longevity assays were performed as previously described in 2.2.2, with some minor differences. Five cohorts were used, instead of three cohorts, containing 10 flies in each cohort. Flies were transferred to new food containing RU486 twice a week and scored for deaths three times a week. Survival was plotted as a Kaplan Meir curve. For statistical analysis a Mantel-Cox test was used.

### 4.2.6 Learning and memory

Short term learning and memory was assessed as has been described previously in Chapter 2. Briefly, flies were tested in cohorts of 15 flies in the APS assay. Flies were tested for phototaxia in 4 trials and were then exposed to the aversive stimulus quinine for 16 trials. Testing of memory was conducted 1 min after the last training trial. Performance index (PI) was calculated as the proportion of flies that avoided the light chamber in the test phase relative to the number of flies found to be phototaxic. A Mann-Whitney statistical test was used to compare experimental flies to non-expressing controls, using GraphPad Prism.

### 4.2.7 Fly sample preparation for western blot

Fly samples for running on western blots were made as follows. Adult flies were snap frozen in liquid nitrogen and heads were dissected. Heads were then homogenised in buffer in a ratio of 1 head: 10 µl buffer (150 mM NaCl, 50 mM MES, 1% Triton-X, protease inhibitor cocktail, 30 mM NaF pH7, 40 mM 2-glycerophosphate pH7, 20 mM Na pyrophosphate, 3.5 mM Na orthovanadate, 1% SDS, 10 µM staurosporine). Samples were centrifuged at 3000 *g* for 2 min and the pellet was discarded. Samples were then made up with 2x SDS sample buffer and boiled at 95°C for 5 min.

### 4.2.8 Western blotting

For western blots using resected human tissue samples, 20 µg of protein was loaded onto SDS-PAGE gels. Western blots were carried out as previously outlined in Chapter 1. The table below (Table 2) shows the primary antibodies used to probe the different samples, including the percentage gel used in each instance. All SDS-PAGE gels were transferred to 0.45 µM nitrocellulose membrane using wet transfer. Blots were blocked for 1 hour at room temperature in 5% BSA 0.1% tween PBS (tPBS). Primary antibody was incubated at 4°C overnight at the dilutions stated in Table 2. Blots were then washed in 0.1% tPBS for 3 x 5 min washes. Blots were then probed with secondary antibodies against rabbit (IRDye, Licor) or mouse (Alexa-Fluor, Invitrogen), both used at 1: 20,000, for 1 hour at room temperature. Blots were visualised using LI-COR.

Table 5. Antibodies used in western blots in Chapter 4.

| Antibody | Dilution | Source | Percentage gel |
|---|---|---|---|
| **pS6K (Thr389)** | 1:500 | Cell Signalling | 10% |
| **S6K** | 1:1,000 | Cell signalling | 10% |
| **pS6 (Ser240/244)** | 1:1,000 | Cell signalling | 10% |
| **S6** | 1:1,000 | Santa Cruz | 10% |
| **p62** | 1:1,000 | ProSci | 10% |
| **Atg8** | 1:500 | Kohler Lab | 15% |
| **eEF2** | 1:1000 | Cell Signalling | 10% |
| **peEF2 (Thr56)** | 1:1000 | Cell Signalling | 10% |
| **Rheb** | 1:1,000 | Abcam | 15% |
| **Human tau** | 1:15,000 | Dako | 10% |
| **PHF-1 human tau** | 1:500 | Peter Davies Lab | 10% |
| **Actin (loading control)** | 1:5,000 | Abcam | 10%/15% |

### 4.2.9 Statistical analysis

Where appropriate values are presented as the mean ± standard error of the mean. To compare differences between groups, statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). The statistical test used for each assay is indicated separately. $p<0.05$ was considered to indicate a statistically significant difference.

## 4.3 Results

### 4.3.1 mTORC1 signalling decreases with age in resected human cortical tissue

It is currently unclear how mTORC1 signalling changes in normal ageing. There are mixed results about how mTORC1 signalling changes with age as reported in (Baar et al., 2016). Very few studies have investigated how mTORC1 signalling changes with age in the brain therefore we attempted to assess this in human brain tissue. It is possible to measure the activity the mTORC1 pathway by measuring the phosphorylation state of downstream components of the pathway, such as S6K and S6. Phosphorylation of proteins have been found to decrease rapidly *post-mortem* (Matsuo et al., 1994), making it difficult to accurately measure signalling pathways such as mTORC1. Therefore, instead of using *post-mortem* tissue this study utilised resected human cortical tissue from living patients. This normally discarded tissue from differently aged patients was processed and run on western blots to be probed for pS6K and pS6. It was found that pS6K levels relative to total S6K levels decreased significantly with age (Figure 4-2 a, b), quantified as a linear regression $r^2$= 0.05031, p=0.0324 (n=9). Levels of pS6 compared to total S6 tended to decrease with age but this was not found to be significant (Figure 4-2 c, d) ($r^2$= 0.03043, p= 0.0983 n=10). As S6K and S6 are phosphorylated with increased activity of the mTORC1 pathway, pS6K and pS6 levels decreasing with age are indicative of decreased mTORC1 signalling in the ageing cortex.

### 4.3.2 dTORC1 signalling also decreases with age in *Drosophila*

As it was demonstrated here that mTOR signalling decreases in the human cortex, and since the next stages of this work included manipulating this pathway in *Drosophila,* it was important to assess whether this age-related decrease in mTORC1 also occurred in flies. For this, peEF2 levels, relative to total eEF2 levels were probed in young (0-3 days) and old (4 weeks old) Elav-GAL4; htau$^{0N3R}$ expressing and non-expressing flies (Figure 4-3 a, b). There was found to be wide variability when this was tested in the flies. However, there was a trend for an increase in peEF2 levels with age in both htau$^{0N3R}$ expressing and non-expressing flies. The trend was stronger in the htau$^{0N3R}$ expressing flies. Increased peEF2 levels are indicative of decreased dTORC1 signalling there this implies that dTORC1 signalling also decreases with age.

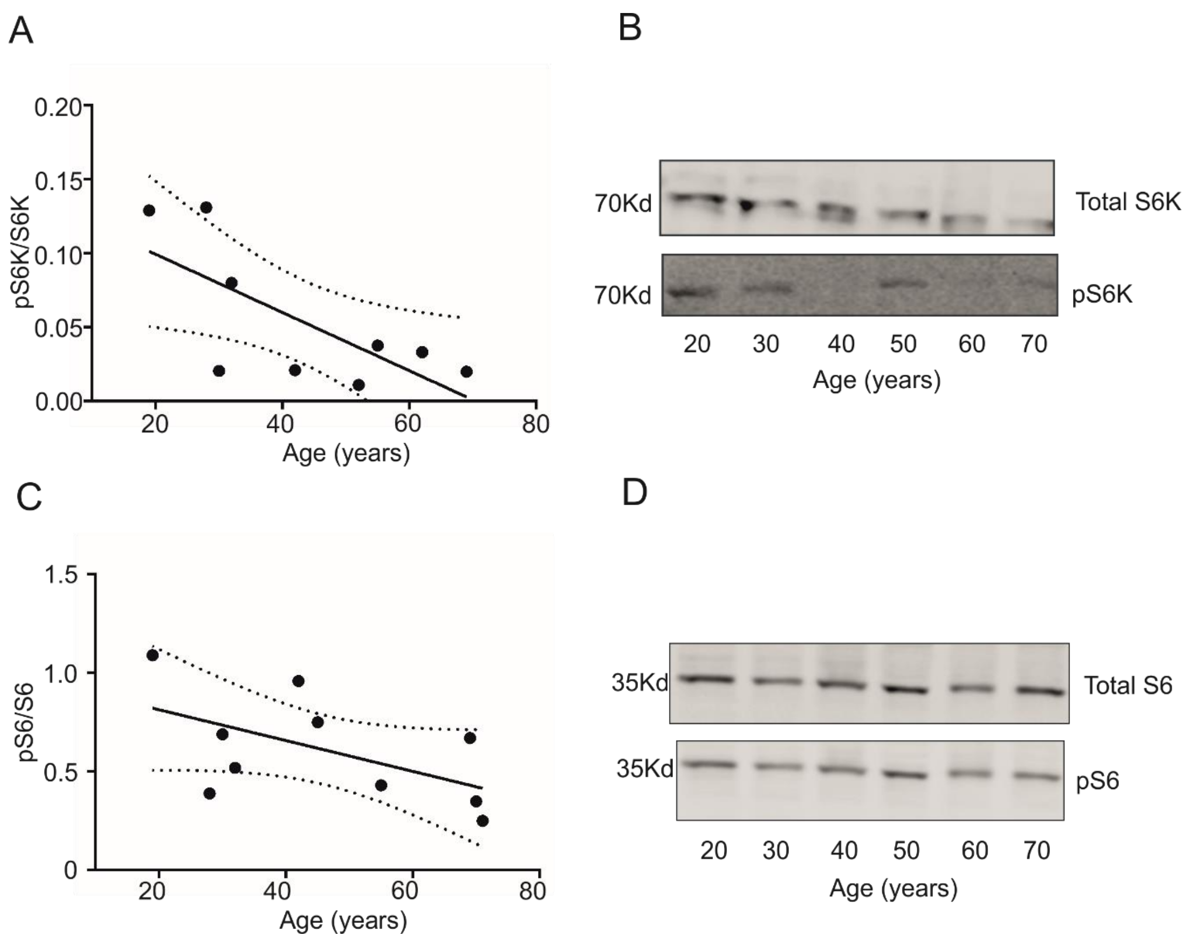



Figure 4-2. mTORC1 signalling activity decreases with age in human resected cortical tissue.
Linear regression showing the ratio of pS6K/total S6K significantly decreases with age, $r^2$=0.5031, p=0.0324 (n=9) (B) representative blots of pS6K/total S6K. (C) Linear regression showing the ratio of pS6/total S6 decreases with age, $r^2$ = 0.3043, p=0.0983 (n=10). (D) Representative blot of pS6/total S6.

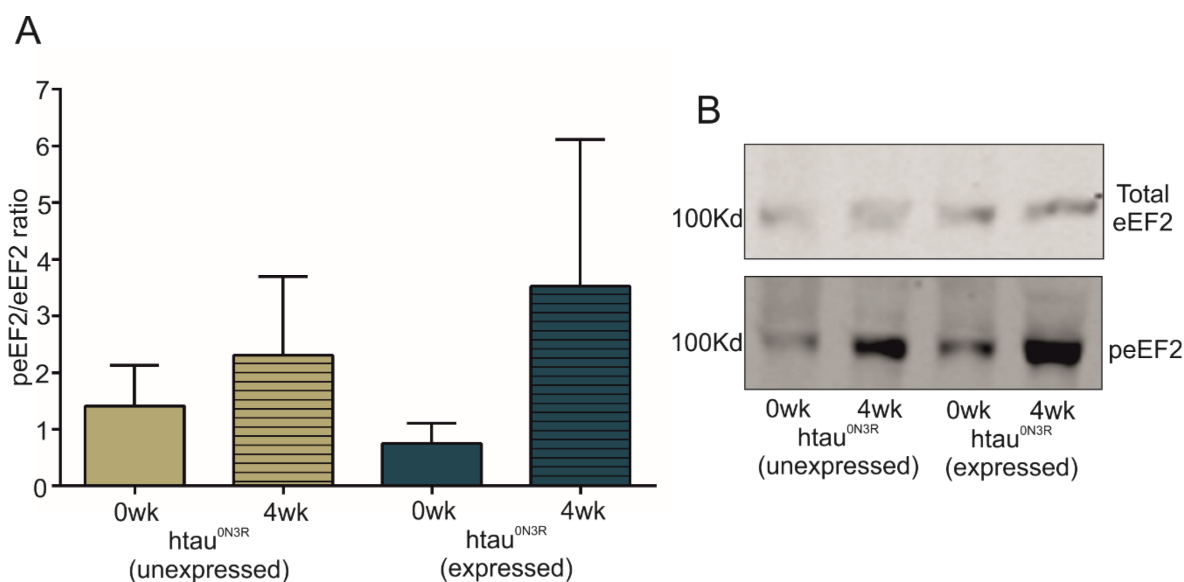



Figure 4-3. dTORC1 signalling decreases with age in *Drosophila*.
Elav GAL4-expressing $htau^{0N3R}$ flies and $htau^{0N3R}$ non-expressing control flies were tested at 0 wk (newly eclosed) and at 4 wks for peEF2 levels relative to total eEF2 levels. (A) Trend seen for an increase in peEF2 levels relative to total eEF2 levels (n=3). (B) Representative blot of total and peEF2.

### 4.3.3 Trend for autophagy to increase with age in resected human cortical tissue

It has been shown here that mTORC1 signalling decreases with age in the human cortex. Therefore, as mTORC1 signalling is known to inhibit autophagy, a decrease in mTORC1 signalling should induce an upregulation of autophagy. To test this, p62 levels were probed in the resected human cortical tissue. p62 is an autophagic adaptor protein that binds to cargo, such as misfolded proteins. Once bound to its cargo, p62 becomes internalised together with the cargo into the autophagosome and degraded in the autolysosome, as depicted in (Figure 4-4a). This means that a decrease in p62 levels would be indicative of increased autophagy. (Figure 4-4 b, c) show that there was a trend for p62 levels, relative to actin levels, to decrease with age, although this was not found to be significant (linear regression $r^2$=0.2766, p=0.1184). These data suggests that autophagy may increase with age, however with the limited number of samples available for this study this was not found to be significant. This data therefore suggests that the basal level of autophagy may also remain constant during normal ageing.

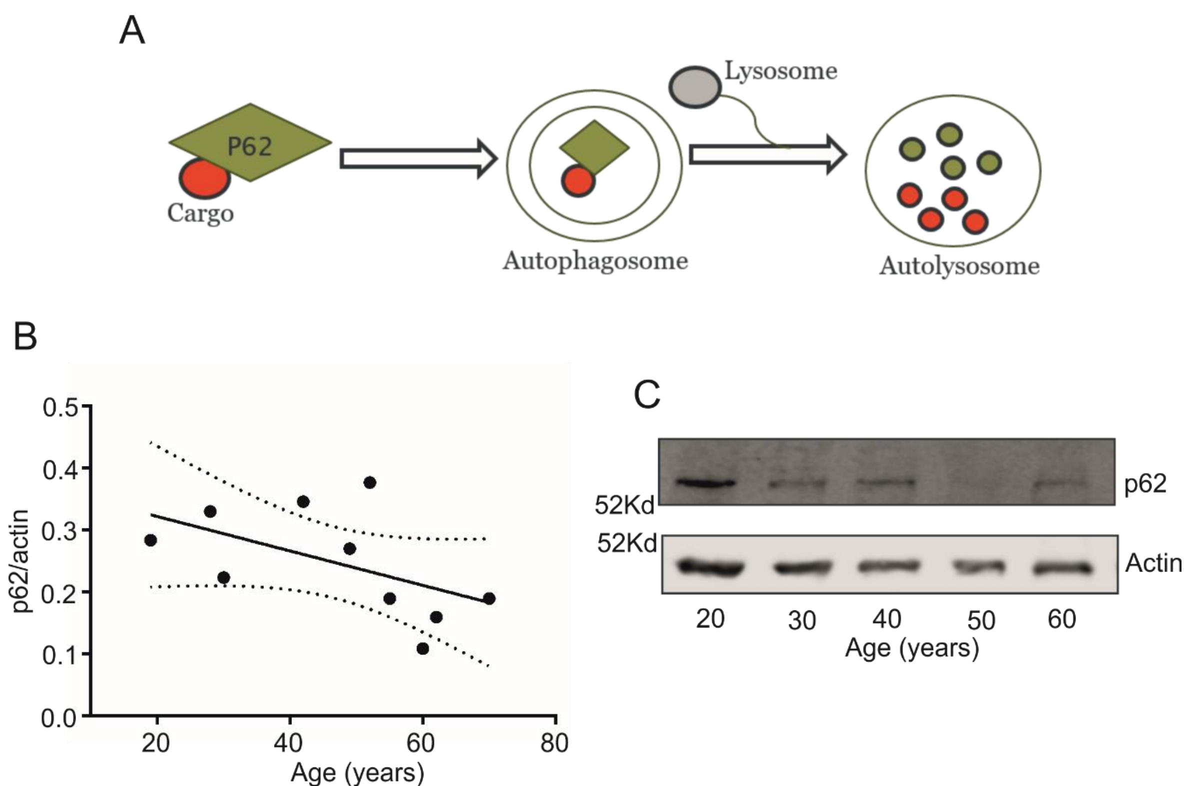



Figure 4-4. A trend seen for autophagy to increase with age in resected human cortical tissue.
(A) p62 is used as a marker of autophagy, p62 binds to cargo destined to be degraded by the autophagic pathway and becomes internalised along with cargo into the autophagosome and degraded in the autolysome, meaning that a decrease in p62 levels is indicative of increased autophagy. (B) A trend for a decrease in p62 levels relative to actin levels is found in resected human cortical tissue with age, $r^2$= 0.2766, p= 0.1184 (not significant (n=10). (C) Representative blot of p62 and actin.

### 4.3.4 Decreased mTORC1 signalling with age is correlated with decreased tau pathology

It is predicted that a decrease in mTORC1 signalling with age should lead to a decrease in the amount of human tau, due to reduced protein translation and/or increased autophagic clearance. Therefore, the next step was to assess how tau protein changed with age in the resected human tissue. To investigate this, both total and phosphorylated tau at S396/S404 sites (PHF-1) were probed. It was found that that there was a decreased level of tau protein, relative to actin levels, with age. A significant correlation between age and total tau levels was observed, linear regression: $r^2$= 0.4178, p=0.0231 (n=12) (Figure 4-5a, b). It was also shown that phospho tau levels relative to total tau decreased with age, with a significant correlation found between age and ptau: $r^2$= 0.3100, p= 0.0386 (n=14) (Figure 4-5c, d). This means that both total and phospho tau levels are decreased in the aged brain correlating with a decrease in mTORC1 signalling.

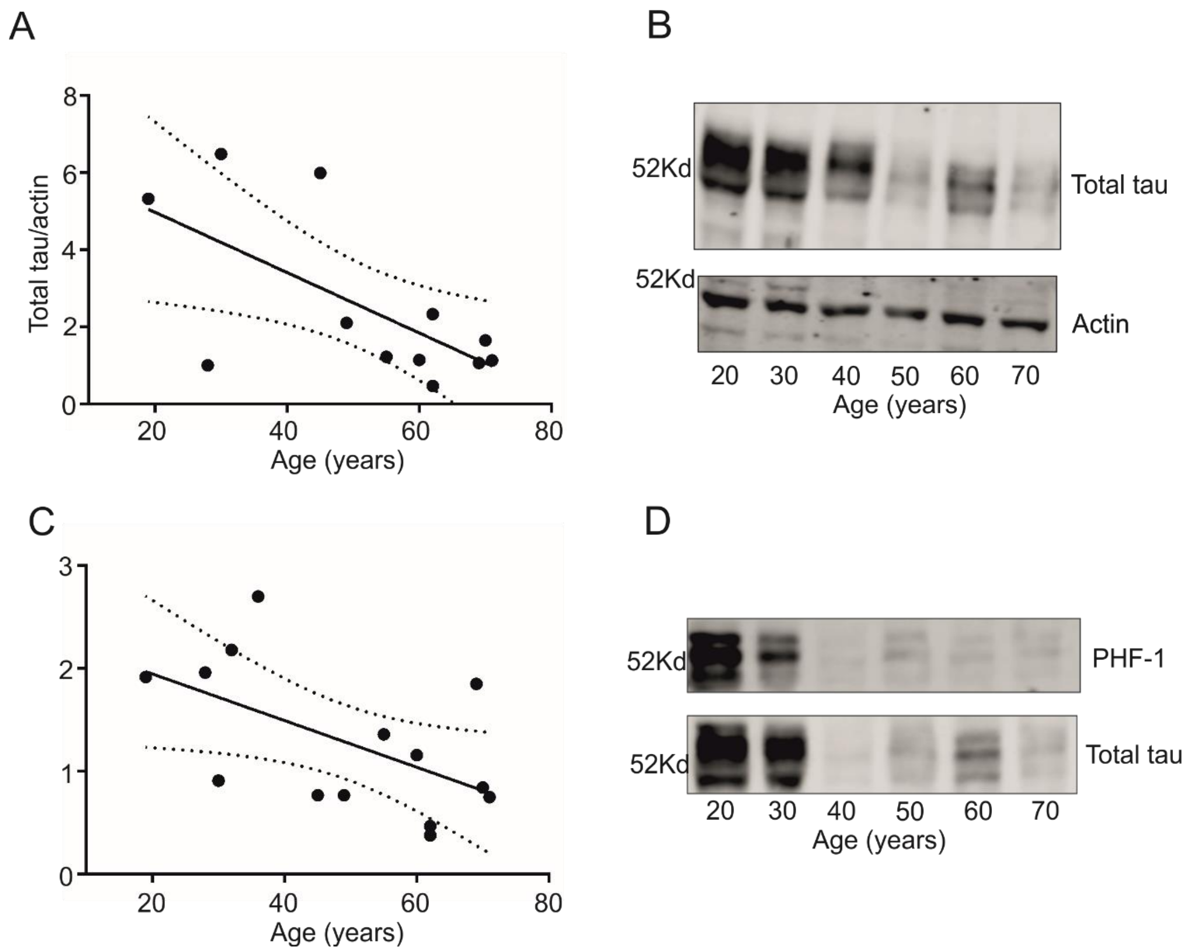



Figure 4-5. Decreased total tau and phosphorylated tau (PHF-1) found, with age, in resected human cortical tissue.
(A) Total tau levels relative to actin found to significantly decrease with age, linear regression $r^2$= 0.4178, p=0.0231 (n=12). (B) Representative blot of total tau/actin. (C) Significant decrease in PHF-1 levels relative to total tau, linear regression, $r^2$= 0.3100, p= 0.0386 (n=14). (D) Representative blot of PHF-1/total tau.

### 4.3.5 GeneSwitch expression system allows for expression of proteins in adulthood

It is shown here that in human cortex, mTORC1 signalling activity significantly decreases with age and this correlates with decreased total and phospho tau protein in healthy ageing cortex. Although there was a trend seen for an increase in autophagy with age, this did not reach significance suggestive of basal levels of autophagy not changing with age. What cannot be evaluated using this tissue from human brains is whether decreased mTORC1 causes this decrease in tau protein. Therefore, it is important to investigate whether this is the case in a model where these pathways can be manipulated and resultant tau phenotypes and tau protein levels can be assessed. Here a *Drosophila* model was used that expresses htau$^{0N3R}$ under GeneSwitch control. This system allows for the conditional expression of proteins when the drug, RU486, is present in the food of the flies (Figure 4-6). This means that htau$^{0N3R}$ expression and the genetic manipulations of the dTORC1/autophagy pathways are induced only in adulthood. This removes any confounding effects that the expression of these proteins could have during development.

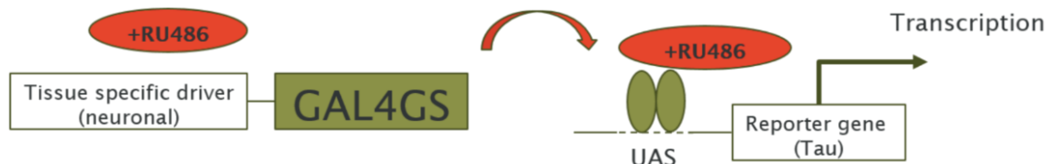
The GeneSwitch system is demonstrated in (Figure 4-6), where Elav-GeneSwitch (ElavGS);htau$^{0N3R}$ flies were fed on RU486 in adulthood. (Figure 4-6 b, c) demonstrate that when these flies were given control diet (without RU486) there was no expression of htau$^{0N3R}$ demonstrated using western blotting. Whereas when these flies were fed on diet containing either 100 µM or 200 µM dose of RU486, this induced expression of htau$^{0N3R}$ in a dose-dependent manner. It was noted that the 200 µM dose of RU486 still induced lower expression than the Elav-GAL4 driver, shown in (Figure 4-6c). Expression of htau$^{0N3R}$ only during adulthood resulted in a subtle yet significant reduction in climbing ability compared to control flies that were fed drug-free diet, (Figure 4-6d), (2-way ANOVA p=0.0291, n=50). Controls flies that only expressed the driver (ElavGS) were also treated RU486 and assessed for climbing over 4 weeks. Addition of RU486 to the diet of controls did not induce any deficits in climbing ability. This demonstrates that there is no direct effect of RU486 on climbing ability, see Appendix C (Figure 6-3).

Longevity was also assessed in ElavGS and ElavGS expressing htau$^{0N3R}$ flies, shown in Appendix C (Figure 6-5). RU486 treatment did not result in a difference in lifespan compared to flies not treated with RU486 for either ElavGS or ElavGS; htau$^{0N3R}$ flies. This is proposed to be because the expression level of using this driver is not high enough to induce a reduction in lifespan. Futher work could include testing higher concentraions of RU486 on htau$^{0N3R}$ expressing flies.

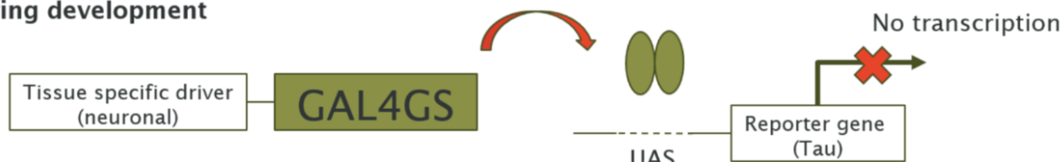
This data demonstrates that the GeneSwitch expression system can be used to express proteins in a dose response manner conditionally expressed only in adulthood. Expression of $htau^{0N3R}$ only in adulthood still produced a deficit in climbing ability but not in longevity. Therefore, it was possible to test whether manipulations of the dTORC1/autophagy pathways were able to impact on $htau^{0N3R}$-mediated climbing.

A

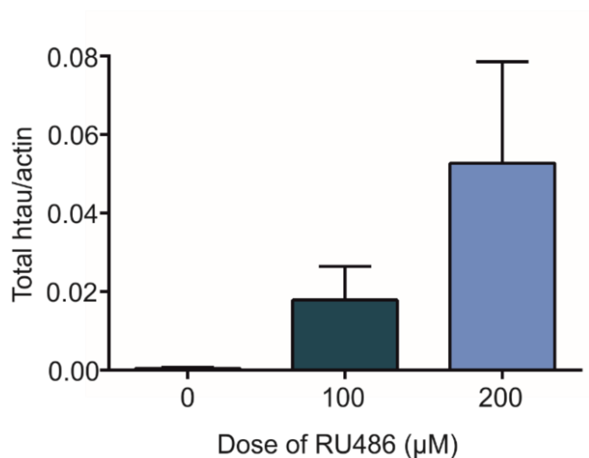
During adulthood



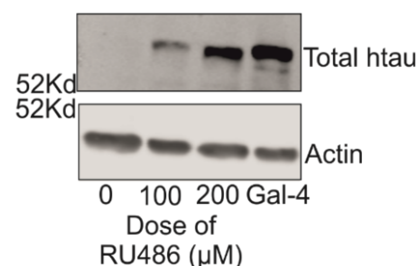

During development




B




C




D

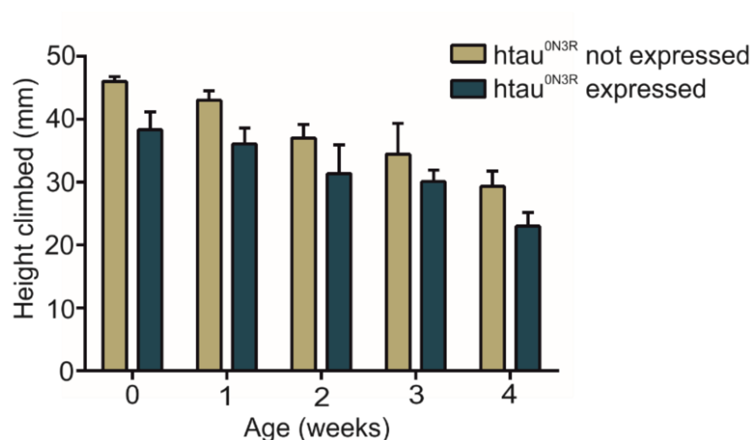



Figure 4-6. GeneSwitch expression system allows for htau$^{0N3R}$ expression in adulthood, in a dose dependent manner.

(A) GeneSwitch allows for transcription only if RU486 is present in the food given to the flies. If RU486 is not present transcription cannot occur and htau$^{0N3R}$ is not expressed. (B) Increasing doses (100 and 200 μM) of RU486, induces increased htau$^{0N3R}$ expression relative to the amount of drug present in the food (n=3 n.s.). (C) Representative blot of total human tau expression. (D) Expression of htau$^{0N3R}$ using 200μM dose of RU486 only in adulthood induces a significant reduction in climbing ability (2-way ANOVA p=0.0291, n=50).

### 4.3.6 Expression of $Rheb_{RNAi}$ improves tau-mediated climbing deficits

As shown above, the GeneSwitch system allows for expression of proteins in adulthood. Expression of $htau^{0N3R}$ in this way causes a subtle yet significant reduction in climbing ability. The next step was to investigate whether manipulation of the dTORC1 signalling pathway would impact on this $htau^{0N3R}$-mediated climbing deficit. To do this, double transgenic flies were made that expressed both $htau^{0N3R}$ and $Rheb_{RNAi}$. Rheb lies upstream of the dTORC1 signalling pathway and has been shown to activate the pathway (Stocker et al., 2003). Therefore, expression of $Rheb_{RNAi}$ should knock down Rheb and consequently cause a reduction in dTORC1 signalling.

Both Rheb and $htau^{0N3R}$ expression was assessed in 2 weeks old $htau^{0N3R}$, $Rheb_{RNAi}$ and $htau^{0N3R}$; $Rheb_{RNAi}$ flies. $htau^{0N3R}$ expression was confirmed in both $htau^{0N3R}$ and $htau^{0N3R}$; $Rheb_{RNAi}$ flies (Figure 4-7). It was not clear from these blots Rheb expression was significantly reduced in $htau^{0N3R}$; $Rheb_{RNAi}$ flies. This could be due to the fact that whole heads were used to probe for Rheb. $Rheb_{RNAi}$ was only expressed here in neuronal cells, therefore as Rheb is an endogenous protein it will be found in other cells in the head. This could well mask any alterations in Rheb expression. To overcome this, two different experiments can be conducted. Firstly, dissected brains can be used instead of whole heads for running on western blots. Secondly, it is possible to express $Rheb_{RNAi}$ using a ubiquitous driver that expresses $Rheb_{RNAi}$ in all cells in the body. Heads of these flies can then be used to test the knockdown of Rheb. Time constraints did not allow for these experiments to be done, however this will be followed up in future work.

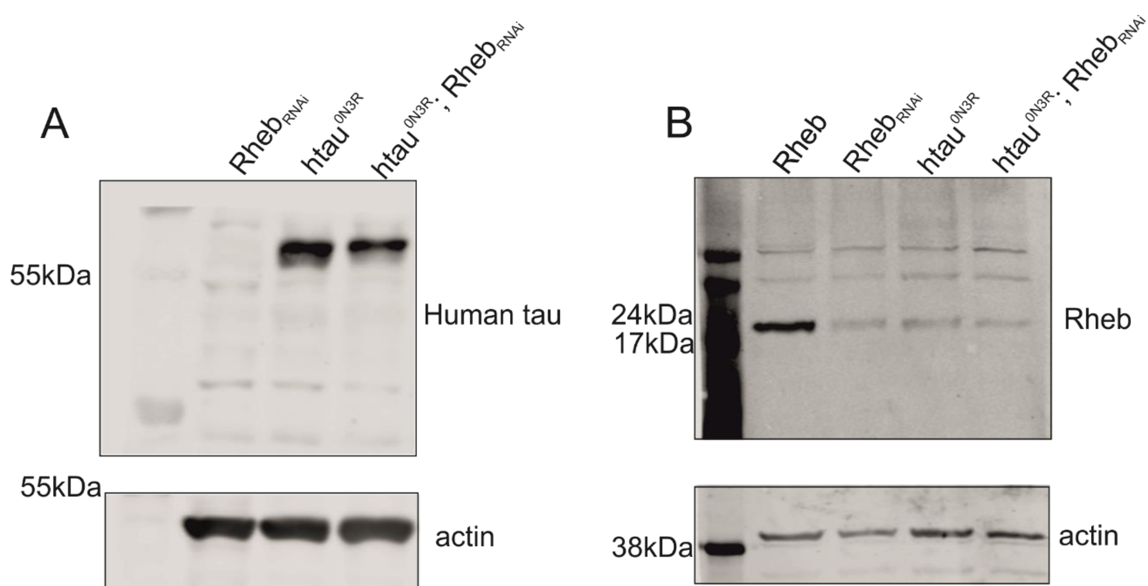



Figure 4-7. Rheb and htau$^{0N3R}$ expression in htau$^{0N3R}$; Rheb$_{RNAi}$ flies.
A) Expression of htau$^{0N3R}$. Both htau$^{0N3R}$ and htau$^{0N3R}$; Rheb$_{RNAi}$ 2 week old flies express htau$^{0N3R}$, no expression seen in Rheb$_{RNAi}$ flies. B) Rheb expression in Rheb$_{RNAi}$, htau$^{0N3R}$ and htau$^{0N3R}$; Rheb$_{RNAi}$ lines. Expression of UAS-Rheb is used as a positive control (far left).

Although knockdown of Rheb expression could not be confirmed here, the climbing behaviour did show a potentially interesting result. (Figure 4-8a) shows that Rheb$_{RNAi}$ expressed alone caused a significant improvement in climbing ability (2-way ANOVA p=0.0033 (n=50)). Post-hoc multiple comparisons revealed that Rheb$_{RNAi}$ expression was significantly different to non-expressing controls at weeks 3 and 4. Interestingly when htau$^{0N3R}$ and Rheb$_{RNAi}$ were expressed together (Figure 4-8bi) there was no significant difference between expressing and non-expressing flies. To be able to compare between the three lines: htau$^{0N3R}$, Rheb$_{RNAi}$ and htau$^{0N3R}$; Rheb$_{RNAi}$ the height that the expressing flies climbed was divided by the average height climbed of their relevant, non-expressing controls. This controls for any potential differences in the genetic background of the different lines, allowing one to compare between the different lines. When comparing the three lines in this way (Figure 4-8bii) expression of Rheb$_{RNAi}$ significantly improved the climbing ability of htau$^{0N3R}$ flies, compared to the expression of htau$^{0N3R}$ alone (2-way ANOVA p=0.0001, n=50), post-hoc multiple comparisons revealed that htau$^{0N3R}$; Rheb$_{RNAi}$ flies were significantly different to htau$^{0N3R}$ at each time point tested. Longevity was conducted on both Rheb and Rheb$_{RNAi}$- expressing flies, with and without co-expression of htau$^{0N3R}$ (Appendix C, Figure 6-6). None of these manipulations caused a difference in longevity relative to non-expressing (-RU486) flies.

These data show that expression of Rheb$_{RNAi}$ appears to decrease tau-mediated defects in climbing. However, as Rheb$_{RNAi}$ expression induces a greater improvement in climbing, when expressed alone, the improvement seen when htau$^{0N3R}$ is also expressed may be due

to a general improvement of the health of the flies. This may not be a specific interaction of the dTORC1 pathway and htau$^{0N3R}$. In addition, Rheb knockdown also requires further investigation in these flies.

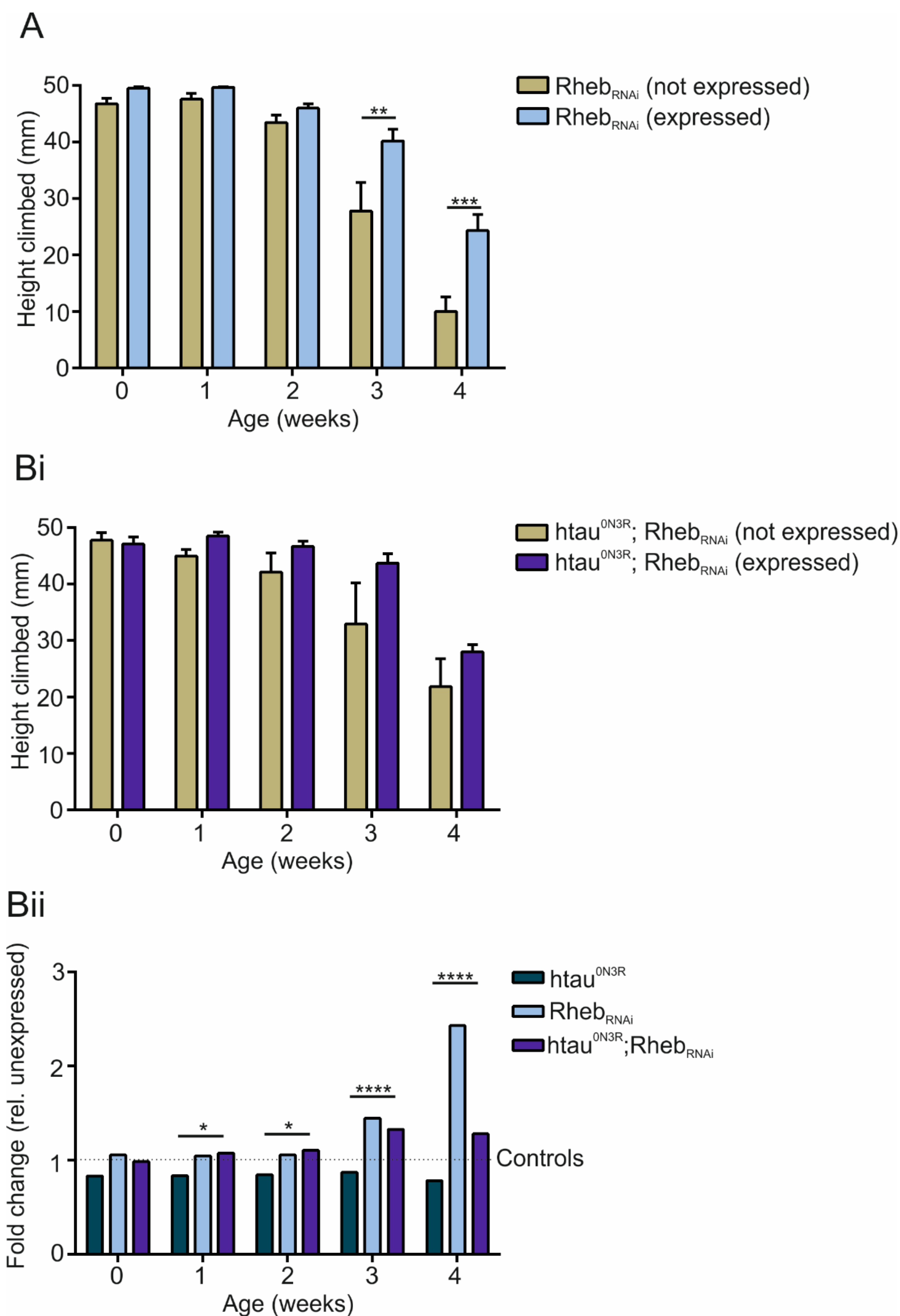



Figure 4-8. Expression of Rheb$_{RNAi}$ improves climbing when expressed alone and also rescues htau$^{0N3R}$ induced deficits in climbing when co-expressed with htau$^{0N3R}$.

(A) Expression of Rheb$_{RNAi}$ alone significantly improves climbing ability p= 0.0033 (n=50) of adult male flies when using Elav-GeneSwitch in adulthood compared to controls, Rheb$_{RNAi}$ not expressed (RU486 not present in food). Multiple comparisons reveal that expression of Rheb$_{RNAi}$ is significantly improved compared to controls at weeks 3 and 4. (Bi) Climbing ability of flies that express both htau$^{0N3R}$ and Rheb$_{RNAi}$. (Bii) Comparison of three lines, htau$^{0N3R}$, Rheb$_{RNAi}$ and htau$^{0N3R}$; Rheb$_{RNAi}$ relative to their relevant non-expressed controls. Co-expression of Rheb$_{RNAi}$ compared with expression of htau$^{0N3R}$ alone significantly improved climbing (2-way ANOVA p=0.0001).

### 4.3.7 Upregulation of autophagy through expression of Atg1 improved tau-mediated deficits in climbing ability

As downregulation of dTORC1 signalling was shown above to improve htau$^{0N3R}$-mediated defects in climbing, it was investigated whether this may be due to an up-regulation of autophagy. To do this Atg1 was co-expressed with htau$^{0N3R}$. Atg1 is the *Drosophila* homolog of the mammalian Ulk1 and is a key initiator of the autophagic clearance pathway, therefore expression of Atg1 should increase the level of autophagy. (Figure 4-9) shows that co-expression of Atg1 caused increased Atg8II compared with expression of htau$^{0N3R}$ alone. Atg8II is the *Drosophila* equivalent of LC3II, levels of which increase when there is increased numbers of autophagosomes, which may be correlated with enhanced autophagosome synthesis. However an increase in Atg8II levels may also be due to a block in ATG8II degradation (Rubinsztein et al., 2009). Therefore to understand fully the state of autophagic flux in these flies future experiments should include the use of electron and fluorescent microscopy to visualise autophagic vesicles.

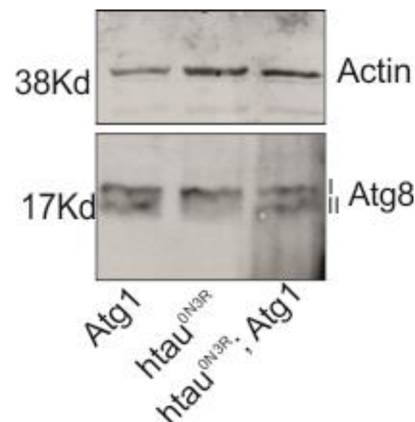



Figure 4-9. Atg8II staining is increased with expression of Atg1, showing activation of autophagy. 4 week old flies were used in this representative blot. Atg1 expressing flies were used as a positive control. Atg8II staining is negligible in htau$^{0N3R}$ expressing flies. Upon co-expression with Atg1 however there was increased Atg8II staining, indicative of increased autophagy in these flies.

(Figure 4-10a) shows that expression of Atg1 alone did not induce alterations to the climbing ability of flies compared to non-expressing control flies. However, (Figure 4-10b) shows that when htau$^{0N3R}$ and Atg1 were co-expressed the climbing ability of the flies was significantly improved compared to non-expressing controls (2-way ANOVA p=0.0481, n=50). Post-hoc multiple comparisons showed that htau$^{0N3R}$; Atg1 expressing flies were significantly different to controls at week 3. Comparing the three lines: htau$^{0N3R}$, Atg1 and htau$^{0N3R}$; Atg1 by dividing by their relevant controls (Figure 4-10bii) it was found that htau$^{0N3R}$; Atg1 flies were significantly different to htau$^{0N3R}$ flies at weeks 2, 3 and 4.

Longevity was conducted on both Atg1 and $Atg1_{RNAi}$ expressing flies, with and without co-expression of $htau^{0N3R}$ (Appendix C, Figure 6-7). Expression of Atg1 alone significantly extended lifespan (Mantel-Cox p=0.0334) (Figure 6-7). However co-expression of $htau^{0N3R}$ with Atg1 significantly reduced lifespan relative to non-expressing controls (p=0.0126) (Figure 6-7c). These data demonstrate that expression of Atg1 induces an improvement in climbing only when htau0N3R is expressed. However longevity Atg1 expression alone extends lifespan but reduces lifespan when co-expressed with htau0N3R. The longevity results are apparently contradictory to the climbing results, however this suggests that upregulation of autophagy has differential effects in earlier life (when climbing assays were conducted) relative to inducing autophagy later in life. This suggests that there is a direct interaction between an upregulation in autophagy and the htau0N3R phenotype. Therefore, the next step was to investigate this interaction.

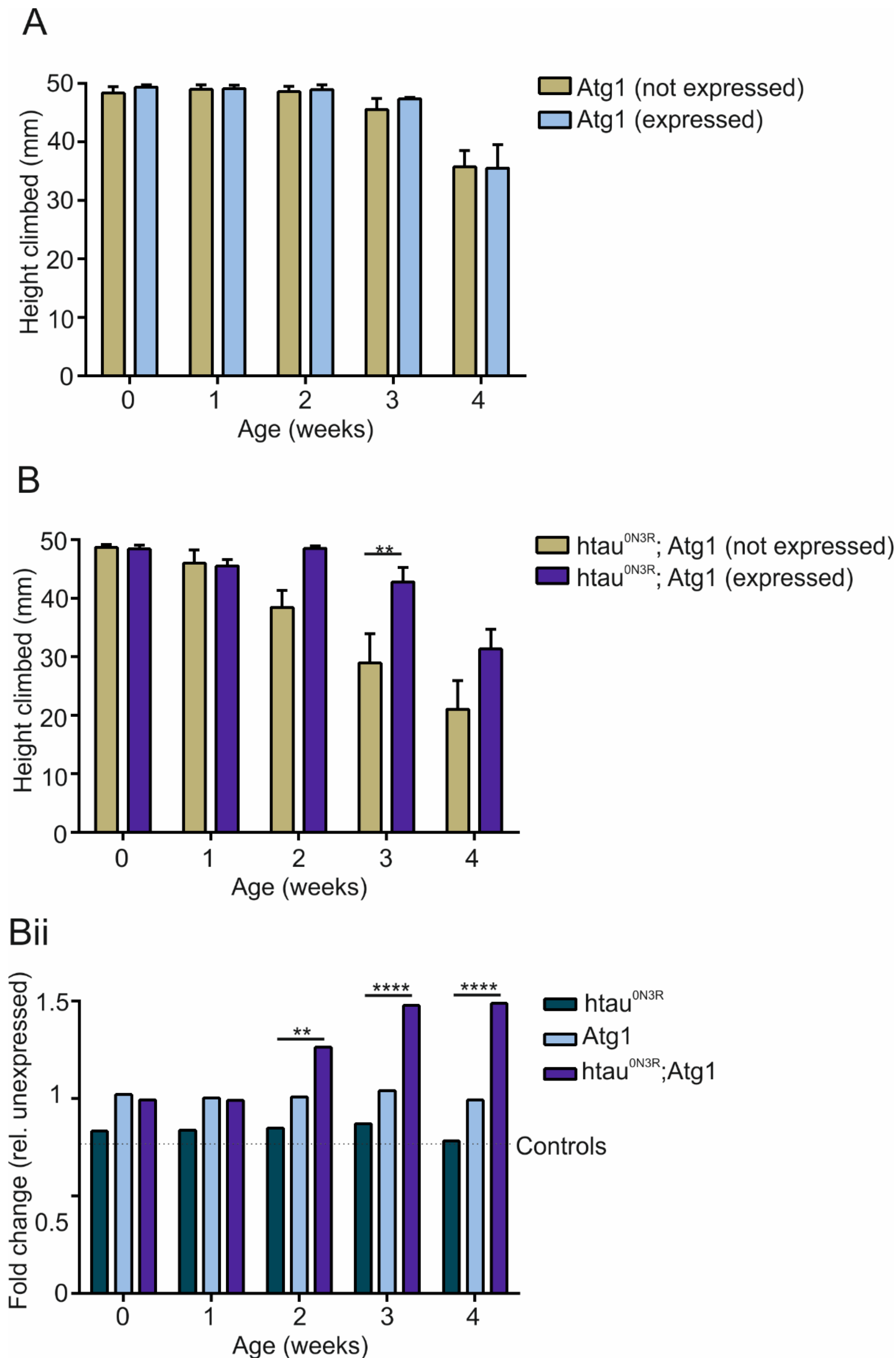



Figure 4-10. Co-expression of Atg1 with htau$^{0N3R}$ improves htau$^{0N3R}$ induced deficits in climbing ability.

(A) Expression of Atg1 alone using Elav-GeneSwitch driver does not alter climbing ability compared to unexpressed Atg1 controls (RU486 not present). (Bi) Co-expression of htau$^{0N3R}$ and Atg1 significantly improves climbing ability (2-way ANOVA p= 0.0481) compared with controls. (Bii) Comparison of the three lines: htau$^{0N3R}$, Atg1 and htau$^{0N3R}$; Atg1 relative to their relevant controls. Co-expression of Atg1 and htau$^{0N3R}$ compared with expression of htau$^{0N3R}$ alone significantly improved climbing ability (2-way ANOVA p=0.0001).

### 4.3.8 Upregulation of autophagy rescues htau$^{0N3R}$-mediated deficits in learning and memory

As co-expression of Atg1 with htau$^{0N3R}$ was demonstrated to improve htau$^{0N3R}$-mediated climbing deficits we also wanted to investigate whether this could additionally improve htau$^{0N3R}$- mediated learning and memory (shown in Chapter 2) As in Chapter 2, an APS assay was used to measure learning and short term memory (1-min post training) of adult flies. The MB GeneSwitch driver was used to express transgenes in the mushroom bodies. Expression was restricted to adulthood by the adding of RU486 to the food once the flies had eclosed. The short term memory of htau$^{0N3R}$ and htau$^{0N3R}$;Atg1 expressing flies were assessed relative to non-expressing controls (Figure 4-11). Htau$^{0N3R}$ has previously been shown in this thesis to cause deficits in learning and memory performance, these data are presented again here for comparison (Figure 4-11a). Expression of Atg1 alone did not cause a significant change in memory compared with controls (Figure 4-11b). Expression of htau$^{0N3R}$; Atg1 also did not alter memory compared with controls (Figure 4-11c). It was noted, however, that the htau$^{0N3R}$; Atg1 controls had a lower PI than htau$^{0N3R}$ and Atg1 controls. When comparing the three lines relative to controls, there was a clear trend for both Atg1 and htau$^{0N3R}$; Atg1 to have improved memory compared with htau$^{0N3R}$, although this was not found to be significant. These data imply that upregulation of autophagy through co-expression of Atg1 reduces htau$^{0N3R}$-mediated deficits in short term memory.

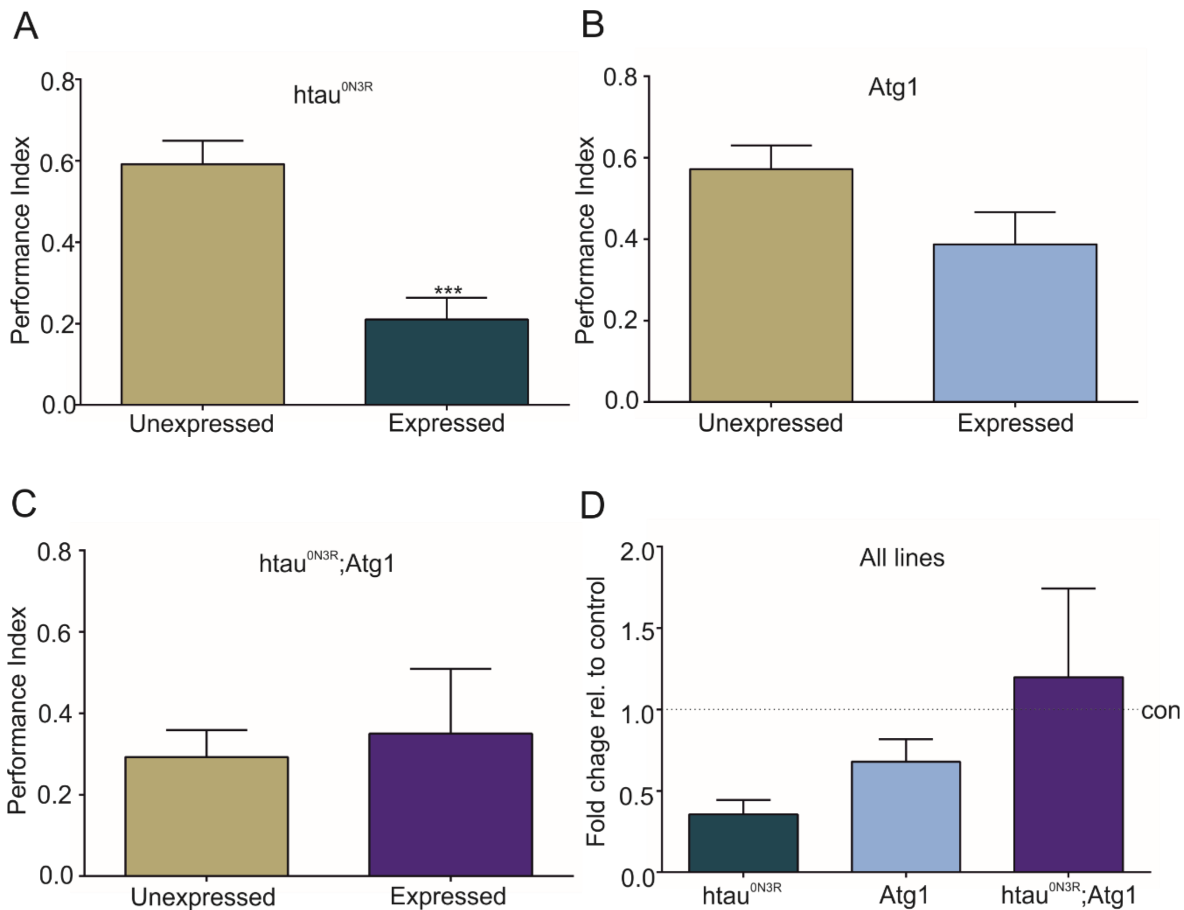



Figure 4-11. Co-expression of Atg1 with htau$^{0N3R}$ improves htau$^{0N3R}$-mediated deficits in learning and memory.
Performance index is the number of flies that are able to associate the quinine with the light chamber, relative to number of phototaxic flies. (A) htau$^{0N3R}$ expressing adult flies (n=105) (+ RU486) compared with non-expressing controls (- RU486) (n=105). (B) Atg1 expressing flies (n=90) compared with non-expressing controls (n=120). (C) Htau$^{0N3R}$; Atg1 expressing adult flies (n=60) compared with non-expressing controls (n=75). (D) Comparison of the 3 lines by dividing the PI of expressed flies by the average of the relative non-expressing controls. Dotted line at 1.0 represents that data has been divided by relative controls.

### 4.3.9 Up-regulation of autophagy reduces age-related accumulation of htau$^{0N3R}$

An up-regulation of autophagy through the co-expression of Atg1 was found to cause an improvement in the htau$^{0N3R}$ climbing phenotype. As up-regulation of autophagy has been shown previously to reduce tau levels in mice (Majumder et al., 2011), it was therefore assessed for whether this was what underpinned the improved climbing phenotype. In Chapter 2, it was shown that htau$^{0N3R}$ accumulates with age when expressed using the Elav-GAL4 driver. To investigate whether this was the case when using the Elav-GeneSwitch driver, total human tau levels were probed in 1, 2 and 4 weeks old htau$^{0N3R}$ flies. It was found that there was a significant increase in expression of htau$^{0N3R}$ from weeks 1-4, (Figure 4-12a and b), 1-way ANOVA p=0.0460, n=3. Next, it was investigated whether this could be attenuated by upregulation of autophagy. When htau$^{0N3R}$ and Atg1 were co-expressed there was no age-related accumulation of htau$^{0N3R}$ (Figure 4-9). However, it was

noted, that there was more htau$^{0N3R}$ expression at weeks 1 and 2 in the double transgenic flies compared to expression of htau$^{0N3R}$ alone. These data suggest that upregulation of the autophagic clearance pathway by over-expression of Atg1 results in reduced accumulation of htau$^{0N3R}$ with age. This correlates with the improvement seen in the htau$^{0N3R}$-mediated climbing phenotype.

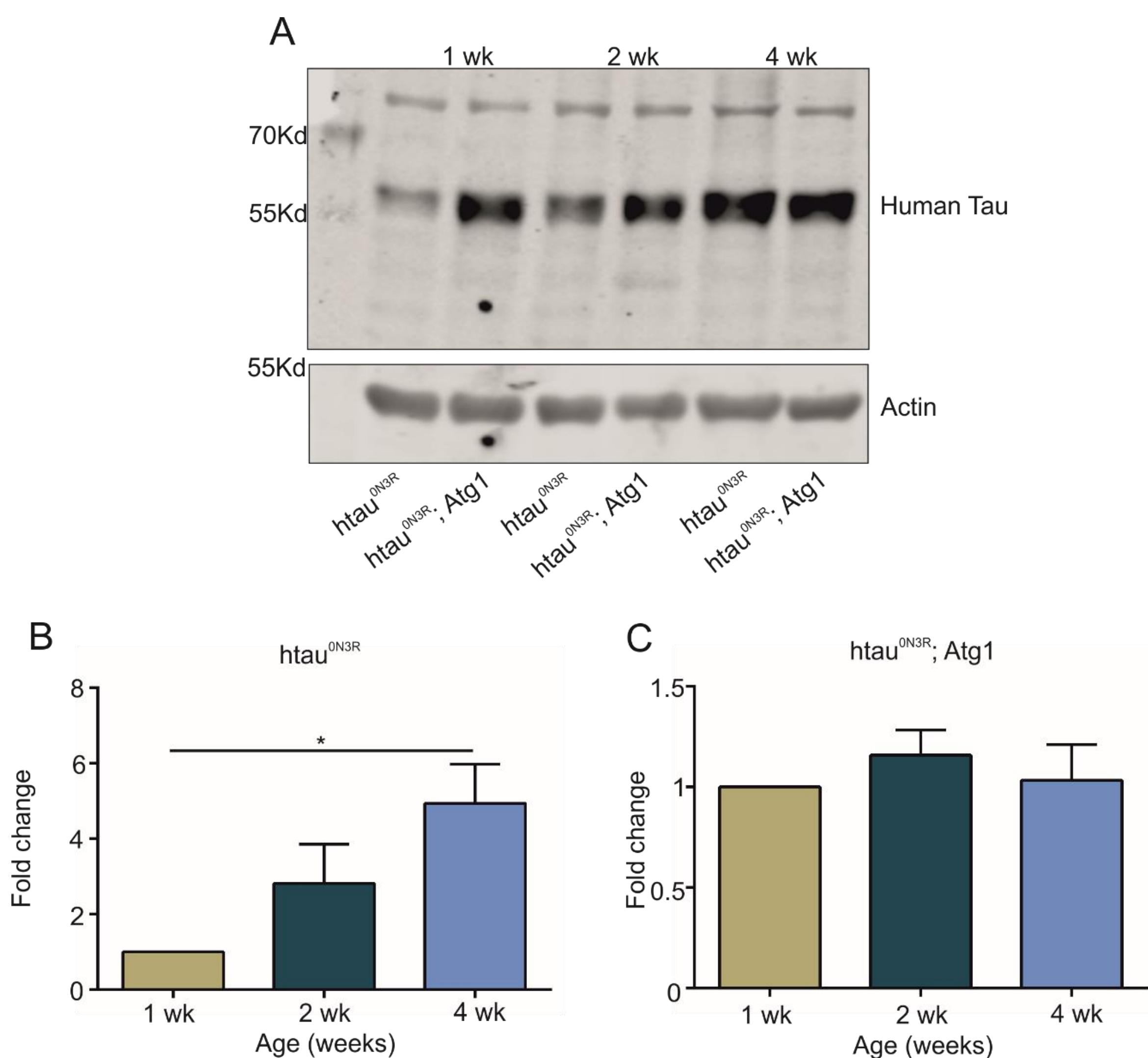



Figure 4-12. Co-expression of Atg1 and htau$^{0N3R}$ prevents age-related accumulation of htau$^{0N3R}$ protein.
(A) Representative blot of htau$^{0N3R}$ and htau$^{0N3R}$; Atg1 adult heads at weeks 1, 2 and 4 of age, probed for total human tau and actin as a loading control. (B) Quantification of htau$^{0N3R}$ expression in htau$^{0N3R}$ adult flies, 1-way ANOVA p=0.0460 (n=3), multiple comparisons revealed that expression is significantly different between the 1 and 4-week time point. (C) Quantification of htau$^{0N3R}$ expression in htau$^{0N3R}$; Atg1 adult fly heads, expression does not change with age.

### 4.3.10 Up-regulation of autophagy reduces age-related accumulation of phosphorylated $htau^{0N3R}$

As upregulation of Atg1 was shown to cause a decrease in tau accumulation, it was investigated next whether this reduced accumulation was due to a reduction in the amount of phosphorylated tau. In the resected human tissue, it was shown that increased autophagy correlated with a reduction in phosphorylated tau at Ser396/Ser404 (PHF-1) sites, therefore this same site of phosphorylation was examined in the double transgenic flies. It was found that there was a trend for an increase in phosphorylated $htau^{0N3R}$ with age in $htau^{0N3R}$-expressing flies (Figure 4-13). In the double transgenic $htau^{0N3R}$; Atg1 flies there was a reduced accumulation of phosphorylated $htau^{0N3R}$ with age. This demonstrates that upregulation of autophagy appears to clear the phosphorylated tau in the double transgenic flies.

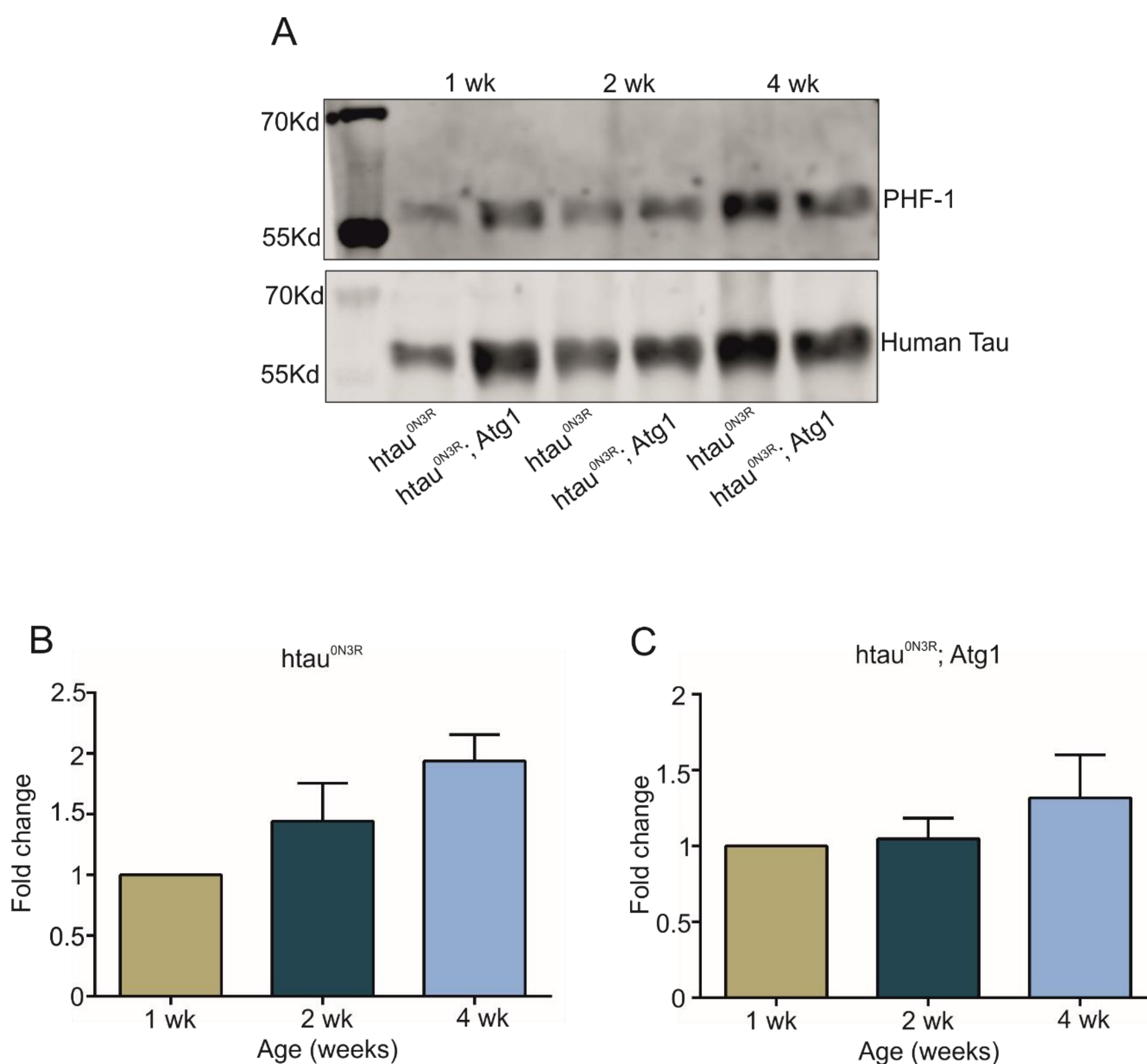



Figure 4-13. Co-expression of Atg1 and htau$^{0N3R}$ decreases age-related accumulation of PHF-1 tau (S396/S404) in htau$^{0N3R}$ fly heads.
(A) Representative blot of htau$^{0N3R}$ and htau$^{0N3R}$; Atg1 adult heads at weeks 1, 2 and 4 of age, probed for PHF-1 tau and total human tau. (B) Quantification of PHF-1 tau expression relative to total human tau levels in htau$^{0N3R}$ expressing flies, a trend for an increase of PHF-1 with age. (C) PHF-1 levels, relative to total tau, increases slightly with age in htau$^{0N3R}$; Atg1 expressing flies but not to the same extent as when htau$^{0N3R}$ is expressed alone.

## 4.4 Discussion

### 4.4.1 Summary

It has been demonstrated here, using resected cortical tissue from living patients, that mTORC1 signalling decreases with age in the human cortex. There was also a trend found for autophagy to increase and a decrease in tau protein with age. What cannot be determined by using this tissue is whether a decrease in mTORC1 directly causes the age-related decrease in both total and phospho tau protein. To understand this interaction further *Drosophila* models were used whereby both the mTORC1 and autophagy pathways were manipulated to recapitulate the decreased mTORC1 and increased autophagy found in the human cortex, with age. $Rheb_{RNAi}$ was used to attempt to knock down Rheb expression, a known activator of the dTORC1 pathway. Co-expression of $Rheb_{RNAi}$ resulted in an improvement in $htau^{0N3R}$ mediated deficits in climbing. It was not, however, clear whether this was due to a general improvement in health of the flies or a direct interaction with $htau^{0N3R}$. Alternatively, when autophagy was increased via co-expression of Atg1 there was no difference in climbing relative to controls. Only when $htau^{0N3R}$ was co-expressed with Atg1 that climbing deficits appeared to be improved. Indeed, biochemical assessment of the tau protein demonstrated that co-expression of Atg1 reduced the age-related accumulation of both total and phospho $htau^{0N3R}$.

### 4.4.2 mTORC1 signalling decreases with age in the human cortex

It was shown here, in human resected cortical tissue, that mTORC1 signalling decreases with age with a trend shown for autophagy to increase with age. How pathways such as mTORC1 and autophagy change with age is a neglected area of research, with few studies investigating this, particularly in the brain. Of the studies that have been conducted in mice, conflicting results have been found. For example, some studies have reported that mTORC1 signalling increases with age in mice (Rice et al., 2005, Sengupta et al., 2010, Leontieva et al., 2014), whereas others have found an age-related decrease in mTORC1 signalling (Linford et al., 2007, Houtkooper et al., 2011). The disparity found between these studies may be due to differences in the tissue used, age of mice, diet regime and gender. Therefore, one study attempted to reconcile these differences by investigating mTORC1 signalling in different tissues in the same mice in both male and females of the same genetic background, C57BL/6J. This study found that there are indeed differences in age-related changes in mTORC1 signalling depending on the tissue being assessed. mTORC1 signalling was found to decrease in both female and male liver tissue but to increase in both

male and female adipose tissue. Whereas in heart tissue mTORC1 signalling was reduced in only females and remained unchanged in males (Baar et al., 2016). These studies collectively demonstrate the complicated nature of this particular pathway and that mTORC1 plays different roles in different tissues. This indicates that further studies are required to understand how this pathway is altered with age in the brain.

There are few studies that have demonstrated how the mTORC1 pathway changes in the normal ageing brain. However, one study did report a decrease in mTORC1 signalling in the hippocampus of ageing mice (Yang et al., 2014), with the authors suggesting that this was due to a decrease in the PI3K/Akt pathway which lies upstream of mTORC1 (Figure 4-1). The decrease in mTORC1 signalling correlated with a decline in the cognitive ability of these mice (Yang et al., 2014). This highlights an important role of the mTORC1 signalling pathway in learning and memory, a process that is reliant on protein synthesis. This has been demonstrated in rats injected with rapamycin injected in the hippocampus, with this found to reduce mTORC1 signalling and consequently reduces the ability of the rats to retain memories long term (Bekinschtein et al., 2007). Corroborating these results, a separate group reported that rapamycin applied to cultured rat hippocampal neurons reduced late phase LTP. LTP is known to require new protein synthesis (Tang et al., 2002). However, a number of other studies have found that rapamycin treatment of mice improves tau and Aβ mediated learning and memory deficits (Caccamo et al., 2010, Majumder et al., 2011). These apparent conflicting results can be potentially explained by the fact that learning and memory requires the right balance of mTORC signalling. These studies highlight the importance of the mTORC1 signalling pathway and its impact on protein synthesis and learning and memory. These studies suggest that a reduction in mTORC1 signalling reduces protein synthesis that is vital for learning and memory. Therefore, it is possible that an age-related decrease in the mTORC1 signalling pathway underlies the decline in cognitive ability found in normal ageing people.

What causes this decrease in mTORC1 signalling is not currently known but various studies have suggested that the BDNF/PI3K/Akt axis also declines with age, and that this is a key regulator of the mTORC1 pathway (Takei et al., 2004). This axis has also been found to decrease with age, as demonstrated in mice (Yang et al., 2014). Oxidative stress has also been implicated in the age-related decline in the mTORC1 pathway. Hydrogen peroxide applied to PC12 cells and primary murine neurons resulted in decreased mTORC1 signalling, as measured by phosphorylation of S6K and 4E-BP (Chen et al., 2010). Similar results were found when hydrogen peroxide was applied to C6 glioma cells, also causing a decrease in mTORC1 signalling activity and activation of autophagy (Byun et

al., 2009). These data suggest that the age-related increase accumulation of ROS and subsequent oxidative damage could well lead to inhibition of the mTORC1 pathway. Future work should investigate in the human brain whether there is a correlation between reduced mTORC1 signalling and upstream mediators of mTORC1 signalling to address what potentially is causing this age-related reduction in mTORC1 signalling.

One possible implication of decreased mTORC1 signalling with age is that this should result in an increase in autophagy, as mTORC1 is a known inhibitor of autophagy. Here it was found that autophagy potentially increases with age in the human brain, shown by decreasing levels of p62, although this did not reach significance. More samples are needed to elucidate whether this is truly the case or whether in fact autophagy levels remain constant throughout normal ageing. Other readouts of the autophagy pathway could also be probed such as LC3II levels and elctron microscopy could be used to visualise autophagic vesicles. These experiments will help to further understand how autophagy changes with age. An age-related increase in autophagy is unexpected as others have found at the transcriptional level, autophagy is down-regulated with age. For example, studies have reported decreased levels of Atg5 and Atg7 gene expression (Lipinski et al., 2010) and decreased levels of Beclin-1 gene expression (Shibata et al., 2006). However, transcriptional down-regulation of autophagy does not necessarily equate to decreased autophagic levels. How lysosomal biology changes with age in the human brain has not been investigated and further studies are required to understand this. Electron and confocal microscopy could be used to visualise whether there is accumulation of autophagic vesicles with age in the brain. This will lead to a better understanding of how autophagy changes in the ageing brain.

### 4.4.3 Age-related decrease in tau

An age-related decrease in mTORC1 signalling and potential increase in autophagy could result in decreased tau protein expression due to decreased tau mRNA translation and increased clearance of tau. Indeed, we have shown here that both total and phospho tau do decrease with age in human cortex. Previous studies have shown that if mTORC1 signalling is down-regulated, this can lead to a reduction in the levels of tau protein. For example, in a *Drosophila* model of tauopathy, rapamycin treatment was found to reduce the tau-mediated rough eye phenotype (Berger et al., 2006). Additionally, in a mouse model that expresses the P301S mutation of tau, rapamycin treatment was shown to reduce NFT pathology, with a decrease seen in both AT8 and AT100 positive staining (Ozcelik et al., 2013, Caccamo et al., 2013). Alternatively, the dTORC1 pathway has been upregulated in *Drosophila* by co-expression of Rheb, which was shown to enhance tau-mediated

neurodegeneration, but this study did not report a reduction in tau levels, perhaps because only one time-point was assessed (Khurana et al., 2006). These studies demonstrate that inhibition of the mTORC1 pathway can reduce tau levels. It is currently unclear whether this is through decreased mRNA translation, increased autophagy or a combination of both.

It is possible that an age-related decrease in tau levels may be caused by an upregulation in autophagy. It has been demonstrated here that if autophagy is upregulated by genetic overexpression of Atg1, then the age-related accumulation of $htau^{0N3R}$ is reduced. Treatment of P301S mice with rapamycin showed that treatment correlated with increased autophagy and reduced levels of tau (Ozcelik et al., 2013, Caccamo et al., 2013). Others have shown that when autophagy was directly upregulated through treatment with trehalose, both total and phospho tau levels were reduced (Rodríguez-Navarro et al., 2010). Collectively, these studies demonstrate that an age-related decrease in mTORC1 signalling and increase in autophagy could well underlie the decreased levels of tau seen with age.

Others have also reported that there is a decrease in tau mRNA expression in human frontal cortex, with age, which is correlated with decreased tau protein with age, similar to what was found here (Lu et al., 2004). Other genes in this study that were observed to be down-regulated with age were grouped into three main cellular roles, including synaptic plasticity, vesicular transport and mitochondrial function. Additionally, they also found an increase in stress responses with age, including antioxidants and genes involved in DNA repair. This was suggested to be due to increased oxidative stress with age, with this leading to increased DNA damage with age that was impacting on particularly vulnerable genes. Indeed, this same study also found increased DNA damage in the promoters of genes reported to be down-regulated, including tau (Lu et al., 2004). Although Lu et al., (2004) did not investigate whether genes involved in the mTORC1 pathway were downregulated, it is possible that this is the case and should be investigated in future work. mTORC1 has been shown to regulate mitochondrial oxidative function; reducing mTORC1 using rapamycin was found to cause a significant decrease in mitochondrial gene expression and consequently mitochondrial respiration (Cunningham et al., 2007). This could imply that a decrease in mTORC1 activity could cause reductions in mitochondrial expression, increase the levels of oxidative damage and of DNA damage, reducing gene expression of vulnerable genes such as tau. This demonstrates an alternative mechanism of how reduced mTORC1 signalling with age may reduce tau levels in the ageing brain.

The implication of reduced tau with age also needs further consideration. Tau's main role is to stabilise microtubules. Therefore, a reduction in tau levels with age could well cause a breakdown of the cytoskeleton. We have demonstrated in our own laboratory that the expression of $htau^{0N3R}$, a highly phosphorylated form of htau with reduced microtubule binding leads to a breakdown of microtubules (Cowan et al., 2010). Little is currently known about how the cytoskeleton changes with age in the human brain during normal ageing, however one study did investigate microtubule density in biopsy control brains. The authors found that there was reduced microtubule density with age (Cash et al., 2003). This would be an important line of investigation for the future, to further understand the implications of decreased tau levels with age.

### 4.4.4 Is mTORC1 signalling dysregulated in AD?

Multiple studies have reported that the mTORC1 pathway is upregulated in the brains of people with AD. For example, increased pS6K was found to correlate with Braak staging and that pS6K co-localises with AT8 positive stained tangles and dystrophic neurites in AD brains (An et al., 2003). Similarly, levels of other downstream components of the mTORC1 pathway such as p4E-BP and peEF2K have been found to be upregulated in AD and that this again correlated with increased levels of phospho tau and total tau levels (Li et al., 2005). It is clear, therefore, that there is a disparity between how the mTORC1 signalling pathway is regulated during ageing and AD. This disparity may underlie why it is that AD is not simply an extension of normal ageing.

Although much of the work surrounding mTORC1 signalling and AD has investigated the impact that manipulation of mTORC1 has on tau and Aβ, there is also evidence to suggest that tau and Aβ can upregulate the mTORC1 pathway. This was demonstrated in a *Drosophila* model that overexpressed human tau with the R406W mutation, which induced increased phosphorylation of S6K (Khurana et al, 2006). It has also been shown in a 3xTg mouse model that mTORC1 signalling is upregulated (Caccammo et al., 2010). How this occurs is currently unknown although it is possible that the expression of Aβ increases the PI3K/Akt pathway in cells (Bhaskar et al., 2009). Although it is not clear that this is how tau upregulates the mTORC1 pathway, this would be important to investigate in future work.

### 4.4.5 Increased autophagy leads to reduced tau-mediated phenotypes and pathology

It has been demonstrated here that upregulation of autophagy, through the expression of Atg1, results in improved tau mediated-climbing, and learning and memory phenotypes. This correlates with a decreased accumulation of both total and phospho tau, in line with a number of previous studies in which rapamycin was used to treat 3xTg mice, with increased autophagy found to correlate with decreased Aβ and tau pathology (Majumder et al., 2011). Interestingly, we have found here an increase in total tau in younger flies (1 and 2 weeks old), with a subsequent decrease in tau levels at 4 weeks. It would be interesting to see if this decrease continued at older ages. Many other studies have not investigated how upregulation of autophagy alters tau levels at different ages, instead just testing at a single time-point (Caccammo et al., 2010, Majumder et al., 2011). Why autophagic clearance would lead to greater levels of tau in the early weeks is not clear, but it is possible that autophagic clearance at early ages increases the levels of amino acids available for protein synthesis. It has been shown here that dTORC1 signalling is increased at younger ages, suggesting greater levels of protein translation occur in younger flies. This, together with increased availability of amino acids from the induction of autophagy in Atg1 expressing flies, may lead to more tau being synthesised in younger ages. At older ages, dTORC1 signalling is decreased meaning that less mRNA translation is occurring, but autophagy is still increased, producing a reduced level of tau at older ages. The increased level of htau$^{0N3R}$ at early ages did not correlate with worsened phenotypes in the flies. We have demonstrated a similar phenomenon before where increased levels of tau correlated with an improvement in phenotype. When htau$^{0N3R}$ flies were treated with lithium, an improvement in larval phenotypes was observed, however an increase in the levels of tau was also seen. This was shown to be caused by an accumulation of non-toxic, non-phosphorylated tau oligomers (Cowan et al., 2015). This demonstrates the importance of the species of tau involved, such as whether this tau is phosphorylated, misfolded or aggregated. Therefore, it is important to further analyse the specific species of htau$^{0N3R}$ being expressed in the flies used here. These data also demonstrate the complex nature of the interplay between autophagic recycling and protein translation that occurs in the cell and how this balance is disrupted with age.

As expression of Atg1 has been reported here to improve htau$^{0N3R}$ phenotypes, it would appear that autophagic flux is either down regulated or dysfunctional in htau$^{0N3R}$ flies. It is possible that the htau$^{0N3R}$ is inhibiting the autophagic pathway in some way. This has been shown previously, where the expression of tau in the *Drosophila* eye was shown to cause the

formation of large autophagic intermediates, which were not seen in control flies (Bakhoum et al., 2014). The authors suggested that this was a tau-induced gridlock of autophagic flux whereby tau was blocking the maturation stage of autophagy. However, the study was conducted in larvae and young adults and so does not indicate the contribution of ageing to this phenotype, which should be investigated in future studies. We reported here that in 4 weeks old htau$^{0N3R}$ flies, there is decreased autophagic flux, exemplified by low Atg8II staining. It could be hypothesised that upregulating autophagy worsens the phenotypes due to increased tau-induced gridlock. However, this does not appear to be the case, with a previous study reporting that rapamycin improved the tau-mediated rough eye phenotype (Bakhoum et al., 2014), and in being shown here, where upregulation of autophagy improved the tau-mediated climbing and memory phenotypes. Therefore, it is possible that tau-induced gridlock of autophagic flux is not toxic and could be a compensatory mechanism to attempt to sequester the pathological tau (Bakhoum et al., 2014). It is clear though, from work presented here and from others (Caccammo et al., 2010, Majumder et al., 2011, Bakhoum et al., 2014), that autophagy is an intriguing target for drugs to prevent or delay the progression of AD.

### 4.4.6 Using human resected brain tissue to study ageing

This work, in addition to demonstrating how mTORC1 signalling and autophagy impact on ageing and tauopathy, has also exemplified the advantages of using resected human tissue. Using this type of tissue has particular advantages over using *post-mortem* tissue, which is more traditionally used for studies of this type. Phosphorylation has been shown to be rapidly reduced upon *post-mortem* delay (Matsuo et al., 1994), for example, in biopsy-derived brain tissue where different phosphorylation epitopes of tau were probed in tissue that was kept at room temperature for different time intervals. It was reported that by 4 hours, almost all phosphorylation was lost from the tau protein (Matsuo et al., 1994). This decrease in phosphorylation could well apply to other proteins as well and so, when assessing phosphorylation of components of the mTORC1 pathway as an indicator of the pathway's activity, resected tissue from living patients is favoured over that from *post-mortem*-brains. However, due to the increased accessibility of brain banks, the amount of available *post-mortem* samples greatly outweighs those that have come from resected tissue or biopsies. A limitation of the work presented here is the relatively low n numbers used to investigate changes with age.

Probing for tau in the resected cortical tissue showed, surprisingly, that young patients had high levels of tau phosphorylated at Ser396/Ser404 (PHF-1). This site is frequently used to

stain for tau in AD brain as it does not react with tau in control brains from *post-mortem* tissue (Bramblett et al., 1993). This demonstrates a disparity in tau phosphorylation in tissue derived from biopsy or resected tissue and that from *post-mortem* material. Matsuo et al. (1994) also found PHF-1 positive tau in biopsy derived brain, along with a number of other traditional AD associated epitopes (Matsuo et al., 1994). In a separate study the Ser262 site was also shown to be phosphorylated in control biopsy brain (Seubert et al., 1995). Various other sites in addition to PHF-1 were tested using the samples presented in this chapter, but very little signal detected using other tau phospho-specific antibodies. This included probing for Ser262 (data not shown). It is possible that this was because these phosphorylated epitopes are present at levels too low to detect. Therefore, if these samples were enriched for tau it may be possible to detect other phospho epitopes. Due to the low level of tissue in the samples used here, this was not possible but would be important to investigate in the future. The data presented here suggest a possible explanation for why tau phosphorylation is increased in AD brains compared with age-matched controls, because tau phosphorylation decreases with age in control brains. An alternative reason suggested for this is due to down-regulation of relevant protein phosphatases in AD brains. In control brains during *post-mortem*, phosphatases rapidly dephosphorylate tau whereas these phosphatases are down-regulated in AD brain tissue (Matsuo et al., 1994). This means that less tau is dephosphorylated during *post-mortem* delays in AD brain compared with control brains. These studies, together with ours, demonstrate the importance of using rapidly processed resected or biopsy brain tissue to accurately determine the biochemical properties of proteins. At a minimum, it is very important to avoid the use of *post-mortem* tissue with long *post-mortem* delays for studies such as this.

### 4.4.7 Conclusions and future directions

It has been discussed here how a decrease in mTORC1 signalling and increase in autophagy with age could underlie the age-related changes in cognitive ability seen in healthy ageing. This down-regulation of the mTORC1 pathway may be driven by increased oxidative stress with age. This study has also shown that there is a disparity in the changes in these pathways during ageing and AD, as the mTORC1 signalling pathway has been shown to have increased activity in AD brains. This study implies that AD is not an inevitable extension of normal ageing and disease-associated mechanisms must cause a switch from a healthy ageing decline in mTORC1 signalling to a disease-related increase. What causes this switch is not clear and warrants further investigation. It is possible that the presence of abnormal proteins such as Aβ and tau could alter the healthy decline in

mTORC signalling. Additionally, it may well be that pathways upstream of mTORC1, such as insulin signalling and oxidative stress, are important in causing this dysregulation in AD. This work has also corroborated what others have found, that increasing autophagic flux can improve tau-mediated phenotypes and this is correlated with decreased levels of tau. It has been shown consistently that upregulation of autophagy can improve AD-associated phenotypes and pathologies. Therefore, autophagy is an exciting area of research for possible novel drug targets for AD and other neurodegenerative diseases.

# Chapter 5: Discussion

The ever growing ageing population has led to a global rise in age-related diseases such as AD. As there is currently no disease modifying treatment for AD there is an increasing need to understand the underlying mechanisms of the disease in order to find new therapeutic interventions. Therefore, investigating how ageing contributes to the mechanisms that underlie AD has the potential to fuel this search for new drugs. The data described in this thesis has contributed to this investigation by exploring cellular pathways that are implicated in ageing and how they may impact on tau pathology. Specifically, pathways involved in mTORC1 signalling and autophagy were investigated for how they change with age and how they impact on tau pathology. This has been investigated here by utilising *Drosophila* models of tauopathy and also human cortical tissue.

## 5.1 Investigating how tau-mediated phenotypes and pathologies evolve with age: identification of isoform specific differences

To be able to understand how pathways such as mTORC1 and autophagy impact on tauopathy it was important to, firstly, explore how tau-mediated phenotypes and pathologies change with age. To do this, *Drosophila* expressing 3R and 4R isoforms of human tau were assessed for age-related changes in longevity and climbing. It was found that expression of $htau^{0N3R}$ caused severe deficits in lifespan and age-related decline in climbing ability. This was correlated with an age-related accumulation in soluble tau protein and age-related changes in phosphorylation. Interestingly, it was found that expression of $htau^{0N3R}$ caused more severe age-related behavioural phenotypes compared with $htau^{0N4R}$-expressing flies. Biochemical analysis of the tau protein demonstrated that both tau isoforms accumulated with age, suggestive of protein turnover becoming dysregulated with age.

We also found that $htau^{0N3R}$ and $htau^{0N4R}$ had different age-related phosphorylation profiles. $htau^{0N3R}$ flies had increased phosphorylation at Ser262 with age. This site has been identified as being key in tau-mediated toxicity (Nishamura et al., 2004) and for binding tau to microtubules (Fischer et al., 2009). Tau phosphorylated at Ser262 has also been found to stabilise de-phosphorylated tau unbound to microtubules (Ando et al., 2016). This may explain why there was decreased phosphorylation, with age, at the Thr212/Ser214 site. Interestingly, these age-related changes in phosphorylation were not found in the $htau^{0N4R}$ flies which did not exhibit as severe age-related phenotypes. These data implicate Ser262 in

mediating tau toxicity and demonstrates the significance of certain phosphorylation sites in isoform-specific tau toxicity. Differences such as these could explain why different tau isoforms are implicated in different tauopathies.

Further investigation in to the different roles and toxicities of the different isoforms of tau will lead to a better understanding of these different tauopathies. For example, it would be interesting to assess the localisation of different isoforms of tau. Tau has been demonstrated to mis-localise to the somatodendritic compartment when phosphorylated (Götz et al., 1995), but it is unclear whether some isoforms of tau are more prone to this mis-localisation than others. In addition, it would be important to investigate the ability of different isoforms to bind to and stabilise microtubules as this was not assessed here. Also it would be interesting to investigate the other isoforms that have the addition of the 1N or 2N repeats as this study has only investigated the 0N isoforms. It has been demonstrated here that *Drosophila* are a useful model for studying tau-mediated phenotypes and pathologies. However, future studies should take into consideration the genetic background of the flies and tau protein expression levels. New lines can be generated that have different UAS-htau isoforms inserted in to the *Drosophila* genome at specific sites. Previously transgenes have been inserted randomly in the *Drosophila* genome whereby different insertions can have dramatic effects on the same transgene. For example, the phiC31 integrase has been used to generate landing sites that are optimised to express transgenes in the nervous system (Knapp et al., 2015). This is potentially a very useful genetic tool for studying tauopathy in *Drosophila* neurons that could be used in future experiments.

## 5.2 What is the species of tau that underlies age-related tau phenotypes?

Investigation of the age-related changes in tau protein, reported here, also provided insight into what the species of tau is that causes toxicity. The data shown in this thesis demonstrated that the tau-mediated phenotypes in the different *Drosophila* models were due to small, soluble species of tau, as very little insoluble tau was found, even in aged flies. What the toxic species of tau are has been the cause of much debate in the AD field. This is because there is evidence for both large aggregates, such as NFTs and PHFs, and also for smaller oligomeric species being responsible for neuronal dysfunction and degeneration, as reviewed in (Cowan & Mudher, 2013). Other studies in *Drosophila* corroborate these findings. Tau-mediated dysfunction and neurodegeneration has been shown to occur in a number of different *Drosophila* models without the appearance of PHFs and NFTs

(Wittmann et al., 2001, Mudher et al., 2004, Mershin et al., 2004, Kosmidis et al., 2010). This is not to say that PHFs and NFTs do not also have roles in inducing tau-mediated dysfunction and degeneration, it is just clear that they are not required for the toxic effects of tau. To understand the relative contributions of different species of tau will be challenging as, both, in humans and animal models a range of different species of tau are present together at the same time. Separating out these different species and testing their relevant functions and toxicities in neurons will be vital to understanding which tau species are important to target for therapeutic intervention.

## 5.3 How does tau affect age-related changes in circadian rhythms?

In addition to studying tau-mediated changes in phenotypes such as longevity and locomotion, circadian rhythms were also assessed in ageing htau$^{0N3R}$ flies. As the *Drosophila* and mammalian molecular clocks that drive circadian rhythms are highly conserved it is possible to use *Drosophila* to model age-related changes in circadian rhythms. Others have found that disruption of the normal oscillation of the molecular clock causes exacerbation of tau-mediated phenotypes (Means et al., 2015), but until now the effect that tau has on circadian rhythms has not been assessed in flies. We demonstrated here that htau$^{0N3R}$ expression causes period lengthening and slowing of the circadian clock. This effect was exacerbated with age in male flies. It was also shown that htau$^{0N3R}$ expressing flies exhibited more nocturnal activity than non-expressing controls. Using the TARGET system, it was possible to restrict expression of htau$^{0N3R}$ to adulthood. We found that 1 week of adult expression was sufficient to cause an increase in period length in male flies. Therefore, this suggests that this is mediated by a soluble non-aggregated tau species. This effect potentially could be due to phosphorylated tau disrupting the axonal transport of PDF. These results are particularly relevant to AD since patients exhibit disruptions in circadian rhythms early on in disease progression (Hahn et al., 2014). Although changes in daily sleep/wake cycles is an early symptom in disease, it is often overlooked due to the debilitating nature of the other symptoms of AD, such as loss of memory. Unravelling the mechanisms that underlie this may provide a biomarker target for early diagnosis of the disease. An important line of enquiry is how Aβ and tau potentially cause dysfunction and degeneration to neurons that control the circadian clock in the brain. These studies provide evidence that expression of these two proteins can disrupt circadian rhythms.

Future work will include investigating the mechanisms of how tau can cause disruptions to clock neurons. For example, it will be important to understand whether tau-mediated dysfunction of Pdf neurons is due to microtubule-destabilisation. *Drosophila* mutants of

other microtubule stabilising proteins can be used to investigate this. If mutations in other microtubule-stabilising proteins, for example, showed a similar period lengthening phenotype it may suggest that microtubule destabilisation does indeed underlie this effect of htau$^{0N3R}$. In addition, it would be important to further characterise changes to Pdf neurons with age. Using confocal microscopy, it would be possible to image how expression of htau$^{0N3R}$ potentially causes degeneration to Pdf neurons with age. These studies will lead to a fuller understanding of how tau can impact on circadian biology and why circadian rhythms become dysregulated in AD.

## 5.4 How do pathways that affect protein turnover change with age?

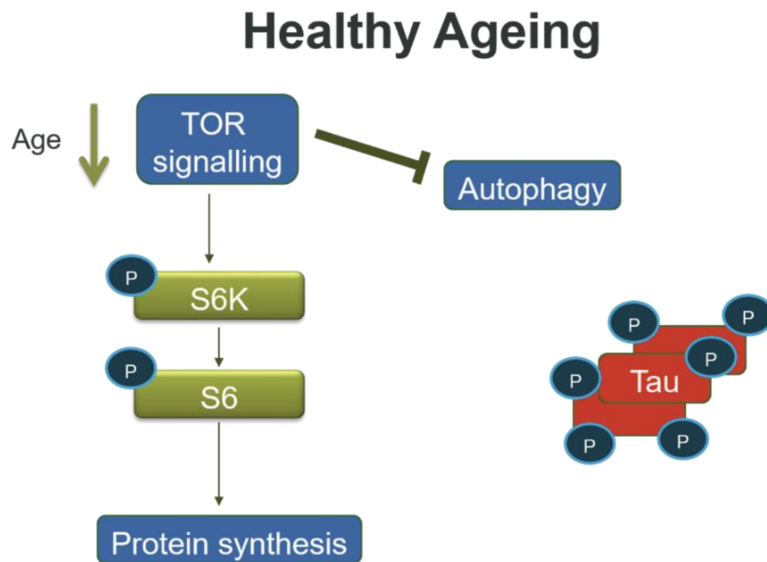
Age-related deficits in *Drosophila* behaviour were also correlated with accumulation of tau protein with age, demonstrated both in Chapters 2 and 4. This suggests that tau turnover becomes dysregulated with age. Protein turnover is regulated by different pathways that control mRNA translation and protein clearance. Specifically, the mTORC1 signalling and autophagy pathways were probed here. To be able to understand whether these pathways contribute to age-related dysregulation of tau turnover it was important to, firstly, understand how these pathways change in normal ageing. To do this, human cortical tissue that was resected from differently aged living patients was used. We found that mTORC1 signalling appeared to decrease and autophagic clearance remained constant although there was a trend for an increase in autophagic flux. This was correlated with an age-related decrease in total and phospho-tau. Therefore, it is possible that an age-related down-regulation of the mTORC1 pathway is a consequence of normal ageing. The fact that neurons are extraordinarily long lived, relative to other cell types in the body, means that they require mechanisms that conserve energy and survival. Decreased mTORC1 signalling with age leads to decreased mRNA translation and protein synthesis and increased autophagic clearance of damaged proteins from the cell. Autophagy was not found here to significantly change with age in the human brain, this suggests that down regulation of mTORC1 signalling effects tau via decreasing mRNA levels rather than through increased clearance via autophagy. It would be important to examine tau mRNA levels using qPCR in the human brain samples to further demonstrate this. Others have shown that inhibition of the TORC1 pathway is associated with extension in lifespan (Kapahi et al., 2004, Hansen et al., 2007, Selman et al., 2009). Is it possible then that human neurons require the mTORC1 pathway to be downregulated with age to be able to survive for many decades?

Alternatively, it is also possible that mTORC1 signalling is downregulated by age-related increases in ROS accumulation. ROS has been reported to reduce the activity of mTORC1

signalling in cells (Chen et al., 2010, Byun et al., 2009). Therefore, it will be important to understand how regulators of the mTORC1 pathway, such as oxidative stress, also change with age in normal ageing, to provide a broader understanding of how the brain ages. This is an ongoing project and the enrolment of more patients to this study will provide a clearer indication of how pathways such as mTORC1 and autophagy change during ageing. Future work, using this tissue, could potentially assess other age-related pathways, such as oxidative stress, insulin signalling and the UPS. In addition, although the tissue used here was classified as "non-pathological" this tissue was resected from patients with a number of different inflictions. Therefore, it will be important to compare tissue between disease types to ensure that any changes seen here are not due in part to the underlying diseases in these patients. It would also be interesting to assess this tissue relative to age matched *post-mortem* tissue to further characterise the differences between using fresh tissue compared with tissue that has been exposed to variable *post-mortem* delays.

## 5.5 Is the mTORC1 signalling pathway dysregulated in AD?

Although mTORC1 signalling has been suggested here to be reduced in normal ageing it has been documented in a number of studies that the mTORC1 pathway has increased activity in AD (Li et al., 2015, An et al., 2003, An et al., 2004). This suggests that mTORC1 signalling becomes dysregulated in the AD brain, resulting in activation of the mTORC1 pathway. Increased activation of mTORC1 signalling could lead to increased tau translation and reduced autophagic clearance, allowing tau to accumulate (Figure 5-1) and form the tau pathology seen in AD. This could occur for a number of different reasons as the mTORC1 pathway is regulated by a wide range of factors. Some of these factors have also been implicated in the pathogenesis of AD such as insulin signalling and oxidative stress. It is also possible that increased levels of tau and Aβ can induce increased mTORC1 activity. This has been shown both in flies (Khurana et al., 2006) and in 3xTg mice (Caccammo et al., 2010). How and why tau and Aβ increase mTORC1 activity is currently unknown, however it has been found that Aβ oligomers can activate the PI3K-Akt signalling transduction pathway which lies upstream of mTORC1 signalling (Bhaskar et al., 2009). Tau's effects on the PI3K-Akt pathway will be an important line of investigation for the future to understand whether tau can also affect mTORC1 signalling in this way.



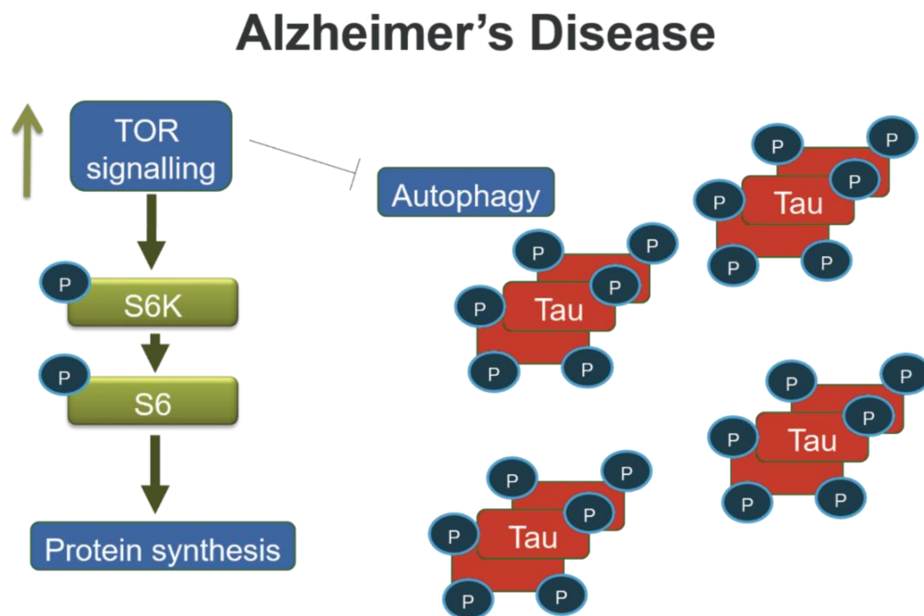




Figure 5-1. Schematic of differential mTORC1 signalling in healthy ageing and AD.
In healthy ageing mTORC1 signalling pathway is downregulated to maintain low levels of protein synthesis and high autophagic clearance, resulting in low levels of tau protein. In AD increased mTORC1 signalling increases protein synthesis and inhibits autophagic clearance, resulting in accumulation of tau pathology.

If increased mTORC1 signalling and decreased autophagy do cause tau pathologies to accumulate in AD, it should be possible to reduce these pathologies by inhibiting the mTORC1 pathway and upregulating autophagy. This was attempted here by using transgenic *Drosophila* that expressed $Rheb_{RNAi}$, however it could not be fully elucidated that Rheb was knocked down in these flies and this will need to be fully investigated in future experiments. However, co-expression of $Rheb_{RNAi}$ did appear to reduce tau-mediated climbing deficits. Other mutants of the dTORC1 pathway could similarly be used such as S6K mutants as used in (Bjedov et al., 2010). In addition, $htau^{0N3R}$ deficits were characterised here in both circadian rhythm and memory assays. These assays can now be used to test these mTORC1 mutants.

## 5.6 How is autophagy involved in the pathogenesis of tauopathies?

Here it was demonstrated that if autophagy initiation is up-regulated by expression of Atg1 in *Drosophila* then $htau^{0N3R}$-mediated deficits in locomotion and memory phenotypes were improved. Expression of Atg1 also caused reduced total and phospho tau accumulation with age. This corroborates what others have found, both in mice (Caccammo et al., 2010, Majumder et al., 2011) and in flies (Bakhoum et al., 2014) that up-regulation of autophagy alleviates tau pathology and improves tau-mediated phenotypes. However, what remains unclear is how autophagy is implicated in the pathogenesis of tauopathies. Others have suggested that autophagy is down-regulated in AD (Pickford et al., 2008). Conversely others have demonstrated that the prescence of tau may trigger autophagy but the process is dysfunctional, unable to clear aggregates of tau. For example, in the human brain markers of autophagy have been found to co-localise with the NFTs found in AD brains (Ma et al., 2010). Bakhoum et al., found that the expression of human tau in *Drosophila* induced the accumulation of large autophagic bodies containing partially digested tau fragments (Bakhoum et al., 2014). This suggests that autophagy becomes dysfunctional in tauopathies. Future work is required here to demonstrate the state of autophagic flux in this model presented here. Bakhoum et al., only demonstrated the prescence of autophagic bodies during a stage in development and not in the adult ageing fly and therefore this would be important to explore. There is some suggestion from the analysis of Atg8II levels in the flies used here that autophagic flux is inhibited, however, this needs to be corroborated using a number of additional readouts of the autophagy pathway. These should include investigating Ref(2)B levels (homolog of p62) and the use of confocal and electron microscopy to visualise potential autophagic bodies.

These studies collectively demonstrate autophagy as a potential therapeutic target for the treatment of tauopathies. This has generated a lot of interest in finding drugs that target autophagy, see review (Levine et al., 2015). Some of these drugs, such as lithium and Metformin, are already FDA approved for other diseases, such as Bipolar Disorder and Diabetes respectively. However, translating these drugs to clinical research in neurodegenerative diseases will be particularly challenging as assessment of autophagic clearance cannot be performed in living people without neurosurgery. A biomarker is therefore required that can measure autophagic flux for it to be possible to assess the efficacy of potential drugs in patients (Levine et al., 2015). However, there is evidence to suggest that autophagic activity can be increased by non-pharmacological interventions. Exercise, for example has been shown to stimulate autophagy in both skeletal muscle (Grumati et al., 2011) and in the cerebral cortex of mice (He et al., 2012). In addition, autophagy via dietary restriction is required to extend lifespan in a number of different species including *C. elegans* (Morselli et al., 2010). These studies imply that diet and exercise can prevent the formation of disease-related pathologies in humans. Autophagy, therefore, remains an enticing target for therapeutic intervention and future research into this could benefit a wide range of diseases that involve the accumulation of toxic aggregated proteins.

## 5.7 Future directions in age-related research in relation to AD

Studying the pathways that underlie ageing and how these pathways interact with age-related disease is highly complex. This is due to there being many inter-connected pathways that regulate both ageing and AD. However, investigating how these pathways converge on both ageing and AD have led to a clearer understanding of why age increases the risk of AD. Here we suggest that the normal age-related reduction in activity of mTORC1 signalling and increased autophagy is dysregulated in AD. This implies that dysregulation of these pathways increases the risk of AD. Further investigation of the pathways that regulate mTORC1 and autophagy will be vital in the discovery of therapies that prevent and treat age-related diseases such as AD.

# Appendices

# Chapter 6: Appendices

## Appendix A Supporting data for Chapter 2

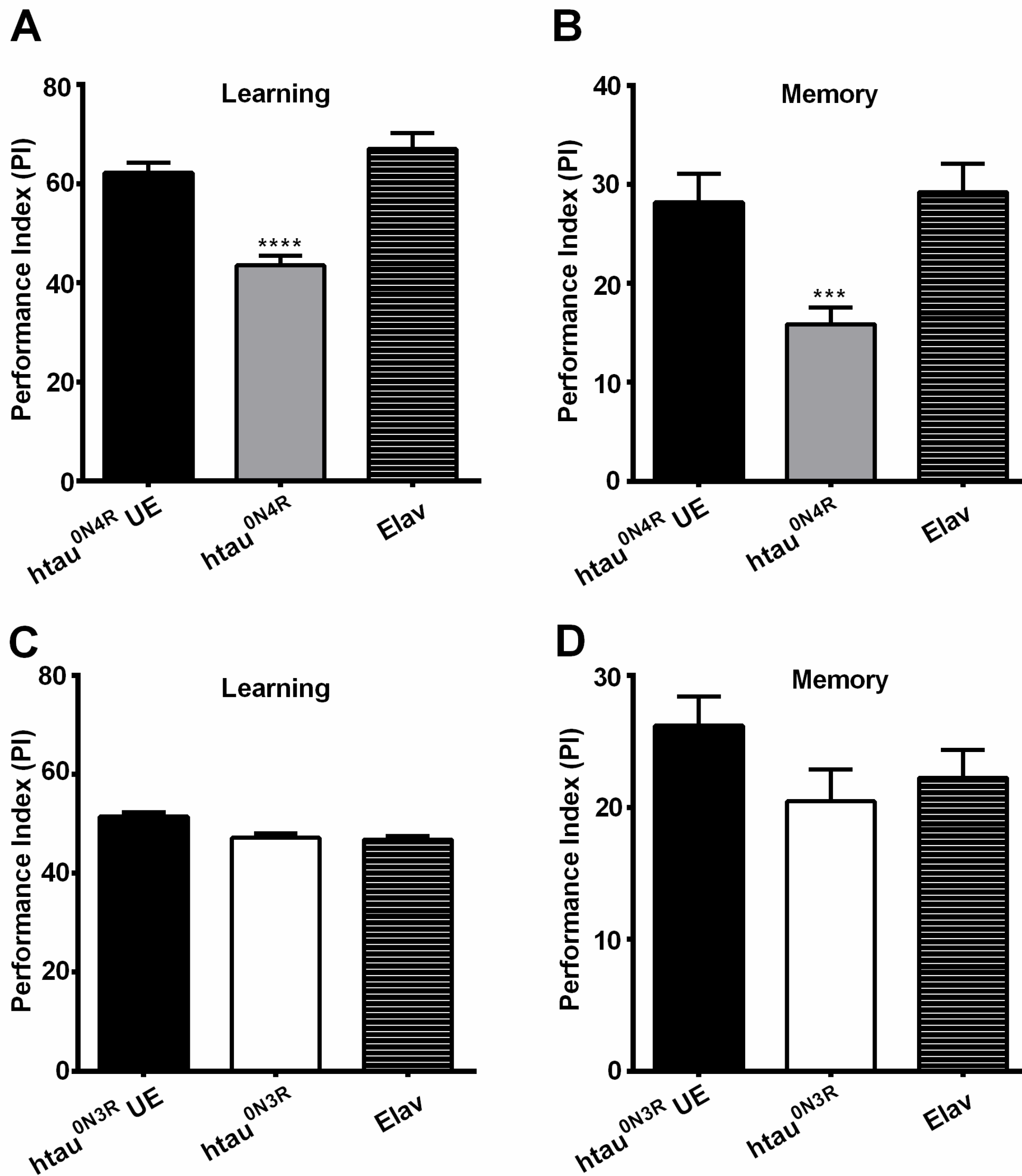



Figure 6-1. Learning and memory in olfactory learning assay of htau$^{0N3R}$ and htau$^{0N4R}$ expressing flies.

Data produced by Skoulakis laboratory. (A+B) Htau$^{0N4R}$ expressing flies show significant impairments in both learning and memory compared to non-expressing htau$^{0N4R}$ flies and Elav controls. (C + D) Htau$^{0N3R}$ expressing flies are not significantly different to either htau$^{0N3R}$ non expressing and Elav controls.

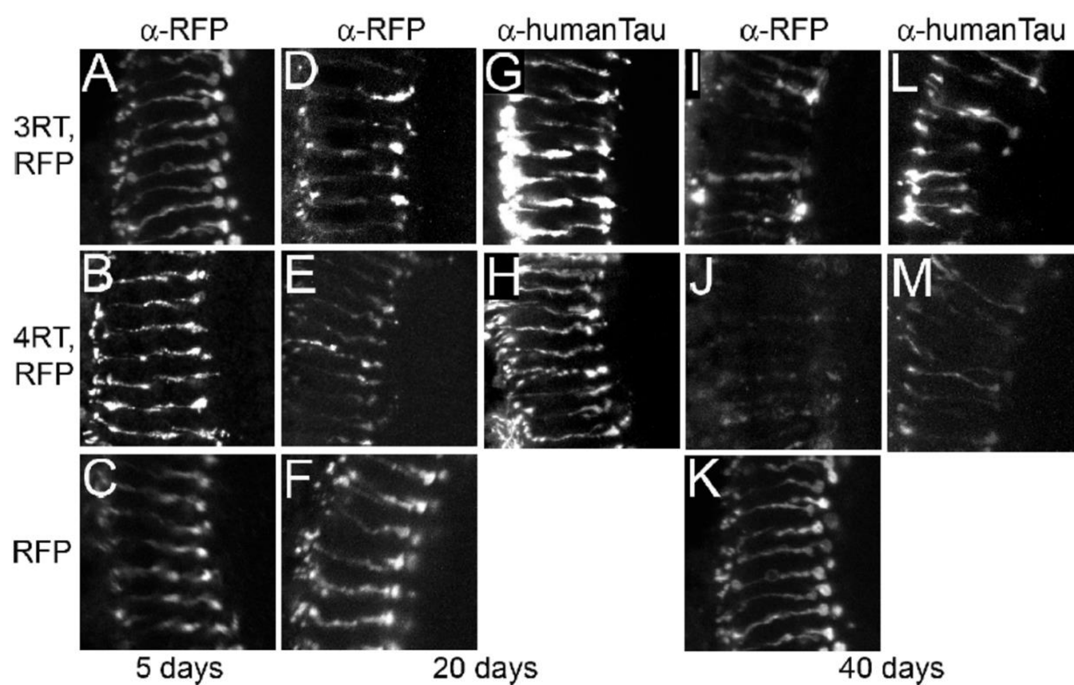



Figure 6-2. $htau^{0N3R}$ and $htau^{0N4R}$ have different toxic effects in the eye.
The outlines of sensory neurons in the eye are visualised by a tagged membrane bound (myr-RFP) was co-expressed with each tau isoform. (A-C) No degeneration was seen at 5 days in either isoform or in myr-RFP controls. (D-F) Day 20 degeneration seen in both isoforms. (I-K) Day 40 $htau^{0N4R}$ neurons have almost completely degenerated but some $htau^{0N3R}$ neurons remain. (G) Increased expression of $htau^{0N3R}$ seen at 20 days relative to (H) $htau^{0N4R}$.

# Appendix B Supporting data for Chapter 3.

Table 6. Circadian data of all lines tested using Pdf-GAL4 driver, both genders for 4 weeks. 1-way ANOVA performed on data with Bonferroni multiple comparisons, statistical significance indicated if Pdf>+ or +>htau$^{0N3R}$ are significantly different to Pdf>htau$^{0N3R}$. * P≤0.05, **P≤0.01, ***P≤0.001, **** P≤0.0001.

| Age of flies | Genotype | Gender | Mean Period length (h) | Mean rhythmic power | Rhythmic % | n |
|---|---|---|---|---|---|---|
| **5-12d** | Pdf>htau$^{0N3R}$ | Males | 24.70 | 1.56 | 87 | 23 |
| | | Females | 24.65 | 1.36 | 62 | 21 |
| | Pdf>+ | Males | 24.18 | 1.48 | 97 | 32 |
| | | Females | 24.70 | 1.50 | 73 | 26 |
| | +>htau$^{0N3R}$ | Males | 23.91* | 2.26**** | 100 | 27 |
| | | Females | 24.29 | 1.78* | 92 | 26 |
| **13-19d** | Pdf>htau$^{0N3R}$ | Males | 25.13 | 1.50 | 100 | 12 |
| | | Females | 24.73 | 1.49 | 88 | 17 |
| | Pdf>+ | Males | 24.50 | 1.72 | 94 | 32 |
| | | Females | 23.55**** | 1.74 | 81 | 26 |
| | +>htau$^{0N3R}$ | Males | 23.59 | 1.93 | 92 | 12 |
| | | Females | 22.90 | 1.87 | 77 | 13 |
| **20-26d** | Pdf>htau$^{0N3R}$ | Males | 25.05 | 1.65 | 91 | 11 |
| | | Females | 24.36 | 1.34 | 69 | 16 |
| | Pdf>+ | Males | 24.10 | 1.76 | 92 | 26 |
| | | Females | 23.84 | 1.68 | 86 | 22 |
| | +>htau$^{0N3R}$ | Males | 23.86 | 1.61 | 82 | 11 |
| | | Females | 23.30 | 1.59 | 50 | 10 |
| **27-33d** | Pdf>htau$^{0N3R}$ | Males | 25.28 | 1.56 | 90 | 10 |
| | | Females | 24.71 | 1.27 | 73 | 11 |
| | Pdf>+ | Males | 24.30* | 1.69 | 92 | 25 |
| | | Females | 23.92 | 1.54 | 63 | 19 |
| | +>htau$^{0N3R}$ | Males | 24.07 | 1.55 | 88 | 8 |
| | | Females | 23.50 | 1.41 | 50 | 6 |

# Appendix C Supporting data for Chapter 4

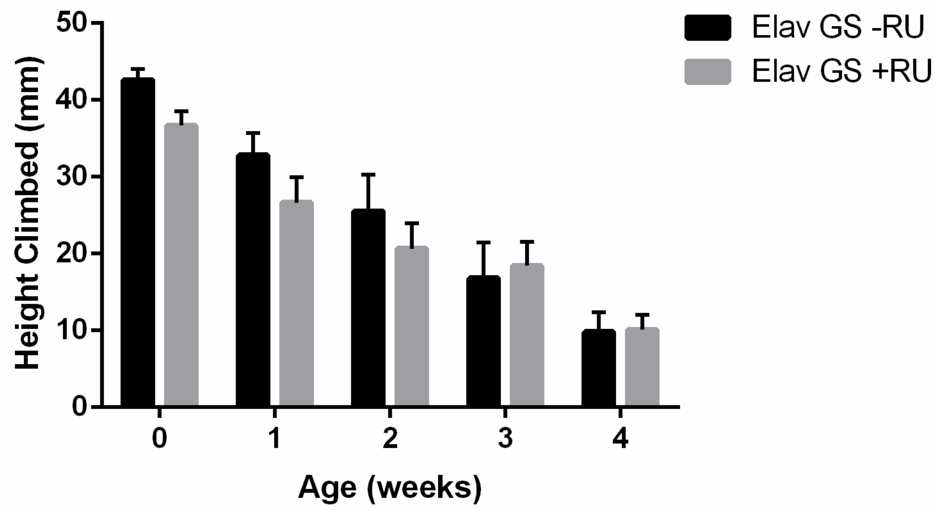



Figure 6-3. The effect of RU486 on control flies' climbing ability.
Flies tested here are the driver line (ElavGS) without being crossed to a UAS-responder. Climbing assessed for 4 weeks (n=50). No significant difference in climbing ability in flies given RU486 and those not given RU486.

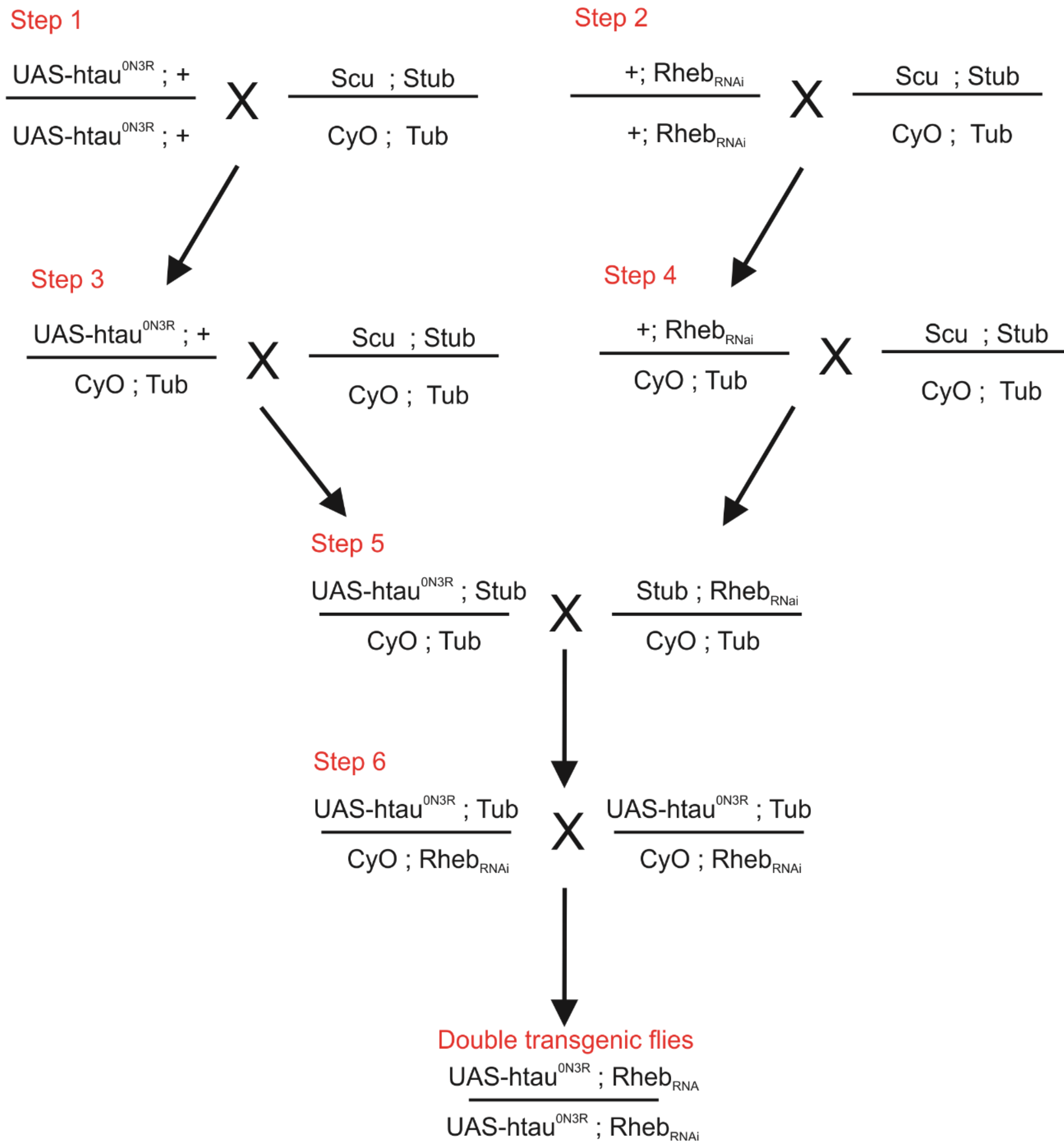



Figure 6-4. Schematic for making double transgenic lines.
Demonstrated here is how htau$^{0N3R}$; RhebRNAi and htau$^{0N3R}$; Atg1 flies were made. but the same scheme was also applied to making the htau$^{0N3R}$; Atg1 flies. UAS-htau$^{0N3R}$ is homozygous on chromosome 2, UAS-Rheb$_{RNAi}$ is homozygous on chromosome 3. The double balancer line w1118/Dp (1; Y) y+; CyO/nub1 b1 snaSco lt1 stw3; MKRS/TM6B, Tb1 was used to track inheritance. On chromosome 2 the balancer chromosomes contains the phenotypic markers; curly wings (CyO) and reduced scutellar bristles (Sco). On chromosome 3, the markers on the balancer chromosomes are stubble bristles (MKRS or Stub) and tubby larvae/pupae (Tb or tub). In steps 1 and 2 each of the homozygous lines is crossed onto the balancer line, with the resultant progeny crossed back onto the double balancer line in steps 3 and 4. The balanced single heterozygotes are then crossed with each other in step 5. The resultant double heterozygote progeny are crossed together in step 6 and progeny are selected that do not show any markers. These are then used to establish the homozygous double transgenic line.

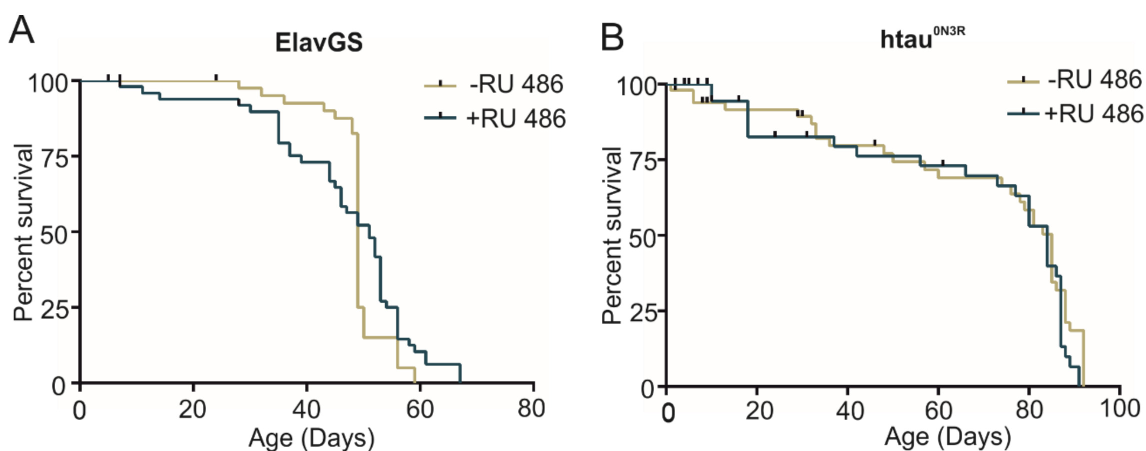



Figure 6-5. Longevity of female ElavGS flies and htau$^{0N3R}$ flies.
Comparison between expressing flies (+RU486) and non expressing flies (-RU486). A) ElavGS flies used as control. +RU486 does not affect longevity. B) ElavGS; htau$^{0N3R}$ flies, expression of htau$^{0N3R}$ does not affect lifespan. Statistical analysis performed using Mantel-Cox test (n=50).

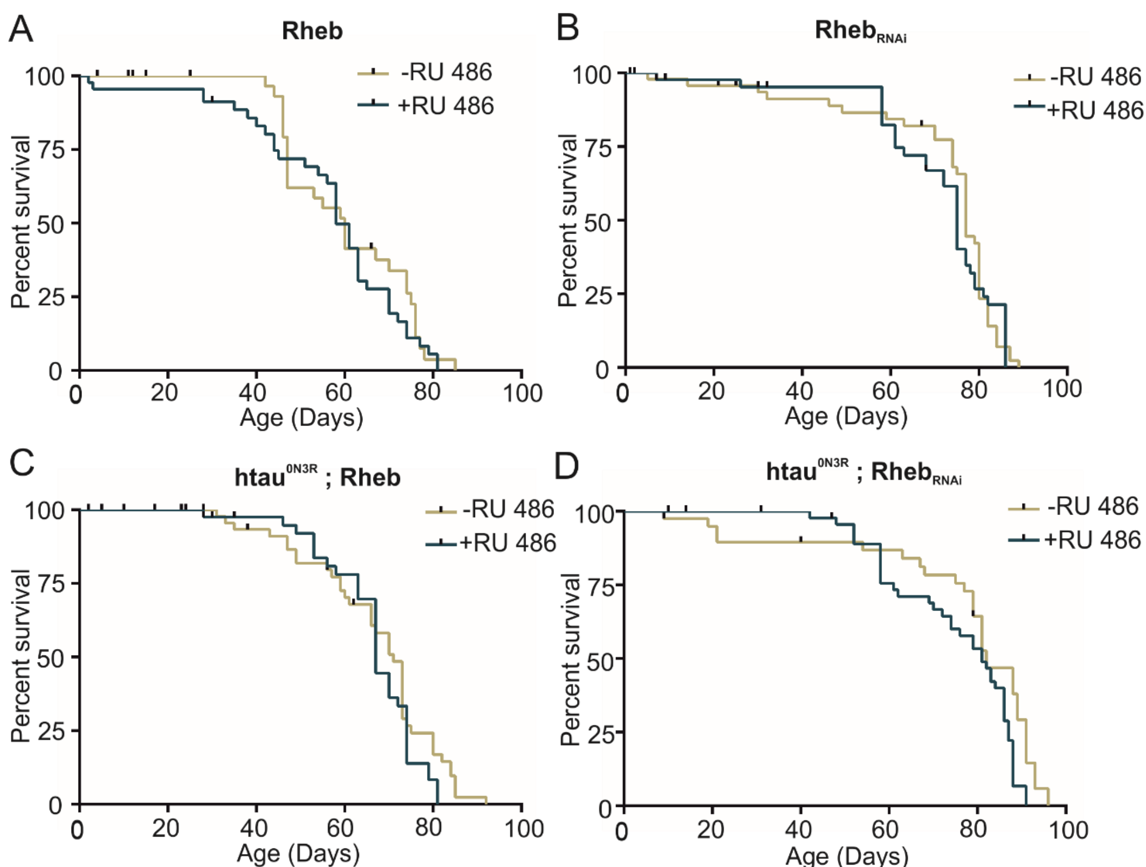



Figure 6-6. Longevity of female Rheb mutant flies.
Comparison between expressing flies (+RU486) and non-expressing flies (-RU486). A) Expression of Rheb does not alter longevity. B) Expression of Rheb$_{RNAi}$ does not alter longevity. C) Co-expression of htau$^{0N3R}$ and Rheb does not alter longevity. D) Co-expression of htau$^{0N3R}$ and Rheb$_{RNAi}$ does not alter longevity. Statistical analysis performed using Mantel-Cox test (n=50).

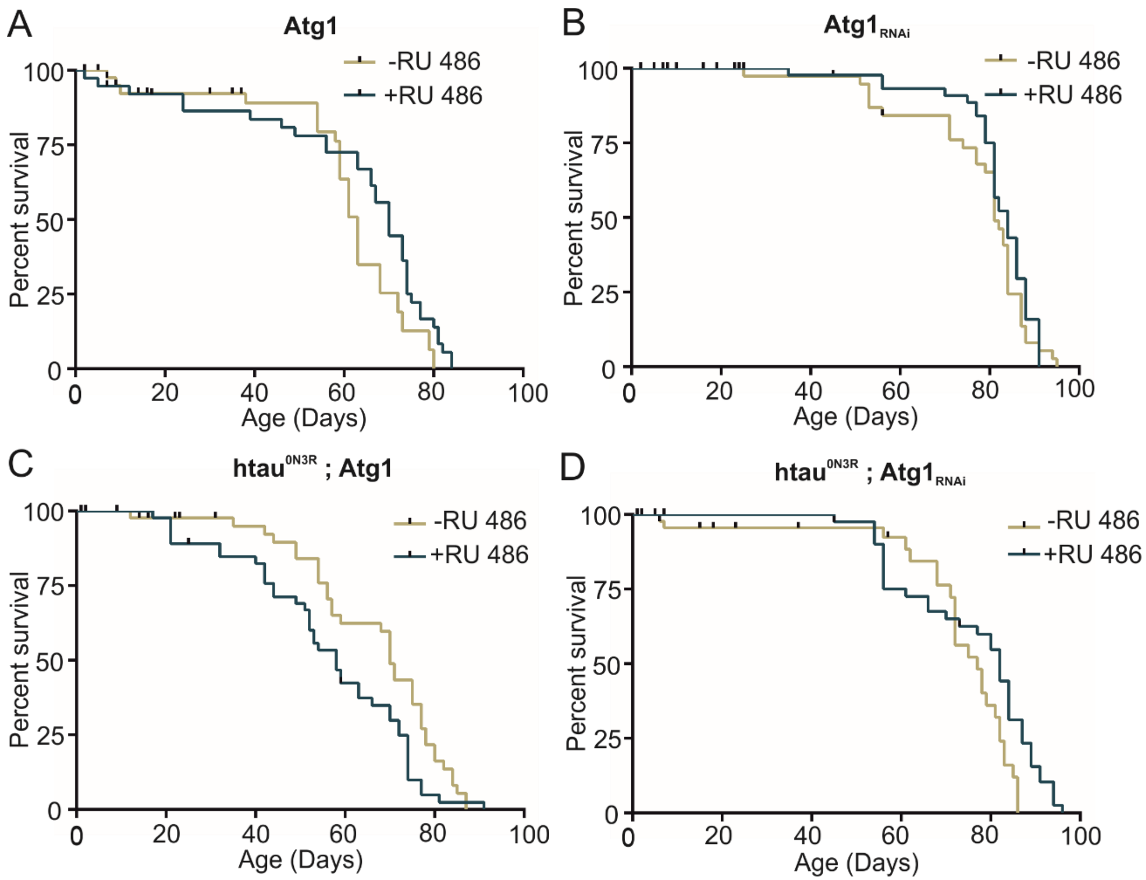



Figure 6-7. Longevity of female Atg1 mutant flies.

Comparison between expressing flies (+RU486) and non-expressing flies (-RU486). A) Expression of Atg1 signifcantly increases lifespan (p=0.0334) B) Expression of $Atg1_{RNAi}$ does not alter longevity. C) Co-expression of $htau^{0N3R}$ and Atg1 significantly reduces lifespan (p=0.0126). D) Co-expression of $htau^{0N3R}$ and $Atg1_{RNAi}$ significantly increased lifespan (p=0.0001). Statistical analysis performed using Mantel-Cox test (n=50).

# Appendix D Additional methods/ recipes

**Standard Bloomington fly media (1L)**

| | |
|---|---|
| $dH_2O$ (ml) | 1 |
| Agar (g) | 6 |
| Yeast (g) | 17.5 |
| Soya flour (g) | 10 |
| Yellow maize meal (g) | 73.1 |
| Light malt extract (g) | 46.2 |
| Dry weight sucrose (g) | 48 |
| Additional $dH_2O$ (ml) | 80 |
| Propionic acid (ml) | 5 |

1. Agar, yeast, soya flour and yellow maize meal are mixed with 1/5 total water into a smooth paste.

2. The remaining water is boiled and malt and sucrose are then added. Mixture stirred until dissolved.

3. Add malt/sucrose solution to agar/yeast/soya flour/maize paste, mix and boil, stirring constantly. Boil for a few minutes.

4. Allow to cool to 60°C and then add propionic acid and stir through.

5. Pour into vials and leave covered to cool (2hrs-overnight) before plugging with cotton wool bungs and storing in fridge.

# Appendix E *Drosophila* genotypes

Table 7. List of fly stocks used in Thesis.

| ***Drosophila* line** | **Genotype** | **Chromosome** | **Obtained from** |
|---|---|---|---|
| **Oregon R** | +;+;+;+ (wild type no inserts) | N/A | Bloomington Stock Centre |
| **Elav GAL4 (pan neuronal driver)** | Elav$^{C155}$-Gal4;+;+;+ | I | Bloomington Stock Centre |
| **MB GeneSwitch driver (drug sensitive)** | +;+;MB-GeneSwitch;+;+ | III | Davies Lab |
| **Elav geneswitch driver (drug sensitive)** | yw;Elav-GeneSwitch;+;+ | II | Bloomington Stock Centre |
| **3 repeat human tau** | w$^{1118}$;UAS- htau$^{0N3R}$;+;+ | II | Bloomington Stock Centre |
| **UAS-Atg1** | y[1] w[*]; P{w[+mC]=UAS-Atg1.S}6B | III | Bloomington Stock Centre |
| **Tau; Atg1/Cyo;Tubby** | w$^{1118}$;UAS-htau$^{0N3R}$/CyO; UAS-Atg1/Tub | II&III | Made by Megan Sealey |
| **4 repeat human tau (low expressor)** | +/+;UAS-htau$^{0N4R}$/Ser | III | Torsten Bossing |
| **4 repeat human tau (high expressor)** | +/+;UAS-htau$^{0N4Rhigh}$/ UAS-htau$^{0N4Rhigh}$;+/+ | III | Skoulakis lab |
| **w$^{1118}$** | +;+;+;+ | N/A | Bloomington Stock Centre |
| **Pdf-GAL4** | Pdf-GAL4;+;+ | II | Herman Wijnen |
| **Pdf-GAL4;tubulinGAL80ts** | Pdf-GAL4;tubulinGAL80$^{ts}$ | II&III | Herman Wijnen |
| **UAS-Atg1$_{RNAi}$** | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02273}attP2 | III | Bloomington Stock Centre |
| **UAS-Rheb** | w[*]; P{w[+mC]=UAS-Rheb.Pa}3 | III | Bloomington Stock Centre |
| **UAS-Rheb$_{RNAi}$** | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00923}attP2 | III | Bloomington Stock Centre |
| **Double balancer line** | w1118/Dp(1;Y)y+;CyO/nub1b 1snaScolt1stw3;MKRS/TM6B, Tb1 | II&III | Bloomington Stock Centre |

# Appendix F Research impact

| **PUBLICATIONS** |
|---|
| Sealey M, Gleeson T, Dickinson B, Mudher A et al., The microtubule-associated protein Tau slows down circadian rhythms when over-expressed in Drosophila clock neurons, *manuscript in preparation.* |
| Cowan CM, Sealey M, Quraishe S, Mudher A, Oxidative stress contributes to tau-mediated neuronal dysfunction in an isoform-specific manner, *manuscript in preparation* |
| Sealey M, Cowan CM, Bossing T, Vourkou E et al, Distinct phenotypes of three-repeat and four-repeat human tau in a transgenic model of tauopathy, *manuscript submitted to Neurobiology of Disease* |
| Quraishe S, Sealey M, Cranfield L, Mudher A (2016), Microtubule stabilising peptides rescue tau phenotypes in vivo, *Scientific Reports*, doi: 1038/srep38224 |
| Cowan CM, Quraishe S, Hands S, Sealey M et al., (2015), Rescue from tau-induced neuronal dysfunction produces insoluble tau oligomers, *Scientific Reports,* doi: 10.1038/srep17191 |
| Sinadinos C, Quraishe S, Sealey M, Samson B et al., (2013) Low Endogenous and Chemical Induced Heat Shock Protein Induction in a 0N3Rtau-Expressing Drosophila Larval Model of Alzheimer's Disease, *J Alzheimer's Dis.,* doi: 10.3233/JAD-2012-121534 |

Note: In addition to the above publications that have either been published or are being prepared for submission we also plan to submit a small paper on the learning and memory assay, a paper on human brain ageing using data from chapter 4 and also a paper on autophagy in the flies, also using data from chapter 4.

## Presentations

| Date | Event/Conference | Type of presentation |
|---|---|---|
| **May 2013** | SoNG-Departmental seminar | Oral |
| **July 2013** | Kerkut Symposium | Oral |
| **September 2013** | SoNG Symposium | Poster |
| **March 2014** | Alzheimer's Research UK | Poster |
| **May 2014** | SoNG Departmental seminar | Oral |
| **July 2014** | UoS Biological Sciences Postgraduate Symposium. | Poster (1st prize) |
| **July 2014** | Kerkut Symposium | Oral |
| **March 2015** | Alzheimer's Research UK | Poster |
| **March 2015** | Alzheimer's & Parkinson's Diseases Congress – AD/PD, Nice | Poster |
| **March 2015** | SoNG-Departmental seminar | Oral |
| **July 2015** | Kerkut Symposium | Oral |
| **July 2015** | UoS Biological Sciences Postgraduate Symposium. | Oral |
| **September 2015** | SoNG Symposium | Poster |
| **March 2016** | Alzheimer's Research UK | Poster |
| **April 2016** | SoNG-Departmental seminar | Oral |

# Bibliography

ALI, Y. O., ESCALA, W., RUAN, K. & ZHAI, R. G. 2011. Assaying locomotor, learning, and memory deficits in Drosophila models of neurodegeneration. *JoVE (Journal of Visualized Experiments)*, e2504-e2504.

ALI, Y. O., RUAN, K. & ZHAI, R. G. 2012. NMNAT suppresses Tau-induced neurodegeneration by promoting clearance of hyperphosphorylated Tau oligomers in a Drosophila model of tauopathy. *Human Molecular Genetics,* 21, 237-250.

ALONSO, A. C., ZAIDI, T., GRUNDKE-IQBAL, I. & IQBAL, K. 1994. Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proceedings of the National Academy of Sciences,* 91, 5562-5566.

AN, W. L., COWBURN, R. F., BJORKDAHL, C., BRAAK, H., ALAFUZOFF, I., IQBAL, K., GRUNDKE-IQBAL, I. & WINBLAD, B. 2004. Association of p70 S6 kinase with neurofibrillary degeneration Alzheimer's disease. *Neurobiology of Aging,* 25, S424-S425.

AN, W. L., COWBURN, R. F., LI, L., BRAAK, H., ALAFUZOFF, I., IQBAL, K., IQBAL, I. G., WINBLAD, B. & PEI, J. J. 2003. Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's disease. *American Journal of Pathology,* 163, 591-607.

ANDO, K., OKA, M., OHTAKE, Y., HAYASHISHITA, M., SHIMIZU, S., HISANAGA, S.-I. & IIJIMA, K. M. 2016. Tau phosphorylation at Alzheimer's disease-related Ser356 contributes to tau stabilization when PAR-1/MARK activity is elevated. *Biochemical and Biophysical Research Communications,* 478, 929-934.

ANDORFER, C. A. & DAVIES, P. 2000. PKA Phosphorylations on Tau: Developmental Studies in the Mouse. *Developmental Neuroscience,* 22, 303-309.

ANISIMOV, V. N., ZABEZHINSKI, M. A., POPOVICH, I. G., PISKUNOVA, T. S., SEMENCHENKO, A. V., TYNDYK, M. L., YUROVA, M. N., ANTOCH, M. P. & BLAGOSKLONNY, M. V. 2010. Rapamycin Extends Maximal Lifespan in Cancer-Prone Mice. *The American Journal of Pathology,* 176, 2092-2097.

ARRIAGADA, P. V., GROWDON, J. H., HEDLEY-WHYTE, E. T. & HYMAN, B. T. 1992. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology,* 42, 631-631.

ASAI, M., YOSHINOBU, Y., KANEKO, S., MORI, A., NIKAIDO, T., MORIYA, T., AKIYAMA, M. & SHIBATA, S. 2001. Circadian profile of Per gene mRNA expression in the suprachiasmatic nucleus, paraventricular nucleus, and pineal body of aged rats. *Journal of neuroscience research,* 66, 1133-1139.

ASUNI, A. A., BOUTAJANGOUT, A., QUARTERMAIN, D. & SIGURDSSON, E. M. 2007. Immunotherapy Targeting Pathological Tau Conformers in a Tangle Mouse Model Reduces Brain Pathology with Associated Functional Improvements. *The Journal of Neuroscience,* 27, 9115-9129.

AUGUSTINACK, J. C., SCHNEIDER, A., MANDELKOW, E.-M. & HYMAN, B. T. 2002. Specific tau phosphorylation sites correlate with severity of neuronal cytopathology in Alzheimer's disease. *Acta Neuropathologica,* 103, 26-35.

BAAR, E. L., CARBAJAL, K. A., ONG, I. M. & LAMMING, D. W. 2016. Sex- and tissue- specific changes in mTOR signaling with age in C57BL/6J mice. *Aging cell,* 15**,** 155-166.

BAKHOUM, M. F., BAKHOUM, C. Y., DING, Z., CARLTON, S. M., CAMPBELL, G. A. & JACKSON, G. R. 2014. Evidence for autophagic gridlock in aging and neurodegeneration. *Translational Research,* 164**,** 1-12.

BALLATORE, C., LEE, V. M. Y. & TROJANOWSKI, J. Q. 2007. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat Rev Neurosci,* 8**,** 663-672.

BANCHER, C., BRAAK, H., FISCHER, P. & JELLINGER, K. A. 1993. Neuropathological staging of Alzheimer lesions and intellectual status in Alzheimer's and Parkinson's disease patients. *Neuroscience letters,* 162**,** 179-182.

BARAZZONI, R., SHORT, K. R. & NAIR, K. S. 2000. Effects of Aging on Mitochondrial DNA Copy Number and Cytochromec Oxidase Gene Expression in Rat Skeletal Muscle, Liver, and Heart. *Journal of Biological Chemistry,* 275**,** 3343-3347.

BASSAN, M., ZAMOSTIANO, R., DAVIDSON, A., PINHASOV, A., GILADI, E., PERL, O., BASSAN, H., BLAT, C., GIBNEY, G., GLAZNER, G., BRENNEMAN, D. E. & GOZES, I. 1999. Complete Sequence of a Novel Protein Containing a Femtomolar-Activity-Dependent Neuroprotective Peptide. *Journal of Neurochemistry,* 72**,** 1283-1293.

BAUMANN, K., MANDELKOW, E. M., BIERNAT, J., PIWNICA-WORMS, H. & MANDELKOW, E. 1993. Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5. *FEBS Letters,* 336**,** 417-424.

BECK, C. D. O., SCHROEDER, B. & DAVIS, R. L. 2000. Learning Performance of Normal and MutantDrosophila after Repeated Conditioning Trials with Discrete Stimuli. *The Journal of Neuroscience,* 20**,** 2944-2953.

BEKINSCHTEIN, P., CAMMAROTA, M., IGAZ, L. M., BEVILAQUA, L. R., IZQUIERDO, I. & MEDINA, J. H. 2007. Persistence of long-term memory storage requires a late protein synthesis-and BDNF-dependent phase in the hippocampus. *Neuron,* 53**,** 261-277.

BERGER, Z., RAVIKUMAR, B., MENZIES, F. M., OROZ, L. G., UNDERWOOD, B. R., PANGALOS, M. N., SCHMITT, I., WULLNER, U., EVERT, B. O., O'KANE, C. J. & RUBINSZTEIN, D. C. 2006. Rapamycin alleviates toxicity of different aggregate-prone proteins. *Human Molecular Genetics,* 15**,** 433-442.

BERGER, Z., RODER, H., HANNA, A., CARLSON, A., RANGACHARI, V., YUE, M., WSZOLEK, Z., ASHE, K., KNIGHT, J., DICKSON, D., ANDORFER, C., ROSENBERRY, T. L., LEWIS, J., HUTTON, M. & JANUS, C. 2007. Accumulation of pathological tau species and memory loss in a conditional model of tauopathy. *J Neurosci,* 27**,** 3650-62.

BHASKAR, K., MILLER, M., CHLUDZINSKI, A., HERRUP, K., ZAGORSKI, M. & LAMB, B. T. 2009. The PI3K-Akt-mTOR pathway regulates Aβ oligomer induced neuronal cell cycle events. *Molecular neurodegeneration,* 4**,** 1.

BINDER, L. I., FRANKFURTER, A. & REBHUN, L. I. 1985. The distribution of tau in the mammalian central nervous system. *The Journal of cell biology,* 101**,** 1371-1378.

BIRKS, J. S. 2006. Cholinesterase inhibitors for Alzheimer's disease. *Cochrane Database of Systematic Reviews*.

BISHOP, N. A., LU, T. & YANKNER, B. A. 2010. Neural mechanisms of ageing and cognitive decline. *Nature,* 464**,** 529-535.

BJEDOV, I., TOIVONEN, J. M., KERR, F., SLACK, C., JACOBSON, J., FOLEY, A. & PARTRIDGE, L. 2010. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. *Cell Metab,* 11**,** 35-46.

BLARD, O., FRÉBOURG, T., CAMPION, D. & LECOURTOIS, M. 2006. Inhibition of proteasome and Shaggy/Glycogen synthase kinase-3β kinase prevents clearance of phosphorylated tau in Drosophila. *Journal of Neuroscience Research,* 84**,** 1107-1115.

BLIWISE, D. L. 1993. Sleep in normal aging and dementia. *Sleep: Journal of Sleep Research & Sleep Medicine*.

BOCHE, D., DONALD, J., LOVE, S., HARRIS, S., NEAL, J. W., HOLMES, C. & NICOLL, J. A. R. 2010. Reduction of aggregated Tau in neuronal processes but not in the cell bodies after Aβ42 immunisation in Alzheimer's disease. *Acta Neuropathologica,* 120**,** 13-20.

BONANNI, E., MAESTRI, M., TOGNONI, G., FABBRINI, M., NUCCIARONE, B., MANCA, M. L., GORI, S., IUDICE, A. & MURRI, L. 2005. Daytime sleepiness in mild and moderate Alzheimer's disease and its relationship with cognitive impairment. *Journal of sleep research,* 14**,** 311-317.

BONAWITZ, N. D., CHATENAY-LAPOINTE, M., PAN, Y. & SHADEL, G. S. 2007. Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. *Cell Metabolism,* 5**,** 265-277.

BOWLING, A. C., MUTISYA, E. M., WALKER, L. C., PRICE, D. L., CORK, L. C. & BEAL, M. H. 1993. Age-Dependent Impairment of Mitochondrial Function in Primate Brain. *Journal of Neurochemistry,* 60**,** 1964-1967.

BRAAK, H. & BRAAK, E. 1995. Staging of alzheimer's disease-related neurofibrillary changes. *Neurobiology of Aging,* 16**,** 271-278.

BRAMBLETT, G. T., GOEDERT, M., JAKES, R., MERRICK, S. E., TROJANOWSKI, J. Q. & LEE, V. M. Y. 1993. Abnormal tau phosphorylation at Ser396 in alzheimer's disease recapitulates development and contributes to reduced microtubule binding. *Neuron,* 10**,** 1089-1099.

BRAND, A. H. & PERRIMON, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *development,* 118**,** 401-415.

BRANDT, R., LÉGER, J. & LEE, G. 1995. Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. *The Journal of Cell Biology,* 131**,** 1327-1340.

BRUNDEN, K. R., ZHANG, B., CARROLL, J., YAO, Y., POTUZAK, J. S., HOGAN, A.-M. L., IBA, M., JAMES, M. J., XIE, S. X., BALLATORE, C., SMITH, A. B., LEE, V. M.-Y. & TROJANOWSKI, J. Q. 2010. Epothilone D Improves Microtubule Density, Axonal Integrity, and Cognition in a Transgenic Mouse Model of Tauopathy. *The Journal of Neuroscience,* 30**,** 13861-13866.

BUÉE, L., BUSSIÈRE, T., BUÉE-SCHERRER, V., DELACOURTE, A. & HOF, P. R. 2000. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders1. *Brain Research Reviews,* 33**,** 95-130.

BURCIN, M. M., O'MALLEY, B. W. & TSAI, S. Y. 1998. A regulatory system for target gene expression. *Front Biosci,* 3**,** c1-7.

BUSSER, J., GELDMACHER, D. S. & HERRUP, K. 1998. Ectopic Cell Cycle Proteins Predict the Sites of Neuronal Cell Death in Alzheimer's Disease Brain. *The Journal of Neuroscience,* 18**,** 2801-2807.

BUTNER, K. A. & KIRSCHNER, M. W. 1991. Tau protein binds to microtubules through a flexible array of distributed weak sites. *J Cell Biol,* 115**,** 717-30.

BYERS, D., DAVIS, R. L. & KIGER, J. A. 1981. Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in Drosophila melanogaster. *Nature,* 289**,** 79-81.

BYUN, Y. J., KIM, S. K., KIM, Y. M., CHAE, G. T., JEONG, S.-W. & LEE, S.-B. 2009. Hydrogen peroxide induces autophagic cell death in C6 glioma cells via BNIP3-mediated suppression of the mTOR pathway. *Neuroscience Letters,* 461**,** 131-135.

CACCAMO, A., BRANCA, C., TALBOOM, J. S., SHAW, D. M., TURNER, D., MA, L., MESSINA, A., HUANG, Z., WU, J. & ODDO, S. 2015. Reducing Ribosomal Protein S6 Kinase 1 Expression Improves Spatial Memory and Synaptic Plasticity in a Mouse Model of Alzheimer's Disease. *The Journal of Neuroscience,* 35**,** 14042-14056.

CACCAMO, A., MAGRÌ, A., MEDINA, D. X., WISELY, E. V., LÓPEZ-ARANDA, M. F., SILVA, A. J. & ODDO, S. 2013. mTOR regulates tau phosphorylation and degradation: implications for Alzheimer's disease and other tauopathies. *Aging Cell,* 12**,** 370-380.

CACCAMO, A., MAJUMDER, S., RICHARDSON, A., STRONG, R. & ODDO, S. 2010. Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: effects on cognitive impairments. *J Biol Chem,* 285**,** 13107-20.

CARMEL, G., MAGER, E. M., BINDER, L. I. & KURET, J. 1996. The Structural Basis of Monoclonal Antibody Alz50's Selectivity for Alzheimer's Disease Pathology. *Journal of Biological Chemistry,* 271**,** 32789-32795.

CASH, A. D., ALIEV, G., SIEDLAK, S. L., NUNOMURA, A., FUJIOKA, H., ZHU, X., RAINA, A. K., VINTERS, H. V., TABATON, M. & JOHNSON, A. B. 2003. Microtubule reduction in Alzheimer's disease and aging is independent of τ filament formation. *The American journal of pathology,* 162**,** 1623-1627.

CASTELLANI, R., HIRAI, K., ALIEV, G., DREW, K. L., NUNOMURA, A., TAKEDA, A., CASH, A. D., OBRENOVICH, M. E., PERRY, G. & SMITH, M. A. 2002. Role of mitochondrial dysfunction in Alzheimer's disease. *Journal of Neuroscience Research,* 70**,** 357-360.

CHANG, Y.-Y. & NEUFELD, T. P. 2010. Autophagy takes flight in Drosophila. *FEBS Letters,* 584**,** 1342-1349.

CHAPMAN, T. & PARTRIDGE, L. 1996. Female Fitness in Drosophila melanogaster: An Interaction between the Effect of Nutrition and of Encounter Rate with Males. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 263**,** 755-759.

CHATTERJEE, S., SANG, T.-K., LAWLESS, G. M. & JACKSON, G. R. 2009. Dissociation of tau toxicity and phosphorylation: role of GSK-3β, MARK and Cdk5 in a Drosophila model. *Human Molecular Genetics,* 18, 164-177.

CHEE, F. C., MUDHER, A., CUTTLE, M. F., NEWMAN, T. A., MACKAY, D., LOVESTONE, S. & SHEPHERD, D. 2005. Over-expression of tau results in defective synaptic transmission in *Drosophila* neuromuscular junctions. *Neurobiology of disease,* 20, 918-928.

CHEN, K.-F., POSSIDENTE, B., LOMAS, D. A. & CROWTHER, D. C. 2014. The central molecular clock is robust in the face of behavioural arrhythmia in a *Drosophila* model of Alzheimer's disease. *Disease Models & Mechanisms,* 7, 445-458.

CHEN, L., XU, B., LIU, L., LUO, Y., YIN, J., ZHOU, H., CHEN, W., SHEN, T., HAN, X. & HUANG, S. 2010. Hydrogen peroxide inhibits mTOR signaling by activation of AMPKα leading to apoptosis of neuronal cells. *Laboratory Investigation,* 90, 762-773.

CHEN, X., LI, Y., HUANG, J., CAO, D., YANG, G., LIU, W., LU, H. & GUO, A. 2007. Study of tauopathies by comparing Drosophila and human tau in Drosophila. *Cell Tissue Res,* 329, 169-78.

CHIPPINDALE, A. K., LEROI, A. M., KIM, S. B. & ROSE, M. R. 1993. Phenotypic plasticity and selection in Drosophila life-history evolution. I. Nutrition and the cost of reproduction. *Journal of Evolutionary Biology,* 6, 171-193.

CLANCY, D. J., GEMS, D., HAFEN, E., LEEVERS, S. J. & PARTRIDGE, L. 2002. Dietary Restriction in Long-Lived Dwarf Flies. *Science,* 296, 319-319.

CLANCY, D. J., GEMS, D., HARSHMAN, L. G., OLDHAM, S., STOCKER, H., HAFEN, E., LEEVERS, S. J. & PARTRIDGE, L. 2001. Extension of Life-Span by Loss of CHICO, a *Drosophila* Insulin Receptor Substrate Protein. *Science,* 292, 104-106.

CLAVAGUERA, F., BOLMONT, T., CROWTHER, R. A., ABRAMOWSKI, D., FRANK, S., PROBST, A., FRASER, G., STALDER, A. K., BEIBEL, M., STAUFENBIEL, M., JUCKER, M., GOEDERT, M. & TOLNAY, M. 2009. Transmission and spreading of tauopathy in transgenic mouse brain. *Nat Cell Biol,* 11, 909-913.

COLODNER, K. J. & FEANY, M. B. 2010. Glial Fibrillary Tangles and JAK/STAT-Mediated Glial and Neuronal Cell Death in a Drosophila Model of Glial Tauopathy. *The Journal of Neuroscience,* 30, 16102-16113.

CONGDON, E. E., WU, J. W., MYEKU, N., FIGUEROA, Y. H., HERMAN, M., MARINEC, P. S., GESTWICKI, J. E., DICKEY, C. A., YU, W. H. & DUFF, K. E. 2012. Methylthioninium chloride (methylene blue) induces autophagy and attenuates tauopathy in vitro and in vivo. *Autophagy,* 8, 609-622.

COWAN, C., BOSSING, T., PAGE, A., SHEPHERD, D. & MUDHER, A. 2010. Soluble hyper-phosphorylated tau causes microtubule breakdown and functionally compromises normal tau in vivo. *Acta Neuropathologica,* 120, 593-604.

COWAN, C. & MUDHER, A. 2013. Are Tau Aggregates Toxic or Protective in Tauopathies? *Frontiers in Neurology,* 4.

COWAN, C. M., QURAISHE, S., HANDS, S., SEALEY, M., MAHAJAN, S., ALLAN, D. W. & MUDHER, A. 2015. Rescue from tau-induced neuronal dysfunction produces insoluble tau oligomers. *Scientific reports,* 5.

CUNNINGHAM, J. T., RODGERS, J. T., ARLOW, D. H., VAZQUEZ, F., MOOTHA, V. K. & PUIGSERVER, P. 2007. mTOR controls mitochondrial oxidative function through a YY1–PGC-1&agr; transcriptional complex. *nature,* 450**,** 736-740.

DAUWALDER, B. & DAVIS, R. 1995. Conditional rescue of the dunce learning/memory and female fertility defects with Drosophila or rat transgenes. *The Journal of Neuroscience,* 15**,** 3490-3499.

DAVIS, R. L. & KIGER, J. A. 1981. Dunce mutants of Drosophila melanogaster: mutants defective in the cyclic AMP phosphodiesterase enzyme system. *The Journal of Cell Biology,* 90**,** 101-107.

DE BELLE, J. & HEISENBERG, M. 1994. Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. *Science,* 263**,** 692-695.

DE CALIGNON, A., POLYDORO, M., SUÁREZ-CALVET, M., WILLIAM, C., ADAMOWICZ, DAVID H., KOPEIKINA, KATHY J., PITSTICK, R., SAHARA, N., ASHE, KAREN H., CARLSON, GEORGE A., SPIRES-JONES, TARA L. & HYMAN, BRADLEY T. 2012. Propagation of Tau Pathology in a Model of Early Alzheimer's Disease. *Neuron,* 73**,** 685-697.

DEMIDENKO, Z. N., ZUBOVA, S. G., BUKREEVA, E. I., POSPELOV, V. A., POSPELOVA, T. V. & BLAGOSKLONNY, M. V. 2009. Rapamycin decelerates cellular senescence. *Cell Cycle,* 8**,** 1888-1895.

DIAS-SANTAGATA, D., FULGA, T. A., DUTTAROY, A. & FEANY, M. B. 2007. Oxidative stress mediates tau-induced neurodegeneration in Drosophila. *J Clin Invest,* 117**,** 236-45.

DICKEY, C., KRAFT, C., JINWAL, U., KOREN, J., JOHNSON, A., ANDERSON, L., LEBSON, L., LEE, D., DICKSON, D., DE SILVA, R., BINDER, L. I., MORGAN, D. & LEWIS, J. 2009. Aging Analysis Reveals Slowed Tau Turnover and Enhanced Stress Response in a Mouse Model of Tauopathy. *The American Journal of Pathology,* 174**,** 228-238.

DING, X.-L., HUSSEMAN, J., TOMASHEVSKI, A., NOCHLIN, D., JIN, L.-W. & VINCENT, I. 2000. The Cell Cycle Cdc25A Tyrosine Phosphatase Is Activated in Degenerating Postmitotic Neurons in Alzheimer's Disease. *The American Journal of Pathology,* 157**,** 1983-1990.

DOBLE, B. W. & WOODGETT, J. R. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. *Journal of Cell Science,* 116**,** 1175-1186.

DREWES, G., TRINCZEK, B., ILLENBERGER, S., BIERNAT, J., SCHMITT-ULMS, G., MEYER, H. E., MANDELKOW, E.-M. & MANDELKOW, E. 1995. Microtubule-associated Protein/Microtubule Affinity-regulating Kinase (p110mark). *Journal of Biological Chemistry,* 270**,** 7679-7688.

DUDAI, Y. 1979. Behavioral plasticity in avDrosophila mutant, dunceDE276. *Journal of comparative physiology,* 130**,** 271-275.

FISCHER, D., MUKRASCH, M. D., BIERNAT, J., BIBOW, S., BLACKLEDGE, M., GRIESINGER, C., MANDELKOW, E. & ZWECKSTETTER, M. 2009.

Conformational Changes Specific for Pseudophosphorylation at Serine 262 Selectively Impair Binding of Tau to Microtubules. *Biochemistry,* 48**,** 10047-10055.

FOLWELL, J., COWAN, C. M., UBHI, K. K., SHIABH, H., NEWMAN, T. A., SHEPHERD, D. & MUDHER, A. 2010. Aβ exacerbates the neuronal dysfunction caused by human tau expression in a Drosophila model of Alzheimer's disease. *Experimental Neurology,* 223**,** 401-409.

FONTANA, L., PARTRIDGE, L. & LONGO, V. D. 2010. Extending Healthy Life Span—From Yeast to Humans. *Science,* 328**,** 321-326.

FROY, O. 2011. Circadian rhythms, aging, and life span in mammals. *Physiology,* 26**,** 225-235.

FULGA, T. A., ELSON-SCHWAB, I., KHURANA, V., STEINHILB, M. L., SPIRES, T. L., HYMAN, B. T. & FEANY, M. B. 2007. Abnormal bundling and accumulation of F-actin mediates tau-induced neuronal degeneration in vivo. *Nat Cell Biol,* 9**,** 139-48.

GARGANO, J. W., MARTIN, I., BHANDARI, P. & GROTEWIEL, M. S. 2005. Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in Drosophila. *Experimental Gerontology,* 40**,** 386-395.

GERSTNER, J. R. & YIN, J. C. P. 2010. Circadian rhythms and memory formation. *Nat Rev Neurosci,* 11**,** 577-588.

GOEDERT, M. & SPILLANTINI, M. G. 2006. A Century of Alzheimer's Disease. *Science,* 314**,** 777-781.

GOEDERT, M., SPILLANTINI, M. G., POTIER, M. C., ULRICH, J. & CROWTHER, R. A. 1989. Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. *The EMBO Journal,* 8**,** 393-399.

GOODE, B. L., CHAU, M., DENIS, P. E. & FEINSTEIN, S. C. 2000. Structural and functional differences between 3-repeat and 4-repeat tau isoforms. Implications for normal tau function and the onset of neurodegenetative disease. *J Biol Chem,* 275**,** 38182-9.

GÖTZ, J., PROBST, A., SPILLANTINI, M. G., SCHÄFER, T., JAKES, R., BÜRKI, K. & GOEDERT, M. 1995. Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform. *The EMBO journal,* 14**,** 1304.

GREEN, A. J. E., HARVEY, R. J., THOMPSON, E. J. & ROSSOR, M. N. 1999. Increased tau in the cerebrospinal fluid of patients with frontotemporal dementia and Alzheimer's disease. *Neuroscience Letters,* 259**,** 133-135.

GRIMA, B., CHELOT, E., XIA, R. & ROUYER, F. 2004. Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. *Nature,* 431**,** 869-873.

GROBER, E., DICKSON, D., SLIWINSKI, M., BUSCHKE, H., KATZ, M., CRYSTAL, H. & LIPTON, R. B. 1999. Memory and mental status correlates of modified Braak staging. *Neurobiology of aging,* 20**,** 573-579.

GRUMATI, P., COLETTO, L., SCHIAVINATO, A., CASTAGNARO, S., BERTAGGIA, E., SANDRI, M. & BONALDO, P. 2011. Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles. *Autophagy,* 7**,** 1415-1423.

GRUNDKE-IQBAL, I., IQBAL, K., QUINLAN, M., TUNG, Y. C., ZAIDI, M. S. & WISNIEWSKI, H. M. 1986a. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *Journal of Biological Chemistry,* 261**,** 6084-6089.

GRUNDKE-IQBAL, I., IQBAL, K., TUNG, Y. C., QUINLAN, M., WISNIEWSKI, H. M. & BINDER, L. I. 1986b. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences,* 83**,** 4913-4917.

GUILLOZET, A. L., WEINTRAUB, S., MASH, D. C. & MESULAM, M. M. 2003. Neurofibrillary tangles, amyloid, and memory in aging and mild cognitive impairment. *Archives of neurology,* 60**,** 729-736.

HAASS, C., SCHLOSSMACHER, M. G., HUNG, A. Y., VIGO-PELFREY, C., MELLON, A., OSTASZEWSKI, B. L., LIEBERBURG, I., KOO, E. H., SCHENK, D. & TEPLOW, D. B. 1992. Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature,* 359**,** 322-325.

HAHN, E. A., WANG, H.-X., ANDEL, R. & FRATIGLIONI, L. 2014. A change in sleep pattern may predict Alzheimer disease. *The American Journal of Geriatric Psychiatry,* 22**,** 1262-1271.

HAMANO, T., GENDRON, T. F., CAUSEVIC, E., YEN, S.-H., LIN, W.-L., ISIDORO, C., DETURE, M. & KO, L.-W. 2008. Autophagic-lysosomal perturbation enhances tau aggregation in transfectants with induced wild-type tau expression. *European Journal of Neuroscience,* 27**,** 1119-1130.

HAMILTON, B., DONG, Y., SHINDO, M., LIU, W., ODELL, I., RUVKUN, G. & LEE, S. S. 2005. A systematic RNAi screen for longevity genes in C. elegans. *Genes & Development,* 19**,** 1544-1555.

HANGER, D. P., BYERS, H. L., WRAY, S., LEUNG, K.-Y., SAXTON, M. J., SEEREERAM, A., REYNOLDS, C. H., WARD, M. A. & ANDERTON, B. H. 2007. Novel Phosphorylation Sites in Tau from Alzheimer Brain Support a Role for Casein Kinase 1 in Disease Pathogenesis. *Journal of Biological Chemistry,* 282**,** 23645-23654.

HANGER, D. P., HUGHES, K., WOODGETT, J. R., BRION, J.-P. & ANDERTON, B. H. 1992. Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: Generation of paired helical filament epitopes and neuronal localisation of the kinase. *Neuroscience Letters,* 147**,** 58-62.

HANSEN, M., TAUBERT, S., CRAWFORD, D., LIBINA, N., LEE, S.-J. & KENYON, C. 2007. Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. *Aging Cell,* 6**,** 95-110.

HARDY, J. & SELKOE, D. J. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science,* 297**,** 353-356.

HARMAN, D. 1955. *Aging: a theory based on free radical and radiation chemistry*, University of California Radiation Laboratory.

HARMAN, D. 2009. Origin and evolution of the free radical theory of aging: a brief personal history, 1954–2009. *Biogerontology,* 10, 773-781.

HARRIS, D. T., KALLMAN, B. R., MULLANEY, B. C. & SCOTT, K. 2015. Representations of taste modality in the Drosophila brain. *Neuron,* 86, 1449-1460.

HARRISON, D. E., STRONG, R., SHARP, Z. D., NELSON, J. F., ASTLE, C. M., FLURKEY, K., NADON, N. L., WILKINSON, J. E., FRENKEL, K., CARTER, C. S., PAHOR, M., JAVORS, M. A., FERNANDEZ, E. & MILLER, R. A. 2009. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature,* 460, 392-395.

HATFIELD, C. F., HERBERT, J., VAN SOMEREN, E. J. W., HODGES, J. R. & HASTINGS, M. H. 2004. Disrupted daily activity/rest cycles in relation to daily cortisol rhythms of home- dwelling patients with early Alzheimer's dementia. *Brain,* 127, 1061-1074.

HAY, N. & SONENBERG, N. 2004. Upstream and downstream of mTOR. *Genes & Development,* 18, 1926-1945.

HE, C., SUMPTER, J., RHEA & LEVINE, B. 2012. Exercise induces autophagy in peripheral tissues and in the brain. *Autophagy,* 8, 1548-1551.

HEIMANS, J. J., VERMORKEN, J. B., WOLBERS, J. G., EELTINK, C. M., MEIJER, O. W., TAPHOORN, M. J. & BEIJNEN, J. H. 1994. Paclitaxel (Taxol) concentrations in brain tumor tissue. *Ann Oncol,* 5, 951-3.

HEISE, C., GARDONI, F., CULOTTA, L., DILUCA, M., VERPELLI, C. & SALA, C. 2014. Elongation factor-2 phosphorylation in dendrites and the regulation of dendritic mRNA translation in neurons. *Frontiers in Cellular Neuroscience,* 8.

HEITMAN, J., MOVVA, N. R. & HALL, M. N. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science,* 253, 905-909.

HEMPEN, B. & BRION, J.-P. 1996. Reduction of Acetylated α-Tubulin Immunoreactivity in Neurofibrillary Tangle-bearing Neurons in Alzheimer's Disease. *Journal of Neuropathology & Experimental Neurology,* 55, 964-972.

HIMMLER, A., DRECHSEL, D., KIRSCHNER, M. W. & MARTIN, D. 1989. Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. *Molecular and Cellular Biology,* 9, 1381-1388.

HIRSCH, J. & BOUDREAU, J. C. 1958. Studies in experimental behavior genetics: I. The heritability of phototaxis in a population of *Drosophila Melanogaster*. *Journal of Comparative and Physiological Psychology,* 51, 647-651.

HOLMES, C., BOCHE, D., WILKINSON, D., YADEGARFAR, G., HOPKINS, V., BAYER, A., JONES, R. W., BULLOCK, R., LOVE, S. & NEAL, J. W. 2008. Long-term effects of Aβ< sub> 42</sub> immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. *The Lancet,* 372, 216-223.

HOOVER, B. R., REED, M. N., SU, J., PENROD, R. D., KOTILINEK, L. A., GRANT, M. K., PITSTICK, R., CARLSON, G. A., LANIER, L. M., YUAN, L.-L., ASHE, K. H. & LIAO, D. 2010. Tau Mislocalization to Dendritic Spines Mediates Synaptic Dysfunction Independently of Neurodegeneration. *Neuron,* 68, 1067-1081.

HOUTKOOPER, R. H., ARGMANN, C., HOUTEN, S. M., CANTÓ, C., JENINGA, E. H., ANDREUX, P. A., THOMAS, C., DOENLEN, R., SCHOONJANS, K. & AUWERX, J. 2011. The metabolic footprint of aging in mice. *Scientific Reports,* 1**,** 134.

HUTTON, M., LENDON, C. L., RIZZU, P., BAKER, M., FROELICH, S., HOULDEN, H., PICKERING-BROWN, S., CHAKRAVERTY, S., ISAACS, A., GROVER, A., HACKETT, J., ADAMSON, J., LINCOLN, S., DICKSON, D., DAVIES, P., PETERSEN, R. C., STEVENS, M., DE GRAAFF, E., WAUTERS, E., VAN BAREN, J., HILLEBRAND, M., JOOSSE, M., KWON, J. M., NOWOTNY, P., CHE, L. K., NORTON, J., MORRIS, J. C., REED, L. A., TROJANOWSKI, J., BASUN, H., LANNFELT, L., NEYSTAT, M., FAHN, S., DARK, F., TANNENBERG, T., DODD, P. R., HAYWARD, N., KWOK, J. B. J., SCHOFIELD, P. R., ANDREADIS, A., SNOWDEN, J., CRAUFURD, D., NEARY, D., OWEN, F., OOSTRA, B. A., HARDY, J., GOATE, A., VAN SWIETEN, J., MANN, D., LYNCH, T. & HEUTINK, P. 1998. Association of missense and 5[prime]-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature,* 393**,** 702-705.

IIJIMA-ANDO, K., ZHAO, L., GATT, A., SHENTON, C. & IIJIMA, K. 2010. A DNA damage-activated checkpoint kinase phosphorylates tau and enhances tau-induced neurodegeneration. *Hum Mol Genet,* 19**,** 1930-8.

IIJIMA, K., GATT, A. & IIJIMA-ANDO, K. 2010. Tau Ser262 phosphorylation is critical for Abeta42-induced tau toxicity in a transgenic Drosophila model of Alzheimer's disease. *Hum Mol Genet,* 19**,** 2947-57.

IKEDA, M., SHOJI, M., KAWARAI, T., KAWARABAYASHI, T., MATSUBARA, E., MURAKAMI, T., SASAKI, A., TOMIDOKORO, Y., IKARASHI, Y., KURIBARA, H., ISHIGURO, K., HASEGAWA, M., YEN, S. H., CHISHTI, M. A., HARIGAYA, Y., ABE, K., OKAMOTO, K., ST GEORGE-HYSLOP, P. & WESTAWAY, D. 2005. Accumulation of filamentous tau in the cerebral cortex of human tau R406W transgenic mice. *Am J Pathol,* 166**,** 521-31.

IQBAL, K., DEL C. ALONSO, A., CHEN, S., CHOHAN, M. O., EL-AKKAD, E., GONG, C.-X., KHATOON, S., LI, B., LIU, F., RAHMAN, A., TANIMUKAI, H. & GRUNDKE-IQBAL, I. 2005. Tau pathology in Alzheimer disease and other tauopathies. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1739**,** 198-210.

ISHIHARA, T., HONG, M., ZHANG, B., NAKAGAWA, Y., LEE, M. K., TROJANOWSKI, J. Q. & LEE, V. M. Y. 1999. Age-Dependent Emergence and Progression of a Tauopathy in Transgenic Mice Overexpressing the Shortest Human Tau Isoform. *Neuron,* 24**,** 751-762.

JACKSON, G. R., WIEDAU-PAZOS, M., SANG, T. K., WAGLE, N., BROWN, C. A., MASSACHI, S. & GESCHWIND, D. H. 2002. Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in Drosophila. *Neuron,* 34**,** 509-19.

JEGANATHAN, S., HASCHER, A., CHINNATHAMBI, S., BIERNAT, J., MANDELKOW, E.-M. & MANDELKOW, E. 2008. Proline-directed Pseudo-phosphorylation at AT8 and PHF1 Epitopes Induces a Compaction of the Paperclip Folding of Tau and Generates a Pathological (MC-1) Conformation. *Journal of Biological Chemistry,* 283**,** 32066-32076.

JEGANATHAN, S., VON BERGEN, M., BRUTLACH, H., STEINHOFF, H.-J. & MANDELKOW, E. 2006. Global Hairpin Folding of Tau in Solution. *Biochemistry,* 45**,** 2283-2293.

JEWELL, J. L. & GUAN, K.-L. 2013. Nutrient signaling to mTOR and cell growth. *Trends in biochemical sciences,* 38**,** 233-242.

KANAAN, N. M., MORFINI, G. A., LAPOINTE, N. E., PIGINO, G. F., PATTERSON, K. R., SONG, Y., ANDREADIS, A., FU, Y., BRADY, S. T. & BINDER, L. I. 2011. Pathogenic Forms of Tau Inhibit Kinesin-Dependent Axonal Transport through a Mechanism Involving Activation of Axonal Phosphotransferases. *The Journal of Neuroscience,* 31**,** 9858-9868.

KAPAHI, P., ZID, B. M., HARPER, T., KOSLOVER, D., SAPIN, V. & BENZER, S. 2004. Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway. *Current Biology,* 14**,** 885-890.

KEITH, C. T. & SCHREIBER, S. L. 1995. PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. *Science,* 270**,** 50.

KELLER, J. N. & MARKESBERY, W. R. 2000. Decreased levels of proteasome activity and proteasome expression in aging spinal cord. *Neuroscience,* 98**,** 149-156.

KEMPF, M., CLEMENT, A., FAISSNER, A., LEE, G. & BRANDT, R. 1996. Tau Binds to the Distal Axon Early in Development of Polarity in a Microtubule- and Microfilament-Dependent Manner. *The Journal of Neuroscience,* 16**,** 5583-5592.

KFOURY, N., HOLMES, B. B., JIANG, H., HOLTZMAN, D. M. & DIAMOND, M. I. 2012. Trans-cellular Propagation of Tau Aggregation by Fibrillar Species. *Journal of Biological Chemistry,* 287**,** 19440-19451.

KHATOON, S., GRUNDKE-IQBAL, I. & IQBAL, K. 1992. Brain Levels of Microtubule-Associated Protein τ Are Elevated in Alzheimer's Disease: A Radioimmuno-Slot-Blot Assay for Nanograms of the Protein. *Journal of Neurochemistry,* 59**,** 750-753.

KHURANA, V., ELSON-SCHWAB, I., FULGA, T. A., SHARP, K. A., LOEWEN, C. A., MULKEARNS, E., TYYNELÄ, J., SCHERZER, C. R. & FEANY, M. B. 2010. Lysosomal Dysfunction Promotes Cleavage and Neurotoxicity of Tau *In Vivo*. *PLoS Genet,* 6**,** e1001026.

KHURANA, V., LU, Y. R., STEINHILB, M. L., OLDHAM, S., SHULMAN, J. M. & FEANY, M. B. 2006. TOR-mediated cell-cycle activation causes neurodegeneration in a Drosophila tauopathy model. *Current Biology,* 16**,** 230-241.

KIRKWOOD, T. B. L. & AUSTAD, S. N. 2000. Why do we age? *Nature,* 408**,** 233-238.

KLIONSKY, D. J. 2005. Autophagy. *Current Biology,* 15**,** R282-R283.

KLOEDEN, P., RÖSSLER, R. & RÖSSLER, O. 1993. Timekeeping in genetically programmed aging. *Experimental gerontology,* 28**,** 109-118.

KNAPP, J.-M., CHUNG, P. & SIMPSON, J. H. 2015. Generating customized transgene landing sites and multi-transgene arrays in Drosophila using phiC31 integrase. *Genetics,* 199**,** 919-934.

KOH, K., EVANS, J. M., HENDRICKS, J. C. & SEHGAL, A. 2006. A Drosophila model for age-associated changes in sleep: wake cycles. *Proceedings of the National Academy of Sciences,* 103**,** 13843-13847.

KOLKER, D. E., FUKUYAMA, H., HUANG, D. S., TAKAHASHI, J. S., HORTON, T. H. & TUREK, F. W. 2003. Aging alters circadian and light-induced expression of clock genes in golden hamsters. *Journal of Biological Rhythms,* 18**,** 159-169.

KONDRATOV, R. V., KONDRATOVA, A. A., GORBACHEVA, V. Y., VYKHOVANETS, O. V. & ANTOCH, M. P. 2006. Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes & development,* 20**,** 1868-1873.

KONOPKA, R. J. & BENZER, S. 1971. Clock Mutants of Drosophila melanogaster. *Proceedings of the National Academy of Sciences,* 68**,** 2112-2116.

KOSIK, K. S., ORECCHIO, L. D., BAKALIS, S. & NEVE, R. L. 1989. Developmentally regulated expression of specific tau sequences. *Neuron,* 2**,** 1389-1397.

KOSMIDIS, S., GRAMMENOUDI, S., PAPANIKOLOPOULOU, K. & SKOULAKIS, E. M. 2010. Differential effects of Tau on the integrity and function of neurons essential for learning in Drosophila. *J Neurosci,* 30**,** 464-77.

KRAMER, J. M. & STAVELEY, B. E. 2003. GAL4 causes developmental defects and apoptosis when expressed in the developing eye of Drosophila melanogaster. *Genetics and molecular research : GMR* [Online], 2. [Accessed 2003].

KRIPKE, D. F. & WYBORNEY, V. G. 1980. Lithium slows rat circadian activity rhythms. *Life sciences,* 26**,** 1319-1321.

KRISHNAN, N., KRETZCHMAR, D., RAKSHIT, K., CHOW, E. & GIEBULTOWICZ, J. M. 2009. The circadian clock gene period extends healthspan in aging Drosophila melanogaster.

KSIEZAK-REDING, H., LIU, W.-K. & YEN, S.-H. 1992. Phosphate analysis and dephosphorylation of modified tau associated with paired helical filaments. *Brain Research,* 597**,** 209-219.

LAPLANTE, M. & SABATINI, DAVID M. 2012. mTOR Signaling in Growth Control and Disease. *Cell,* 149**,** 274-293.

LE BOURG, É. & BUECHER, C. 2002. Learned suppression of photopositive tendencies inDrosophila melanogaster. *Animal learning & behavior,* 30**,** 330-341.

LEE, G., THANGAVEL, R., SHARMA, V. M., LITERSKY, J. M., BHASKAR, K., FANG, S. M., DO, L. H., ANDREADIS, A., VAN HOESEN, G. & KSIEZAK-REDING, H. 2004. Phosphorylation of Tau by Fyn: Implications for Alzheimer's Disease. *The Journal of Neuroscience,* 24**,** 2304-2312.

LEONTIEVA, O. V., PASZKIEWICZ, G. M. & BLAGOSKLONNY, M. V. 2014. Fasting levels of hepatic p-S6 are increased in old mice. *Cell Cycle,* 13**,** 2656-2659.

LESAUTER, J. & SILVER, R. 1993. Lithium lengthens the period of circadian rhythms in lesioned hamsters bearing SCN grafts. *Biological psychiatry,* 34**,** 75-83.

LEVINE, B., PACKER, M. & CODOGNO, P. 2015. Development of autophagy inducers in clinical medicine. *The Journal of clinical investigation,* 125**,** 14-24.

LEVY, E., CARMAN, M. D., FERNANDEZ-MADRID, I. J., POWER, M. D., LIEBERBURG, I., VAN DUINEN, S. G., BOTS, G., LUYENDIJK, W. & FRANGIONE, B. 1990. Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science,* 248**,** 1124-1126.

LEWIS, J., MCGOWAN, E., ROCKWOOD, J., MELROSE, H., NACHARAJU, P., VAN SLEGTENHORST, M., GWINN-HARDY, K., MURPHY, M. P., BAKER, M., YU, X., DUFF, K., HARDY, J., CORRAL, A., LIN, W.-L., YEN, S.-H., DICKSON, D. W., DAVIES, P. & HUTTON, M. 2000. Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nat Genet,* 25**,** 402-405.

LI, H. & SATINOFF, E. 1998. Fetal tissue containing the suprachiasmatic nucleus restores multiple circadian rhythms in old rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 275**,** R1735-R1744.

LI, X., ALAFUZOFF, I., SOININEN, H., WINBLAD, B. & PEI, J. J. 2005. Levels of mTOR and its downstream targets 4E-BP1, eEF2, and eEF2 kinase in relationships with tau in Alzheimer's disease brain. *Febs Journal,* 272**,** 4211-4220.

LINDWALL, G. & COLE, R. D. 1984. Phosphorylation affects the ability of tau protein to promote microtubule assembly. *Journal of Biological Chemistry,* 259**,** 5301-5.

LINFORD, N. J., BEYER, R. P., GOLLAHON, K., KRAJCIK, R. A., MALLOY, V. L., DEMAS, V., BURMER, G. C. & RABINOVITCH, P. S. 2007. Transcriptional response to aging and caloric restriction in heart and adipose tissue. *Aging cell,* 6**,** 673-688.

LIPINSKI, M. M., ZHENG, B., LU, T., YAN, Z., PY, B. F., NG, A., XAVIER, R. J., LI, C., YANKNER, B. A., SCHERZER, C. R. & YUAN, J. 2010. Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. *PNAS,* 107**,** 14164-14169.

LIU, F., GRUNDKE-IQBAL, I., IQBAL, K. & GONG, C.-X. 2005. Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *European Journal of Neuroscience,* 22**,** 1942-1950.

LONG, D. M., BLAKE, M. R., DUTTA, S., HOLBROOK, S. D., KOTWICA-ROLINSKA, J., KRETZSCHMAR, D. & GIEBULTOWICZ, J. M. 2014. Relationships between the Circadian System and Alzheimer's Disease-Like Symptoms in Drosophila. *PLoS ONE,* 9**,** e106068.

LOVESTONE, S., HARTLEY, C. L., PEARCE, J. & ANDERTON, B. H. 1996. Phosphorylation of tau by glycogen synthase kinase-3β in intact mammalian cells: The effects on the organization and stability of microtubules. *Neuroscience,* 73**,** 1145-1157.

LU, T., PAN, Y., KAO, S.-Y., LI, C., KOHANE, I., CHAN, J. & YANKNER, B. A. 2004. Gene regulation and DNA damage in the ageing human brain. *Nature,* 429**,** 883-891.

LUCAS, J. J., HERNÁNDEZ, F., GÓMEZ- RAMOS, P., MORÁN, M. A., HEN, R. & AVILA, J. 2001. Decreased nuclear β- catenin, tau hyperphosphorylation and neurodegeneration in GSK- 3β conditional transgenic mice. *The EMBO Journal,* 20**,** 27-39.

LUO, W. & SEHGAL, A. 2012. Regulation of circadian behavioral output via a MicroRNA-JAK/STAT circuit. *Cell,* 148**,** 765-779.

MA, J. F., HUANG, Y., CHEN, S. D. & HALLIDAY, G. 2010. Immunohistochemical evidence for macroautophagy in neurones and endothelial cells in Alzheimer's disease. *Neuropathol Appl Neurobiol,* 36**,** 312-9.

MAEDA, J., JI, B., IRIE, T., TOMIYAMA, T., MARUYAMA, M., OKAUCHI, T., STAUFENBIEL, M., IWATA, N., ONO, M., SAIDO, T. C., SUZUKI, K., MORI, H., HIGUCHI, M. & SUHARA, T. 2007. Longitudinal, quantitative assessment of amyloid, neuroinflammation, and anti-amyloid treatment in a living mouse model of Alzheimer's disease enabled by positron emission tomography. *J Neurosci,* 27**,** 10957-68.

MAH, V. H., ESKIN, T. A., KAZEE, A. M., LAPHAM, L. & HIGGINS, G. A. 1992. In situ hybridization of calcium/calmodulin dependent protein kinase II and tau mRNAs: species differences and relative preservation in Alzheimer's disease. *Molecular Brain Research,* 12**,** 85-94.

MAJUMDER, S., RICHARDSON, A., STRONG, R. & ODDO, S. 2011. Inducing Autophagy by Rapamycin Before, but Not After, the Formation of Plaques and Tangles Ameliorates Cognitive Deficits. *PLoS ONE,* 6**,** e25416.

MAKRIDES, S. C. 1983. PROTEIN SYNTHESIS AND DEGRADATION DURING AGING AND SENESCENCE. *Biological Reviews,* 58**,** 343-422.

MALIK, B. R. & HODGE, J. J. 2014. Drosophila adult olfactory shock learning. *Journal of visualized experiments: JoVE*.

MARTIN, J., MARLER, M., SHOCHAT, T. & ANCOLI-ISRAEL, S. 2000. Circadian rhythms of agitation in institutionalized patients with Alzheimer's disease. *Chronobiology International,* 17**,** 405-418.

MARTINEK, S., INONOG, S., MANOUKIAN, A. S. & YOUNG, M. W. 2001. A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. *Cell,* 105**,** 769-779.

MASLIAH, E., MALLORY, M., ALFORD, M., DETERESA, R., HANSEN, L. A., MCKEEL, D. W. & MORRIS, J. C. 2001. Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. *Neurology,* 56**,** 127-129.

MASTERS, C. L., SIMMS, G., WEINMAN, N. A., MULTHAUP, G., MCDONALD, B. L. & BEYREUTHER, K. 1985. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proceedings of the National Academy of Sciences,* 82**,** 4245-4249.

MATECIC, M., SMITH JR, D. L., PAN, X., MAQANI, N., BEKIRANOV, S., BOEKE, J. D. & SMITH, J. S. 2010. A microarray-based genetic screen for yeast chronological aging factors. *PLoS genetics,* 6**,** e1000921.

MATSUO, E. S., SHIN, R.-W., BILLINGSLEY, M. L., VAN DEVOORDE, A., O'CONNOR, M., TROJANOWSKI, J. Q. & LEE, V. M. 1994. Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. *Neuron,* 13**,** 989-1002.

MCCAY, C., CROWELL, M. F. & MAYNARD, L. 1935. The Effect of Retarded Growth Upon the Length of Life Span and Upon the Ultimate Body Size One Figure. *The Journal of Nutrition,* 10**,** 63-79.

MCGUIRE, S., LE, P., OSBORN, A., MATSUMOTO, K. & DAVIS, R. Temporal and regional gene expression targeting with the conventional GAL4/UAS system in Drosophila. A Dros Res Conf, 2003. 153.

MCGUIRE, S. E., DESHAZER, M. & DAVIS, R. L. 2005. Thirty years of olfactory learning and memory research in Drosophila melanogaster. *Progress in Neurobiology,* 76**,** 328-347.

MCGUIRE, S. E., LE, P. T. & DAVIS, R. L. 2001. The Role of *Drosophila* Mushroom Body Signaling in Olfactory Memory. *Science,* 293**,** 1330-1333.

MEANS, J. C., VENKATESAN, A., GERDES, B., FAN, J.-Y., BJES, E. S. & PRICE, J. L. 2015. Drosophila Spaghetti and Doubletime Link the Circadian Clock and Light to Caspases, Apoptosis and Tauopathy. *PLoS Genet,* 11**,** e1005171.

MELÉNDEZ, A., TALLOCZY, Z., SEAMAN, M., ESKELINEN, E.-L., HALL, D. H. & LEVINE, B. 2003. Autophagy genes are essential for dauer development and life-span extension in C. elegans. *Science,* 301**,** 1387-1391.

MERSHIN, A., PAVLOPOULOS, E., FITCH, O., BRADEN, B. C., NANOPOULOS, D. V. & SKOULAKIS, E. M. C. 2004. Learning and Memory Deficits Upon TAU Accumulation in Drosophila Mushroom Body Neurons. *Learning & Memory,* 11**,** 277-287.

MEYER, T. E., KOVÁCS, S. J., EHSANI, A. A., KLEIN, S., HOLLOSZY, J. O. & FONTANA, L. 2006. Long-term caloric restriction ameliorates the decline in diastolic function in humans. *Journal of the American College of Cardiology,* 47**,** 398-402.

MILLER, R. A., HARRISON, D. E., ASTLE, C. M., BAUR, J. A., BOYD, A. R., DE CABO, R., FERNANDEZ, E., FLURKEY, K., JAVORS, M. A., NELSON, J. F., ORIHUELA, C. J., PLETCHER, S., SHARP, Z. D., SINCLAIR, D., STARNES, J. W., WILKINSON, J. E., NADON, N. L. & STRONG, R. 2011. Rapamycin, But Not Resveratrol or Simvastatin, Extends Life Span of Genetically Heterogeneous Mice. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 66A**,** 191-201.

MIZUSHIMA, N. 2007. Autophagy: process and function. *Genes & Development,* 21**,** 2861-2873.

MONDRAGÓN-RODRÍGUEZ, S., BASURTO-ISLAS, G., SANTA-MARIA, I., MENA, R., BINDER, L. I., AVILA, J., SMITH, M. A., PERRY, G. & GARCÍA-SIERRA, F. 2008. Cleavage and conformational changes of tau protein follow phosphorylation during Alzheimer's disease. *International Journal of Experimental Pathology,* 89**,** 81-90.

MORSELLI, E., MAIURI, M., MARKAKI, M., MEGALOU, E., PASPARAKI, A., PALIKARAS, K., CRIOLLO, A., GALLUZZI, L., MALIK, S. & VITALE, I. 2010. Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy. *Cell death & disease,* 1**,** e10.

MUDHER, A., SHEPHERD, D., NEWMAN, T., MILDREN, P., JUKES, J., SQUIRE, A., MEARS, A., BERG, S., MACKAY, D. & ASUNI, A. 2004. GSK-3β inhibition reverses axonal transport defects and behavioural phenotypes in Drosophila. *Molecular psychiatry,* 9**,** 522-530.

MUSIEK, E. S., XIONG, D. D. & HOLTZMAN, D. M. 2015. Sleep, circadian rhythms, and the pathogenesis of Alzheimer disease. *Experimental & molecular medicine,* 47**,** e148.

NACHARAJU, P., LEWIS, J., EASSON, C., YEN, S., HACKETT, J., HUTTON, M. & YEN, S.-H. 1999. Accelerated filament formation from tau protein with specific FTDP-17 missense mutations. *FEBS Letters,* 447**,** 195-199.

NEVE, R. L., HARRIS, P., KOSIK, K. S., KURNIT, D. M. & DONLON, T. A. 1986. Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Molecular Brain Research,* 1**,** 271-280.

NIGHORN, A., HEALY, M. J. & DAVIS, R. L. 1991. The cyclic AMP phosphodiesterase encoded by the drosophila dunce gene is concentrated in the mushroom body neuropil. *Neuron,* 6**,** 455-467.

NISHIMURA, I., YANG, Y. & LU, B. 2004. PAR-1 Kinase Plays an Initiator Role in a Temporally Ordered Phosphorylation Process that Confers Tau Toxicity in Drosophila. *Cell,* 116**,** 671-682.

NOBLE, W., HANGER, D., MILLER, C. & LOVESTONE, S. 2013. The Importance of Tau Phosphorylation for Neurodegenerative Diseases. *Frontiers in Neurology,* 4.

NOBLE, W., PLANEL, E., ZEHR, C., OLM, V., MEYERSON, J., SULEMAN, F., GAYNOR, K., WANG, L., LAFRANCOIS, J., FEINSTEIN, B., BURNS, M., KRISHNAMURTHY, P., WEN, Y., BHAT, R., LEWIS, J., DICKSON, D. & DUFF, K. 2005. Inhibition of glycogen synthase kinase-3 by lithium correlates with reduced tauopathy and degeneration in vivo. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 6990-6995.

NUKINA, N., KOSIK, K. S. & SELKOE, D. J. 1988. The monoclonal antibody, Alz 50, recognizes tau proteins in Alzheimer's disease brain. *Neuroscience Letters,* 87**,** 240-246.

ODDO, S., CACCAMO, A., SHEPHERD, J. D., MURPHY, M. P., GOLDE, T. E., KAYED, R., METHERATE, R., MATTSON, M. P., AKBARI, Y. & LAFERLA, F. M. 2003. Triple-Transgenic Model of Alzheimer's Disease with Plaques and Tangles: Intracellular Aβ and Synaptic Dysfunction. *Neuron,* 39**,** 409-421.

OZCELIK, S., FRASER, G., CASTETS, P., SCHAEFFER, V., SKACHOKOVA, Z., BREU, K., CLAVAGUERA, F., SINNREICH, M., KAPPOS, L. & GOEDERT, M. 2013. Rapamycin attenuates the progression of tau pathology in P301S tau transgenic mice. *PloS one,* 8**,** e62459.

PANDA, D., SAMUEL, J. C., MASSIE, M., FEINSTEIN, S. C. & WILSON, L. 2003. Differential regulation of microtubule dynamics by three- and four-repeat tau: Implications for the onset of neurodegenerative disease. *Proceedings of the National Academy of Sciences,* 100**,** 9548-9553.

PANDA, S., HOGENESCH, J. B. & KAY, S. A. 2002. Circadian rhythms from flies to human. *Nature,* 417**,** 329-335.

PARK, J. H., HELFRICH-FÖRSTER, C., LEE, G., LIU, L., ROSBASH, M. & HALL, J. C. 2000. Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. *Proceedings of the National Academy of Sciences,* 97**,** 3608-3613.

PARTRIDGE, L., ALIC, N., BJEDOV, I. & PIPER, M. D. W. 2011. Ageing in Drosophila: The role of the insulin/Igf and TOR signalling network. *Experimental Gerontology,* 46, 376-381.

PARTRIDGE, L. & GEMS, D. 2007. Benchmarks for ageing studies. *Nature,* 450, 165-167.

PARTRIDGE, L., PIPER, M. D. W. & MAIR, W. 2005. Dietary restriction in Drosophila. *Mechanisms of Ageing and Development,* 126, 938-950.

PEI, J.-J., TANAKA, T., TUNG, Y.-C., BRAAK, E., IQBAL, K. & GRUNDKE-IQBAL, I. 1997. Distribution, Levels, and Activity of Glycogen Synthase Kinase-3 in the Alzheimer Disease Brain. *Journal of Neuropathology & Experimental Neurology,* 56, 70-78.

PERRY, G., FRIEDMAN, R., SHAW, G. & CHAU, V. 1987. Ubiquitin is detected in neurofibrillary tangles and senile plaque neurites of Alzheimer disease brains. *Proceedings of the National Academy of Sciences,* 84, 3033-3036.

PICKFORD, F., MASLIAH, E., BRITSCHGI, M., LUCIN, K., NARASIMHAN, R., JAEGER, P. A., SMALL, S., SPENCER, B., ROCKENSTEIN, E., LEVINE, B. & WYSS-CORAY, T. 2008. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. *J Clin Invest,* 118, 2190-9.

POORKAJ, P., BIRD, T. D., WIJSMAN, E., NEMENS, E., GARRUTO, R. M., ANDERSON, L., ANDREADIS, A., WIEDERHOLT, W. C., RASKIND, M. & SCHELLENBERG, G. D. 1998. Tau is a candidate gene for chromosome 17 frontotemporal dementia. *Annals of Neurology,* 43, 815-825.

POWERS, R. W., KAEBERLEIN, M., CALDWELL, S. D., KENNEDY, B. K. & FIELDS, S. 2006. Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes & Development,* 20, 174-184.

PRICE, J. L., BLAU, J., ROTHENFLUH, A., ABODEELY, M., KLOSS, B. & YOUNG, M. W. 1998. double-time Is a Novel Drosophila Clock Gene that Regulates PERIOD Protein Accumulation. *Cell,* 94, 83-95.

PRINCE, M., WIMO, A., GUERCHET, M., ALI, G.-C., WU, Y.-T. & PRINA, M. 2015. World Alzheimer Report 2015, The Global Impact of Dementia: An analysis of prevalence, incidence, cost and trends. *Alzheimer's Disease International.*

PRINZ, P. N., PESKIND, E. R., VITALIANO, P. P., RASKIND, M. A., EISDORFER, C., ZEMCUZNIKOV, H. N. & GERBER, C. J. 1982. Changes in the Sleep and Waking EEGs of Nondemented and Demented Elderly Subjects. *Journal of the American Geriatrics Society,* 30, 86-92.

PROFENNO, L. A., PORSTEINSSON, A. P. & FARAONE, S. V. 2010. Meta-Analysis of Alzheimer's Disease Risk with Obesity, Diabetes, and Related Disorders. *Biological Psychiatry,* 67, 505-512.

PROUD, C. G. 2014. Control of the translational machinery by amino acids. *The American Journal of Clinical Nutrition,* 99, 231S-236S.

PYO, J.-O., YOO, S.-M., AHN, H.-H., NAH, J., HONG, S.-H., KAM, T.-I., JUNG, S. & JUNG, Y.-K. 2013. Overexpression of Atg5 in mice activates autophagy and extends lifespan. *Nat Commun,* 4.

QURAISHE, S., COWAN, C. M. & MUDHER, A. 2013. NAP (davunetide) rescues neuronal dysfunction in a Drosophila model of tauopathy. *Mol Psychiatry,* 18**,** 834-842.

REFINETTI, R., CORNÉLISSEN G., & HALBERG F. 2007. Procedures for numerical analysis of circadian rhythms. *Biological Rhythm Research*, 38, 275-325.

REITER, L. T., POTOCKI, L., CHIEN, S., GRIBSKOV, M. & BIER, E. 2001. A Systematic Analysis of Human Disease-Associated Gene Sequences In Drosophila melanogaster. *Genome Research,* 11**,** 1114-1125.

RENN, S. C. P., PARK, J. H., ROSBASH, M., HALL, J. C. & TAGHERT, P. H. 1999. A pdf Neuropeptide Gene Mutation and Ablation of PDF Neurons Each Cause Severe Abnormalities of Behavioral Circadian Rhythms in Drosophila. *Cell,* 99**,** 791-802.

RICE, K., KINNARD, R., WRIGHT, G. & BLOUGH, E. 2005. Aging alters vascular mechanotransduction: pressure-induced regulation of p70S6k in the rat aorta. *Mechanisms of ageing and development,* 126**,** 1213-1222.

ROBIDA-STUBBS, S., GLOVER-CUTTER, K., LAMMING, DUDLEY W., MIZUNUMA, M., NARASIMHAN, SRI D., NEUMANN-HAEFELIN, E., SABATINI, DAVID M. & BLACKWELL, T. K. 2012. TOR Signaling and Rapamycin Influence Longevity by Regulating SKN-1/Nrf and DAF-16/FoxO. *Cell Metabolism,* 15**,** 713-724.

RODRÍGUEZ-NAVARRO, J. A., RODRÍGUEZ, L., CASAREJOS, M. J., SOLANO, R. M., GÓMEZ, A., PERUCHO, J., CUERVO, A. M., DE YÉBENES, J. G. & MENA, M. A. 2010. Trehalose ameliorates dopaminergic and tau pathology in parkin deleted/tau overexpressing mice through autophagy activation. *Neurobiology of disease,* 39**,** 423-438.

ROGAWSKI, M. A. & WENK, G. L. 2003. The Neuropharmacological Basis for the Use of Memantine in the Treatment of Alzheimer's Disease. *CNS Drug Reviews,* 9**,** 275-308.

ROHN, T. T., HEAD, E., SU, J. H., ANDERSON, A. J., BAHR, B. A., COTMAN, C. W. & CRIBBS, D. H. 2001. Correlation between Caspase Activation and Neurofibrillary Tangle Formation in Alzheimer's Disease. *The American Journal of Pathology,* 158**,** 189-198.

ROY, B. & JACKSON, G. R. 2014. Interactions between Tau and α-synuclein augment neurotoxicity in a Drosophila model of Parkinson's disease. *Human Molecular Genetics.*

RUBINSZTEIN, DAVID C., MARIÑO, G. & KROEMER, G. 2011. Autophagy and Aging. *Cell,* 146**,** 682-695.

SABATINI, D. M., ERDJUMENT-BROMAGE, H., LUI, M., TEMPST, P. & SNYDER, S. H. 1994. RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell,* 78**,** 35-43.

SANCHEZ-SORIANO, N., TRAVIS, M., DAJAS-BAILADOR, F., GONÇALVES-PIMENTEL, C., WHITMARSH, A. J. & PROKOP, A. 2009. Mouse ACF7 and drosophila short stop modulate filopodia formation and microtubule organisation during neuronal growth. *J Cell Sci,* 122**,** 2534-2542.

SANTACRUZ, K., LEWIS, J., SPIRES, T., PAULSON, J., KOTILINEK, L., INGELSSON, M., GUIMARAES, A., DETURE, M., RAMSDEN, M., MCGOWAN, E., FORSTER, C., YUE, M., ORNE, J., JANUS, C., MARIASH, A., KUSKOWSKI, M., HYMAN, B., HUTTON, M. & ASHE, K. H. 2005. Tau Suppression in a Neurodegenerative Mouse Model Improves Memory Function. *Science,* 309**,** 476-481.

SARBASSOV, D. D., ALI, S. M., KIM, D.-H., GUERTIN, D. A., LATEK, R. R., ERDJUMENT-BROMAGE, H., TEMPST, P. & SABATINI, D. M. 2004. Rictor, a Novel Binding Partner of mTOR, Defines a Rapamycin-Insensitive and Raptor-Independent Pathway that Regulates the Cytoskeleton. *Current Biology,* 14**,** 1296-1302.

SARBASSOV, D. D., ALI, S. M., SENGUPTA, S., SHEEN, J.-H., HSU, P. P., BAGLEY, A. F., MARKHARD, A. L. & SABATINI, D. M. 2006. Prolonged Rapamycin Treatment Inhibits mTORC2 Assembly and Akt/PKB. *Molecular Cell,* 22**,** 159-168.

SCHEUNER, D., ECKMAN, C., JENSEN, M., SONG, X., CITRON, M., SUZUKI, N., BIRD, T., HARDY, J., HUTTON, M. & KUKULL, W. 1996. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature medicine,* 2**,** 864-870.

SCHINDOWSKI, K., BRETTEVILLE, A., LEROY, K., BÉGARD, S., BRION, J.-P., HAMDANE, M. & BUÉE, L. 2006. Alzheimer's Disease-Like Tau Neuropathology Leads to Memory Deficits and Loss of Functional Synapses in a Novel Mutated Tau Transgenic Mouse without Any Motor Deficits. *The American Journal of Pathology,* 169**,** 599-616.

SCHNEIDER, A., BIERNAT, J., VON BERGEN, M., MANDELKOW, E. & MANDELKOW, E. M. 1999. Phosphorylation that Detaches Tau Protein from Microtubules (Ser262, Ser214) Also Protects It against Aggregation into Alzheimer Paired Helical Filaments. *Biochemistry,* 38**,** 3549-3558.

SCHWEERS, O., SCHÖNBRUNN-HANEBECK, E., MARX, A. & MANDELKOW, E. 1994. Structural studies of tau protein and Alzheimer paired helical filaments show no evidence for beta-structure. *Journal of Biological Chemistry,* 269**,** 24290-24297.

SCOTT, R. C., JUHÁSZ, G. & NEUFELD, T. P. 2007. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Current Biology,* 17**,** 1-11.

SCUTERI, A., NICOLINI, G., MILOSO, M., BOSSI, M., CAVELLTI, G., WINEBANK, A. & TREDICI, G. 2006. Paclitaxel Toxicity in Post-mitotic Dorsal Root Ganglion (DRG) Cells. *Anticancer Research,* 26**,** 1065-1070.

SELMAN, C., TULLET, J., WIESER, D., IRVINE, E. & LINGARD, S. 2009. Ribosomal protein S6 Kinase 1 signaling regulates mammalian life span. *Science,* 326**,** 140-144.

SENGUPTA, A., KABAT, J., NOVAK, M., WU, Q., GRUNDKE-IQBAL, I. & IQBAL, K. 1998. Phosphorylation of Tau at Both Thr 231 and Ser 262 Is Required for Maximal Inhibition of Its Binding to Microtubules. *Archives of Biochemistry and Biophysics,* 357**,** 299-309.

SENGUPTA, S., PETERSON, T. R. & SABATINI, D. M. 2010. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Molecular cell,* 40**,** 310-322.

SERGEANT, N., DELACOURTE, A. & BUÉE, L. 2005. Tau protein as a differential biomarker of tauopathies. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1739**,** 179-197.

SEUBERT, P., MAWAL-DEWAN, M., BARBOUR, R., JAKES, R., GOEDERT, M., JOHNSON, G. V., LITERSKY, J. M., SCHENK, D., LIEBERBURG, I. & TROJANOWSKI, J. Q. 1995. Detection of phosphorylated Ser262 in fetal tau, adult tau, and paired helical filament tau. *Journal of Biological Chemistry,* 270**,** 18917-18922.

SEUGNET, L., SUZUKI, Y., STIDD, R. & SHAW, P. J. 2009. Aversive phototaxic suppression: evaluation of a short-term memory assay in Drosophila melanogaster. *Genes, Brain and Behavior,* 8**,** 377-389.

SHIBATA, M., LU, T., FURUYA, T., DEGTEREV, A., MIZUSHIMA, N., YOSHIMORI, T., MACDONALD, M., YANKNER, B. & YUAN, J. 2006. Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. *Journal of Biological Chemistry,* 281**,** 14474-14485.

SHIBATANI, T., NAZIR, M. & WARD, W. F. 1996. Alteration of Rat Liver 20S Proteasome Activities by Age and Food Restriction. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 51A**,** B316-B322.

SHIPTON, O. A., LEITZ, J. R., DWORZAK, J., ACTON, C. E. J., TUNBRIDGE, E. M., DENK, F., DAWSON, H. N., VITEK, M. P., WADE-MARTINS, R., PAULSEN, O. & VARGAS-CABALLERO, M. 2011. Tau Protein Is Required for Amyloid β-Induced Impairment of Hippocampal Long-Term Potentiation. *The Journal of Neuroscience,* 31**,** 1688-1692.

SIMONSEN, A., CUMMING, R. C., BRECH, A., ISAKSON, P., SCHUBERT, D. R. & FINLEY, K. D. 2008. Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistnace in adult Drosophila. *Autophagy,* 4**,** 176-184.

SINADINOS, C., COWAN, C. M., WYTTENBACH, A. & MUDHER, A. 2012. Increased throughput assays of locomotor dysfunction in Drosophila larvae. *J Neurosci Methods,* 203**,** 325-34.

SPILLANTINI, M. G., MURRELL, J. R., GOEDERT, M., FARLOW, M. R., KLUG, A. & GHETTI, B. 1998. Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proceedings of the National Academy of Sciences,* 95**,** 7737-7741.

SPILMAN, P., PODLUTSKAYA, N., HART, M. J., DEBNATH, J., GOROSTIZA, O., BREDESEN, D., RICHARDSON, A., STRONG, R. & GALVAN, V. 2010. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS One,* 5**,** e9979.

SPIRES-JONES, T. L., DE CALIGNON, A., MATSUI, T., ZEHR, C., PITSTICK, R., WU, H.-Y., OSETEK, J. D., JONES, P. B., BACSKAI, B. J., FEANY, M. B., CARLSON, G. A., ASHE, K. H., LEWIS, J. & HYMAN, B. T. 2008. In vivo imaging reveals dissociation between caspase activation and acute neuronal death in tangle-bearing neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 28**,** 862-867.

SPIRES, T. L., ORNE, J. D., SANTACRUZ, K., PITSTICK, R., CARLSON, G. A., ASHE, K. H. & HYMAN, B. T. 2006. Region-specific Dissociation of Neuronal Loss and Neurofibrillary Pathology in a Mouse Model of Tauopathy. *The American Journal of Pathology,* 168**,** 1598-1607.

STADELMANN, C., DECKWERTH, T. L., SRINIVASAN, A., BANCHER, C., BRÜCK, W., JELLINGER, K. & LASSMANN, H. 1999. Activation of Caspase-3 in Single Neurons and Autophagic Granules of Granulovacuolar Degeneration in Alzheimer's Disease: Evidence for Apoptotic Cell Death. *The American Journal of Pathology,* 155**,** 1459-1466.

STEINHILB, M. L., DIAS-SANTAGATA, D., FULGA, T. A., FELCH, D. L. & FEANY, M. B. 2007. Tau phosphorylation sites work in concert to promote neurotoxicity in vivo. *Mol Biol Cell,* 18**,** 5060-8.

STELZMANN, R. A., NORMAN SCHNITZLEIN, H. & REED MURTAGH, F. 1995. An English translation of Alzheimer's 1907 paper,"Über eine eigenartige Erkankung der Hirnrinde". *Clinical anatomy,* 8**,** 429-431.

STERNICZUK, R., DYCK, R. H., LAFERLA, F. M. & ANTLE, M. C. 2010. Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 1. Circadian changes. *Brain research,* 1348**,** 139-148.

STOCKER, H., RADIMERSKI, T., SCHINDELHOLZ, B., WITTWER, F., BELAWAT, P., DARAM, P., BREUER, S., THOMAS, G. & HAFEN, E. 2003. Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. *Nature cell biology,* 5**,** 559-566.

SUNDERLAND, T., LINKER, G., MIRZA, N. & ET AL. 2003. DEcreased β-amyloid1-42 and increased tau levels in cerebrospinal fluid of patients with alzheimer disease. *JAMA,* 289**,** 2094-2103.

SUSTER, M. L., SEUGNET, L., BATE, M. & SOKOLOWSKI, M. B. 2004. Refining GAL4-driven transgene expression in Drosophila with a GAL80 enhancer-trap. *genesis,* 39**,** 240-245.

SWAAB, D. F., FLIERS, E. & PARTIMAN, T. 1985. The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. *Brain research,* 342**,** 37-44.

TAKEI, N., INAMURA, N., KAWAMURA, M., NAMBA, H., HARA, K., YONEZAWA, K. & NAWA, H. 2004. Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. *The Journal of neuroscience,* 24**,** 9760-9769.

TANG, S. J., REIS, G., KANG, H., GINGRAS, A.-C., SONENBERG, N. & SCHUMAN, E. M. 2002. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. *Proceedings of the National Academy of Sciences,* 99**,** 467-472.

TATEBAYASHI, Y., MIYASAKA, T., CHUI, D.-H., AKAGI, T., MISHIMA, K.-I., IWASAKI, K., FUJIWARA, M., TANEMURA, K., MURAYAMA, M., ISHIGURO, K., PLANEL, E., SATO, S., HASHIKAWA, T. & TAKASHIMA, A. 2002. Tau filament formation and associative memory deficit in aged mice expressing mutant (R406W) human tau. *Proceedings of the National Academy of Sciences,* 99**,** 13896-13901.

TERRY, R. D., MASLIAH, E., SALMON, D. P., BUTTERS, N., DETERESA, R., HILL, R., HANSEN, L. A. & KATZMAN, R. 1991. Physical basis of cognitive alterations in alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Annals of Neurology,* 30**,** 572-580.

THORNTON, C., BRIGHT, NICOLA J., SASTRE, M., MUCKETT, PHILLIP J. & CARLING, D. 2011. AMP-activated protein kinase (AMPK) is a tau kinase, activated in response to amyloid β-peptide exposure. *Biochemical Journal,* 434**,** 503-512.

TÓTH, M. L., SIGMOND, T., BORSOS, É., BARNA, J., ERDÉLYI, P., TAKÁCS-VELLAI, K., OROSZ, L., KOVÁCS, A. L., CSIKÓS, G. & SASS, M. 2008. Longevity pathways converge on autophagy genes to regulate life span in Caenorhabditis elegans. *Autophagy,* 4**,** 330-338.

TRANAH, G. J., BLACKWELL, T., STONE, K. L., ANCOLI- ISRAEL, S., PAUDEL, M. L., ENSRUD, K. E., CAULEY, J. A., REDLINE, S., HILLIER, T. A. & CUMMINGS, S. R. 2011. Circadian activity rhythms and risk of incident dementia and mild cognitive impairment in older women. *Annals of neurology,* 70**,** 722-732.

UBHI, K. K., SHAIBAH, H., NEWMAN, T. A., SHEPHERD, D. & MUDHER, A. 2007. A comparison of the neuronal dysfunction caused by Drosophila tau and human tau in a Drosophila model of tauopathies. *Invertebrate Neuroscience,* 7**,** 165-171.

VERNACE, V. A., ARNAUD, L., SCHMIDT-GLENEWINKEL, T. & FIGUEIREDO-PEREIRA, M. E. 2007. Aging perturbs 26S proteasome assembly in Drosophila melanogaster. *The FASEB Journal,* 21**,** 2672-2682.

VIÑA, J., BORRÁS, C. & MIQUEL, J. 2007. Theories of ageing. *IUBMB Life,* 59**,** 249-254.

VISSCHER, M., DE HENAU, S., WILDSCHUT, MATTHEUS H. E., VAN ES, ROBERT M., DHONDT, I., MICHELS, H., KEMMEREN, P., NOLLEN, ELLEN A., BRAECKMAN, BART P., BURGERING, BOUDEWIJN M. T., VOS, HARMJAN R. & DANSEN, TOBIAS B. 2016. Proteome-wide Changes in Protein Turnover Rates in C. elegans Models of Longevity and Age-Related Disease. *Cell Reports,* 16**,** 3041-3051.

VOLICER, L., HARPER, D. G., MANNING, B. C., GOLDSTEIN, R. & SATLIN, A. 2001. Sundowning and Circadian Rhythms in Alzheimer's Disease. *American Journal of Psychiatry,* 158**,** 704-711.

WALKER, G., HOUTHOOFD, K., VANFLETEREN, J. R. & GEMS, D. 2005. Dietary restriction in C. elegans: From rate-of-living effects to nutrient sensing pathways. *Mechanisms of Ageing and Development,* 126**,** 929-937.

WANG, K. & KLIONSKY, D. J. 2011. Mitochondria removal by autophagy. *Autophagy,* 7**,** 297-300.

WANG, X., LI, W., WILLIAMS, M., TERADA, N., ALESSI, D. R. & PROUD, C. G. 2001. Regulation of elongation factor 2 kinase by p90RSK1 and p70 S6 kinase. *The EMBO Journal,* 20**,** 4370-4379.

WANG, Z., SINGHVI, A., KONG, P. & SCOTT, K. 2004. Taste representations in the Drosophila brain. *Cell,* 117**,** 981-991.

WEAVER, C. L., ESPINOZA, M., KRESS, Y. & DAVIES, P. 2000. Conformational change as one of the earliest alterations of tau in Alzheimer's disease. *Neurobiology of Aging,* 21**,** 719-727.

WEINGARTEN, M. D., LOCKWOOD, A. H., HWO, S. Y. & KIRSCHNER, M. W. 1975. A protein factor essential for microtubule assembly. *Proceedings of the National Academy of Sciences,* 72**,** 1858-1862.

WEITZMAN, E. D., MOLINE, M. L., CZEISLER, C. A. & ZIMMERMAN, J. C. 1982. Chronobiology of aging: Temperature, sleep-wake rhythms and entrainment. *Neurobiology of Aging,* 3**,** 299-309.

WELSH, D. K. & MOORE-EDE, M. C. 1990. Lithium lengthens circadian period in a diurnal primate, Saimiri sciureus. *Biological psychiatry,* 28**,** 117-126.

WINBLAD, B., AMOUYEL, P., ANDRIEU, S., BALLARD, C., BRAYNE, C., BRODATY, H., CEDAZO-MINGUEZ, A., DUBOIS, B., EDVARDSSON, D., FELDMAN, H., FRATIGLIONI, L., FRISONI, G. B., GAUTHIER, S., GEORGES, J., GRAFF, C., IQBAL, K., JESSEN, F., JOHANSSON, G., JÖNSSON, L., KIVIPELTO, M., KNAPP, M., MANGIALASCHE, F., MELIS, R., NORDBERG, A., RIKKERT, M. O., QIU, C., SAKMAR, T. P., SCHELTENS, P., SCHNEIDER, L. S., SPERLING, R., TJERNBERG, L. O., WALDEMAR, G., WIMO, A. & ZETTERBERG, H. 2016. Defeating Alzheimer's disease and other dementias: a priority for European science and society. *The Lancet Neurology,* 15**,** 455-532.

WITTING, W., MIRMIRAN, M., BOS, N. P. A. & SWAAB, D. F. 1994. The Effect of Old Age on the Free-Running Period of Circadian Rhythms in Rat. *Chronobiology International,* 11**,** 103-112.

WITTMANN, C. W., WSZOLEK, M. F., SHULMAN, J. M., SALVATERRA, P. M., LEWIS, J., HUTTON, M. & FEANY, M. B. 2001. Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. *Science,* 293**,** 711-4.

WU, J. J., QUIJANO, C., CHEN, E., LIU, H., CAO, L., FERGUSSON, M. M., ROVIRA, I. I., GUTKIND, S., DANIELS, M. P. & KOMATSU, M. 2009. Mitochondrial dysfunction and oxidative stress mediate the physiological impairment induced by the disruption of autophagy. *Aging (Albany NY),* 1**,** 425-437.

WYSE, C. A. & COOGAN, A. N. 2010. Impact of aging on diurnal expression patterns of CLOCK and BMAL1 in the mouse brain. *Brain research,* 1337**,** 21-31.

XIONG, Y., ZHAO, K., WU, J., XU, Z., JIN, S. & ZHANG, Y. Q. 2013. HDAC6 mutations rescue human tau-induced microtubule defects in Drosophila. *Proceedings of the National Academy of Sciences,* 110**,** 4604-4609.

YAMAGUCHI, H., ISHIGURO, K., UCHIDA, T., TAKASHIMA, A., LEMERE, A. C. & IMAHORI, K. 1996. Preferential labeling of Alzheimer neurofibrillary tangles with antisera for tau protein kinase (TPK) I/glycogen synthase kinase-3β and cyclin-dependent kinase 5, a component of TPK II. *Acta Neuropathologica,* 92**,** 232-241.

YANG, F., CHU, X., YIN, M., LIU, X., YUAN, H., NIU, Y. & FU, L. 2014. mTOR and autophagy in normal brain aging and caloric restriction ameliorating age-related cognition deficits. *Behavioural Brain Research,* 264**,** 82-90.

YIANNOPOULOU, K. G. & PAPAGEORGIOU, S. G. 2013. Current and future treatments for Alzheimer's disease. *Therapeutic Advances in Neurological Disorders,* 6**,** 19-33.

ZHANG, B., MAITI, A., SHIVELY, S., LAKHANI, F., MCDONALD-JONES, G., BRUCE, J., LEE, E. B., XIE, S. X., JOYCE, S., LI, C., TOLEIKIS, P. M., LEE, V. M.-Y. & TROJANOWSKI, J. Q. 2005. Microtubule-binding drugs offset tau sequestration by stabilizing microtubules and reversing fast axonal transport deficits in a tauopathy model. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 227-231.

ZHDANOVA, I., YU, L., LOPEZ-PATINO, M., SHANG, E., KISHI, S. & GUELIN, E. 2008. Aging of the circadian system in zebrafish and the effects of melatonin on sleep and cognitive performance. *Brain research bulletin,* 75**,** 433-441.