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AUTOPHAGY GRIDLOCK IN TAUOPATHY

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AUTOPHAGY GRIDLOCK IN TAUOPATHY

by

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Dedication

This dissertation is dedicated to …

my precious wife Christine

for her endless support, care, and unfailing encouragement

And to my loving family

for always providing me with the best means of education

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AUTOPHAGY GRIDLOCK IN TAUOPATHY

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ABSTRACT

Tauopathies are a group of neurodegenerative diseases characterized by the presence of intracellular aggregates containing the microtubule associated protein tau. Bulk degradation of aggregation-prone proteins can occur via autophagy. Here, we utilized the previously developed model of human tauopathy in *Drosophila* to examine how autophagy modulates human tau toxicity and to study the effects of human tau on the autophagic machinery. We showed that caloric restriction and rapamycin feeding suppressed the human tau-induced toxic phenotype, with a decrease in the soluble levels of human tau. Genetic upregulation of the autophagy/lysosomal system also alleviated human tau toxicity, whereas its downregulation exerted the opposite effect. We also showed

that human tau misexpression induced the accumulation of autophagic intermediates which was correlated with a slight increase in macroautophagy induction. The autophagic intermediates were immunoreactive for Blue Cheese, the fly homolog of autophagy-linked FYVE protein, ALFY. A blockage in autophagic flux also contributed to the accumulation of autophagic intermediates with subsequent formation of large spherical bodies, which we termed giant autophagic bodies (GAB). In contrast to mature autolysosomes, GAB were significantly larger in size, higher in pH and contained a mixture of digested and undigested material indicating a blockage in the degradative capacity of macroautophagy. We demonstrated that lowering basal autophagy levels reduced the number of GAB. On the other hand, augmenting autophagy resulted in the formation of large autophagic intermediates that were poorly acidified and caused a decrease in the number of mature autolysosomes, implying an autophagy gridlock preventing the maturation of autophagic intermediates. Collectively, these results show that human tau misexpression both impaired and its toxicity was regulated by autophagy. Early activation of autpophagy suppressed tauopathy, likely through sequestration of soluble human tau; however, tauopathy also appeared to be associated with a gridlock in autophagic flux leading to the formation of large and poorly acidified autophagic intermediates.

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CHAPTER I

MODELING NEURODEGENERATION IN FLIES

1.1 Why *Drosophila*

Neurodegenerative diseases are characterized by loss of specific neuronal populations, with resulting signs and symptoms corresponding to these affected cells. Most of these diseases share a common pathological feature: abnormal protein accumulations variously referred to as aggregates or inclusions. Misfolding of disease-associated proteins may alter their function and disrupt crucial cellular pathways, eventually leading to neurodegeneration. Familial forms of some neurodegenerative diseases have been linked to mutations in specific genes. These disease-associated mutations have facilitated the development of many animal models for neurodegenerative diseases, some of which have utilized the simple vinegar fly, *Drosophila melanogaster*.

Over the last decade, *Drosophila melanogaster* has emerged as a widely used model for human disease via targeted misexpression of human disease-associated proteins (Bilen and Bonini, 2005; Celotto and Palladino, 2005; Marsh and Thompson, 2006; Sang and Jackson, 2005). The chief advantage of creating such models is that once a suitable phenotype has been obtained, the genetic toolkit of fly genetics can be used to dissect underlying disease pathways. Although some critics of this approach have argued that it has not generated many novel insights, fly models of human neurodegenerative disorders have

provided valuable information when viewed within the context of other models and systems of analysis. *Drosophila* is an attractive organism for establishing models of human disease and studying genetic interactions for several reasons. First of these reasons is the functional similarity between the genomes of *Homo sapiens* and *Drosophila*; more than 75% of genes associated with human diseases have a homolog in *Drosophila* (Reiter et al., 2001). Given this high similarity between human genes and their *Drosophila* counterparts, fly models using misexpression of human genes or mutations of their fly homologs have been engineered for many classes of human diseases, including cancer and developmental, cardiac, immunological, and neurodegenerative disorders (Bier, 2005). Second, many well-studied biological pathways are conserved between humans and *Drosophila*; these include endocrine, intracellular signaling, cell death, and pattern formation pathways, e.g., the transforming growth factor-β (TGF-β)/*decapentaplegic* pathway, the *wingless*/Wnt pathway, insulin signaling, and the *Notch* pathway (Adams et al., 2000). A third attractive feature of using *Drosophila* is the versatile genetic toolkit that has been developed over more than a century of use. Genetic manipulation of *Drosophila* can generate a wide variety of mutants and transgenes. Gene loss of function can be accomplished via RNAi, transposon insertion or imprecise excision, as well as by chemical and X ray mutagenesis (Bellen et al., 2004). Gain of function effects can be studied using various systems including the binary yeast transcription activator protein GAL4/ upstream activation sequence (UAS) system (Brand and Perrimon, 1993)

which has been utilized in flies to allow overexpression in a tissue-specific manner (Venken and Bellen, 2007). The mechanism of the GAL4/UAS binary system is flies is described in **figure 1.1A**. Briefly, the GAL4 transgene is fused to a tissue-specific promoter so that GAL4 expression is under the temporal and spatial properties of the promoter. The transgenic fly carrying the GAL4 is crossed to another carrying the target transgene fused to an upstream activating sequence (UAS). The progeny will have the transgene expressed under the spatial and temporal expression pattern of the tissue-specific promoter used. Introducing a temperature-sensitive GAL80 can also be used to modulate the timing of expression of the transgene. As shown in **figure 1.1B**, a temperature sensitive GAL80 ($GAL80^{ts}$) (Johnston, 1987; Lohr et al., 1995) is expressed ubiquitously under a tubulin promoter. At low temperatures such as 18^0C, $GAL80^{ts}$ binds to GAL4 preventing it from binding the UAS and the transcription of the transgene does not occur. However, at higher temperatures such as 29^0C, $GAL80^{ts}$ does not bind efficiently to GAL4, which is free to bind to the UAS, and the transgene is transcribed.

The relatively short life cycle of the fly (shown in **figure 1.2**) also provides advantages for genetic modeling. It takes 9-10 days for an embryo to reach the adult stage and a single cross yields abundant progeny, facilitating generation of genetic variants and the efficient production of *Drosophila* models for human diseases. Moreover, the relatively short lifespan of the fly (no more than 120

days, depending upon conditions) facilitates examination of gene effects in aged individuals.

1.2 The fly eye as a model system

Generation of a useful fly model for a disease requires the development of a morphological, behavioral, or physiological phenotype in response to a transgene or mutation. Several factors determine the potential for success of a model. First, the similarity between the phenotype and the clinical signs observed in patients is considered by many to be a critical test of a model. An additional factor that can be used to assess the quality of a model is the degree of similarity between pathology formed in the fly and that observed in humans with regard to tissue specificity, formation of inclusions or aggregates, and cell dysfunction or death. The ease with which the phenotype can be screened and the specificity of scoring are important additional factors in designing screens for genetic modifiers.

The *Drosophila* eye has been widely used as a model for neurodegenerative diseases and is well suited to this purpose for several reasons. The retina is of neural origin and contains photoreceptor neurons similar to their mammalian counterparts. The final assembly of the adult compound eye requires precisely coordinated developmental events in larvae and pupae (Kumar, 2012). These developmental stages are well characterized, and each stage can be analyzed at a single cell level. It is believed that at least

2/3 of *Drosophila* vital genes play a role in pattern formation and/or connectivity of the adult retina (Thaker and Kankel, 1992); thus if a genetic manipulation (e.g., misexpression of a human gene) impacts even one such vital pathway it may reasonably be expected to yield an abnormal eye phenotype (Thaker and Kankel, 1992; Thomas and Wassarman, 1999). Under laboratory conditions, the eye is dispensable for survival; thus, knockdown of vital genes does not affect overall survival or fertility of the fly. Finally, many eye phenotypes can be readily scored under the dissecting microscope or using techniques such as the deep pseudopupil[1] or optical neutralization (Banerjee et al., 1987; Franceschini and Kirschfeld, 1971).

The compound eye of *Drosophila* is comprised of approximately 760 repeating subunits called ommatidia. Each ommatidium includes photoreceptor neurons, cone cells and pigment cells, as well as interommatidial cells formed in early developmental stages and removed by cell death. Because patterning in the eye results from a wave of morphogenesis that starts in the larval stage,

---

[1] Disrupting the rhabdomeric pattern can cause a deep pseudopupil disturbance Franseschini, N., 1975 Sampling the visual environment by the compound eye of the fly: fundamentals and applications, pp. 98-125 in ***Photoreceptor Optics,*** edited by A.W. Snyder and R. Menzel. Springer-Verlag, Berlin. Quoted from Thaker, H.M., and Kankel, D.R. (1992). Mosaic analysis gives an estimate of the extent of genomic involvement in the development of the visual system in Drosophila melanogaster. Genetics *131*, 883-894.

identical cells are not derived from a common precursor cell. Therefore, different genetic clones can be analyzed in the same eye, i.e., clones homozygous for a lethal mutation can be identified adjacent to wild type clones, facilitating phenotypic analysis. The eye is developed from the eye disc, which arises from about 20 cells of the optic primordiumin in the embryo. During the late larval stage, a dorsal-ventral invagination called the morphogenetic furrow develops from posterior to anterior. This wave of differentiation is driven by hedgehog expression and inhibited by wingless expression at the dorsal and ventral margins. Hedgehog is expressed by the developing photoreceptors and in turn drives the expression of decapentaplegic (dpp) and atonal (ato). The axons of the developing photoreceptors project to the larval laminal plexus in a retinotopic fashion (Blackman et al., 1991; Ma and Moses, 1995; Ma et al., 1993; Technau, 2008). Disruption of these well-orchestrated events could result in the failure of the adult eye to develop properly. A phenotype commonly used to score degeneration in the eye is the “rough” eye. Many transgenes induce a rough eye phenotype, which consists of ommatidial fusion and loss of interommatidial bristles. The rough phenotype varies in severity and (ideally) is dosage-sensitive, making it suitable for genetic screening.

1.3 Modeling human tauopathies in flies[2]

Tauopathies are a group of neurodegenerative diseases characterized by pathologic accumulation of tau; Alzheimer Disease (AD) is one such tauopathy. Dominant mutations in tau have been identified in frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17, now also referred to as frontotemporal lobar degeneration-tau (FTLD-T)) (D'Souza et al., 1999). In other tauopathies, such as AD, Pick disease, corticobasal degeneration and progressive supranuclear palsy, tau mutations have not been identified, although neurofibrillary tangles (NFT) and other forms of tau accumulations are common pathological hallmark of the diseases (Ballatore et al., 2007). Recent genome-wide association studies also have identified tau polymorphisms as important contributors to risk for sporadic Parkinson disease (Edwards et al., 2010; Satake et al., 2009; Simon-Sanchez et al., 2009).

In *Drosophila*, several models of tauopathy have been developed. One of the early models used the binary GAL4/UAS expression system to drive the expression of human tau (Williams et al., 2000). In this model, targeted expression of human tau resulted in age-dependent loss of sensory axons and axonal pathology such as abnormal axonal bundling and swelling (beading).

[2] This is a brief overview of the history of the making of fly models of human tauopathies. A more detailed explanation of the different models and the pathogenesis of human tau in *Drosophila* can be found in the introduction of chapters 3 and 4.

Feany and colleagues reported more robust toxicity of FTLD-associated R406W and V337M mutations as compared to wild type tau; phenotypes included early mortality and vacuolization of brain but without formation of obvious NFT pathology (Wittmann et al., 2001).

Jackson and colleagues used a direct fusion construct driving expression of wild type human tau in the *Drosophila* eye without the need for GAL4, which produced a dosage-sensitive rough eye phenotype (Jackson et al., 2002). In this model, overexpression of the *Drosophila* glycogen synthase kinase-3β (GSK-3β) homolog *shaggy* exacerbated the toxic effects of tau. It also induced the formation of discrete flame-shaped aggregates which were immunoreactive for AT100, a specific marker of NFT (Augustinack et al., 2002; Hoffmann et al., 1997; Zheng-Fischhofer et al., 1998).

The engineering of these models has provided useful reagents for genetic screens. Kinases and phosphatases have been identified as modifiers of tau toxicity (Shulman and Feany, 2003), lending credence to the assertion that tau phosphorylation plays an important role in mediating its toxicity. This assertion is further supported by the finding that a phosphorylation-incompetent form of tau, obtained by mutating all the kinase sites to alanine, has reduced neurotoxicity as compared to wild type tau (Steinhilb et al., 2007). Pseudo-hyperphosphorylated tau produced by substituting all serines and threonines with glutamate, has increased toxicity (Fulga et al., 2007). The Jackson laboratory, on the other hand, found that a tau mutant resistant to GSK-3β retained substantial toxicity, perhaps

because of an increased affinity for microtubules (Chatterjee et al., 2009). Findings by Lecourtois and colleagues, however, suggested that human tau, unlike *Drosophila* tau, has low affinity for *Drosophila* microtubules (Feuillette et al., 2010). Mudher and coworkers recently demonstrated that soluble, non-filamentous hyperphosphorylated tau causes toxicity through disruption of axonal transport (Cowan et al., 2010b).

The effects of human tau misexpression are not only limited to the fly eye, Skoulakis and colleagues recently investigated the role of tau phosphorylation in the *Drosophila* CNS. Wild type tau overexpression causes developmental mushroom body ablation in the fly (Kosmidis et al., 2010). Tau phosphorylation at Ser^{238} and Thr^{245} was necessary for toxicity in this model. These flies exhibited severe impairment of associative learning and memory. FTLD-linked mutants were significantly less toxic to the mushroom body than wild type tau. Another recent tau model which may be useful for modifier screening was developed by Yeh, et al. using a GAL4 driver to express tau in the dorsal mesothorax (notum) of *Drosophila.* Overexpression of tau caused loss of mechanosensory bristles on the dorsal surface of the fly. The phenotype was enhanced by tau phosphorylation and was suppressed by reduction of shaggy dosage (Yeh et al., 2010).

1.4 Other neurodegeneration models in flies

Tauopathies are among many other neurodegenerative diseases to be modeled in flies. These include AD (non-tau models), Parkinson disease, polyglutamine diseases and amyotrophic lateral sclerosis. Here, I will briefly highlight the main fly models that have contributed to our knowledge in the field of neurodegeneration. In the subsequent chapters I will refer to some of these models and their internal pathology when discussing autophagic dysfunction in neurodegeneration models.

Aβ models

Mutations in the amyloid precursor protein (APP) cause familial AD (Bertram et al., 2010). Amyloid plaques are mainly composed of $A\beta_{40}$ or $A\beta_{42}$ amino acid peptides derived from APP cleavage. Amyloidogenic Aβ peptides arise from APP cleavage through β- and γ-secretase (Hardy, 2006). Presenilins, which have a single fly homolog (Psn), are a component of the γ-secretase complex. Mutations in PS1 and PS2 cause early onset familial AD (Bertram et al., 2010). *Drosophila* homologs of other components of the γ-secretase, including nicastrin, Pen-2, and Aph-1 have been identified (Hu and Fortini, 2003). The fly homolog of β-secretase, dBACE, recently has been identified (Carmine-Simmen et al., 2009); this enzyme cleaves human APP at a different site than that at which human β-secretase acts. However, dBACE overexpression cleaves dAPPl, the amyloid precursor protein (APP) fly homolog, and produces an amyloidogenic

form that aggregates. It is noteworthy that dAppl does not contain the Aβ domain found in human APP. This suggests that although the sequence is not conserved between humans and *Drosophila*, amyloidogenic processing may still be conserved (Carmine-Simmen et al., 2009). Loss of dAPPl is similar to loss of APP, since large deletions of dAppl result in reduced locomotion and phototaxis deficiency that are rescued by human APP (Luo et al., 1992). Aβ toxicity in flies also has been modeled by directed overexpression of Aβ fragments. $A\beta_{42}$ (but not $A\beta_{40}$) overexpression reduces lifespan in flies; however, both Aβ fragments cause progressive loss of associative learning. Aβ overexpression in the eye causes retinal phenotypes (Iijima et al., 2004). Moreover, Aβ overexpression causes long-term depression. Chiang et al. showed through immunostaining of PI3K that Aβ induces PI3K hyperactivity. Knockdown or pharmacologic inhibition of PI3K function led to rescue of the long-term depression phenotype (Chiang et al., 2010). Crowther et al. reported that Aβ forms non-amyloidogenic aggregates that resemble diffuse plaques. Immunohistochemistry, using a conformation-dependent antibody, indicates that oligomers may actually be the entities mediating neurotoxic effects (Crowther et al., 2005). The downstream effects of Aβ have not been identified with certainty, although recent findings suggest tau phosphorylation to be an important downstream effect of Aβ-induced neurotoxicity. A recent paper by Iijima et al. showed that co-expression of $A\beta_{42}$ with tau increased tau phosphorylation at Ser262, an AD-related phosphoepitope (Iijima et al., 2010). Ser262 phosphorylation enhanced tau-induced neurotoxicity,

whereas co-expression of $A\beta_{42}$ and a non-phosphorylatable form of tau, Ser262Ala, did not cause any neurodegenerative phenotypes.

Parkinson disease models

Parkinson disease (PD) is characterized by bradykinesia, tremor, postural instability and rigidity (Jankovic, 2008). Pathological hallmarks of PD include degeneration of dopaminergic neurons in the substantia nigra pars compacta and the presence of Lewy bodies, eosinophilic cytoplasmic inclusions comprised in part of α-synuclein (Braak et al., 2003). Although PD is usually sporadic, mutations have been linked to familial forms of PD. Synucleins α-, β- and γ- are soluble proteins found primarily in neural tissue; α-synuclein is associated with PD. Autosomal dominant PD is caused by the A53T (Polymeropoulos et al., 1997) and A30P (Krüger et al., 1998) mutations in SNCA/α-synuclein; the E46K mutation appears to be associated with a more widespread synucleinopathy called dementia with Lewy bodies (Zarranz et al., 2004). Genomic duplication (Ibáñez et al., 2004) and triplication (Singleton et al., 2003) of α-synuclein have also been identified as causes for familial PD. Overexpression of either wild type or mutant forms of α-synuclein in the fly leads to loss of tyrosine hydroxylase-immunoreactive neurons (Auluck et al., 2002; Feany and Bender, 2000). Moreover, intracytoplasmic accumulation of α-synuclein suggestive of Lewy bodies is observed. These transgenic flies also exhibit age-dependent motor impairment and mild retinal abnormalities. Climbing defects are more severe in

the A30P mutant as compared to wild type α-synuclein or the A53T mutant form (Feany and Bender, 2000).

Polyglutamine disease models

Polyglutamine diseases refer to a group of neurodegenerative disorders resulting from a CAG expansion leading to an expanded polyglutamine tract within the protein. This expansion mutation within the coding region of an affected gene causes a conformation change to a β-sheet structure (Perutz, 1999). *Drosophila* models of polyglutamine diseases share several characteristics of human disease, including intraneuronal ubiquitinated aggregates and nuclear inclusions in neurons. Also, *Drosophila* models share length-dependent pathology (Chan et al., 2000).

Amyotrophic lateral sclerosis models

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease in the US or Charcot's disease in Europe, affects upper and lower motor neurons. 10% of cases are familial (Rowland and Shneider, 2001). Superoxide dismutase (SOD) mutations have been identified in familial cases, and SOD neuronal inclusions are found in ALS cases suggesting that oxidative stress plays an important role in the pathogenesis of the disease (Rowland and Shneider, 2001). *Drosophila* with a null SOD allele exhibit increased sensitivity to oxidative stress, as well as reduced longevity (Phillips et al., 1989). Mutant or wild type human

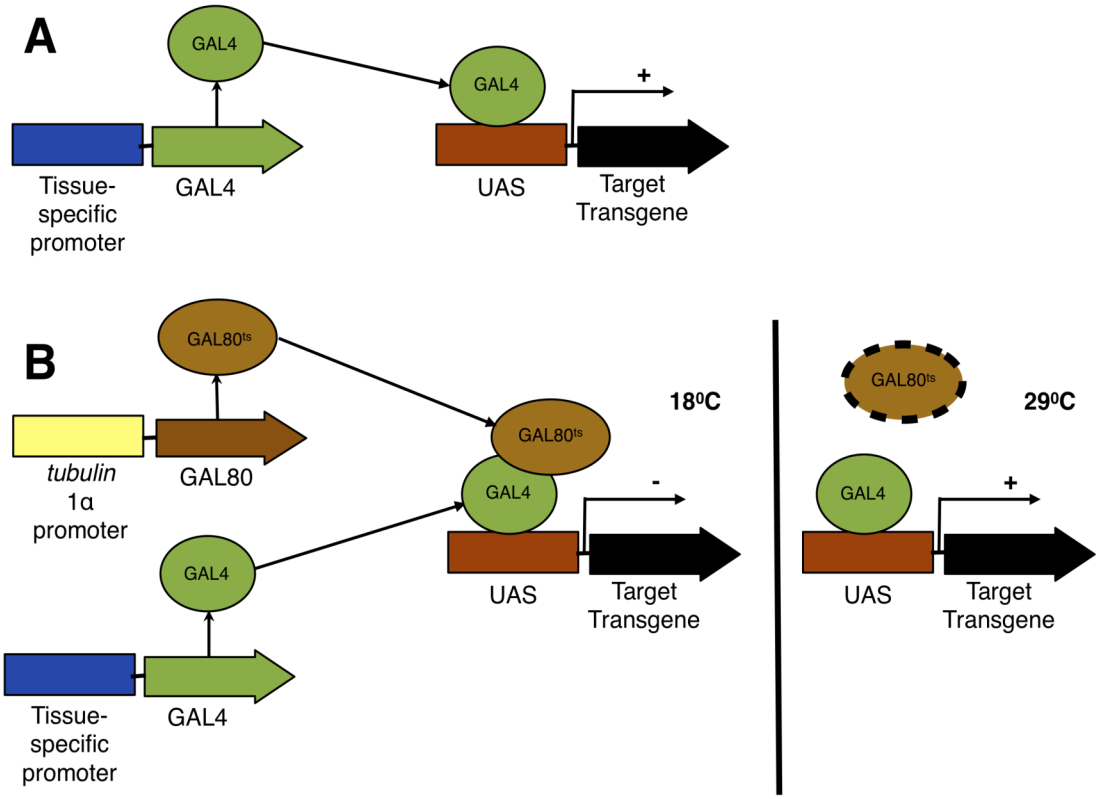
SOD expression in *Drosophila* resulted in neuronal toxicity and aggregation (Phillips et al., 1989; Watson et al., 2008).

1.5 Concluding remarks

The pathological hallmarks of many neurodegenerative diseases have been well described for decades, and the last two decades have seen major strides forward in understanding the molecular bases of these diseases; nonetheless, the mechanisms underlying neurodegenerative disorders remain largely unclear. The purpose of this chapter was to show that *Drosophila* models of neurodegeneration have proven useful in recapitulating many aspects of human neurodegenerative disorders. The utility of such models has not proven to be in uncovering startling new mechanistic pathways, but rather in contributing - along with other models - to incremental advances in understanding of neurodegenerative disorders.

FIGURES

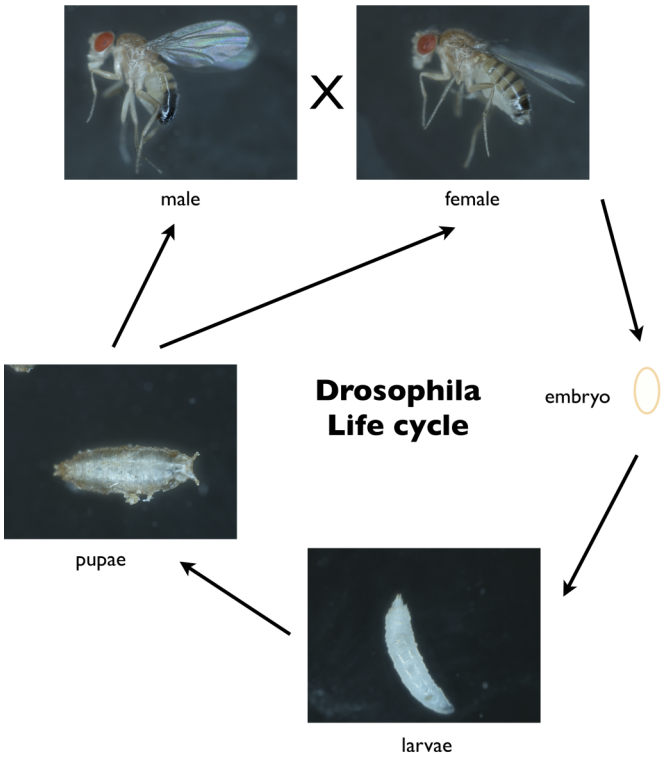
Figure 1.1. The GAL4/UAS binary system



A) The yeast transcription activator protein (GAL4) transgene (green) is fused to a tissue-specific promoter (blue) so that GAL4 expression is under the temporal and spatial properties of the promoter. The target transgene is also fused to an upstream activating sequence (UAS). GAL4 binds to the UAS and drives the expression of the transgene under the spatial and temporal expression pattern of the tissue-specific promoter used. **B)** Introducing a temperature-sensitive GAL80 can also be used to modulate the timing of expression of the transgene. A temperature sensitive GAL80 ($GAL80^{ts}$) is expressed ubiquitously (brown) under the control of a tubulin promoter (yellow). At low temperatures such as 18°C,

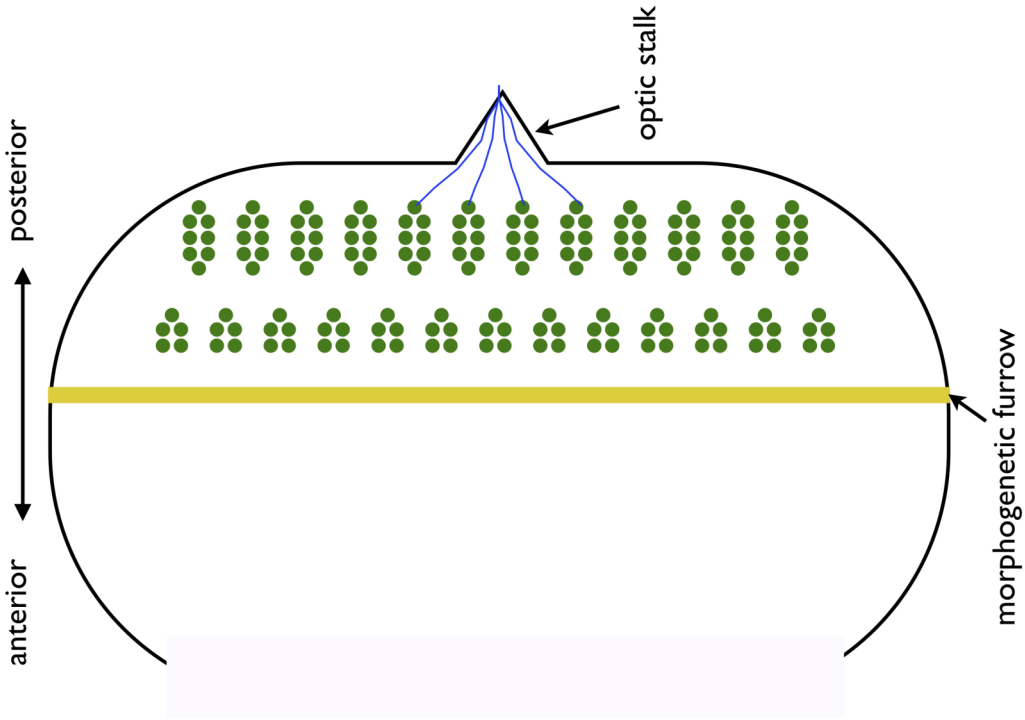
GAL80^{ts} binds to GAL4 preventing it from binding the UAS and the transcription of the transgene does not occur. However, at higher temperatures (e.g., 29ºC), GAL80^{ts} does not bind efficiently to GAL4, which is free to bind to the UAS, and the transgene is transcribed.

Figure 1.2. The *Drosophila* life cycle



The fruitfly *Drosophila* melanogaster has a relatively short life cycle. After mating, the females lay eggs. The embryo then develops into larvae. There are three different larval stages (first, second and third instar). The third instar larvae acquire a puparium in a process called metamorphosis. Upon maturation, pupae eclose from the thickened puparium. The whole cycle can take from 10 to 14 days depending upon the incubation temperature.

Figure 1.3. The wave of differentiation in the eye disc



During the late larval stage, a dorsal-ventral invagination called the morphogenetic furrow (yellow) develops. The axons of the developing photoreceptors project to the larval laminal plexus in a retinotopic fashion. Only few axons (blue) are shown.

CHAPTER II

MATERIALS AND METHODS

2.1 *Drosophila* stocks and genotypes

The glass-tau flies

All experiments and crosses were carried out at 25^0C unless specified otherwise. The glass-tau flies were previously engineered by our lab (Jackson et al., 2002). Briefly, a cDNA encoding the full length, four-repeat, wild type human tau was inserted into the glass multimer reporter (GMR) expression vector. Embryos were injected and yielded several transgenic lines, from which line 1.1, which exhibits a strong phenotype, was used. The full genotype for this line is w^{1118}; glass-$tau^{1.1}$/CyO. The balancer on the second chromosome was only used to maintain a stable stock and was omitted during phenotypic or immunohistochemical analysis. The GFP-tagged glass-tau flies were generated using the same method. A GFP cDNA was added to the N terminus of the human tau cDNA and was inserted into the GMR expression vector. The genotype of the resulting flies was w^{1118}; glass-eGFP.$tau^{1.1}$. The use of a GMR direct fusion with human tau, as opposed to using the yeast binary GAL4/UAS system, resulted in a stable human tau expression when co-expressed with other transgenes.

Genotypes

The following stocks were obtained from the Bloomington stock center; Canton S, *w*^{1118}, *w*^{1118}; P[EP]Atg2^{EP3697}/TM6B, Tb^{1}, *w*^{1118}; P[UAS-S6k.KQ]2, *w*^{1118}; P[EP]cathD^{EP2151}, *w*^{1118}; Mi[ET1]Atg4^{MB03551}, *ry*^{506} P[PZ]Atg6^{00096}/TM3, *ry*^{RK} Sb^{1} Ser^{1}, *w*^{1118} Mi[ET1]HDAC6^{MB06564}, *w*^{1118}; +; UAS-Atg5.GFP, *w*^{1118}; UAS-LC3.GFP, *w*^{1118}; +; Atg1^{d30}/TM6B, Tb^{1}, *y*^{1}*w*^{1118}; UAS-Rab5.S43N, *y*^{1}*w*^{1118}; +; UAS-Rab7.T22N and *y*^{1}*w*^{1118}; UAS-Rab14.S49N. The following stock was a gift from Dr. Rachel Kraut (Nanyang Technological University); bchs^{17} (blue cheese). The following stocks were gifts from Dr. Robin Hiesinger (University of Texas Southwestern); *vha*^{100} and *w*^{1118}; UAS-dAtg8.mCherry.eGFP.

2.2 Caloric restriction and rapamycin feeding

All stocks and crosses were grown on fresh vials containing Jazz-Mix food (Fisher Scientific) unless a chemical such as rapamycin or DMSO were added. In the latter case, instant food media was used to avoid heating which may affect the stability of the compounds. All crosses with the same experimental group were grown on vials prepared from the same food batch. Caloric restriction was achieved by feeding flies on vials containing 30% of the caloric content. This was achieved either by diluting the Jazz-Mix prepared food to about 30% in agar, or by preparing the food from its basic ingredients.

Rapamycin feeding

For the phenotypic analysis, rapamycin diluted in DMSO or ethanol was added to an instant food media. Similar concentration of DMSO was added to the control vials. Flies were grown at 25ºC and the phenotype of the progeny was analyzed. For analyzing the effects of rapamycin in larval eye discs, larvae were grown on food containing 5 µM rapamycin then transferred to a dish containing 10 µM rapamycin where they were incubated for one hour. Eye discs were then dissected and either a live LysoTracker stain or an immunohistochemical stain was performed as will be described later.

2.3 Immunohistochemistry

Immunohistochemistry of adult fly eyes

Adult flies, 3 to 5 days old, were anesthetized and decapitated. Females were used unless otherwise specified. An incision was made along the anterior and posterior borders of the cornea to dissect out the eye as shown in **figure 2.1**. The eye, containing both the retina and the lamina, was incubated in PBS with 0.2% Triton-X for no more than 15 minutes until all eyes had been dissected. They were then transferred to a fixative solution containing 4% paraformaldehyde in PBS, and left for 45 minutes at room temperature, followed by 4 washes in PBS for 10 minutes each. The eyes were incubated in PBS solution with 2% Triton-X overnight at 4ºC to reduce autofluorescence. Blocking solution, consisting of 1% normal goat serum and 3% bovine serum albumin in PBS, was

added for 1 hour at room temperature. The eyes were transferred to the primary antibody solution containing the diluted antibody in the blocking solution where they were incubated overnight at 4^0C. After 4 washes in PBS for 10 minutes each, the secondary antibodies diluted in the blocking solution were added. Finally, the eyes were washed 4 times in PBS for 10 minutes each and were mounted on a slide with high borders to preserve the anatomy, using vectashield. All images were taken using confocal microscopy and the parameters, such as the fluorescence gain, were kept constant for the experimental and control samples.

The following antibodies were used at the designated dilution; anti-Bchs, a gift from Dr. Kraut (1:600), anti-GFP-FITC, Abcam (1:200), phalloidin 568, Invitrogen (1:25), phalloidin 647, Invitrogen (1:25) and Hoechst, Invitrogen (1:1000). The secondaries were all Alexafluor from Invitrogen and were used at a concentration 1:200.

Immunohistochemistry of eye discs

Third instar larvae were transferred to Ringer solution where the larval brain and imaginal discs were dissected out. Females were used unless otherwise specified. The brain was removed and the eye-antennal disc complex was left attached to the mouth hook. The eye-antennal disc was then placed in a fixative solution containing 4% paraformaldehyde in PBS, and left for 30 minutes at room temperature, followed by 4 washes in PBS for 10 minutes each. Next, a

PBS solution containing 2% Triton-X was added for 20 minutes at room temperature. The primary and secondary antibodies incubations were done similar to the adult eye staining. The following antibodies were used at the designated dilution; anti-GFP-FITC, Abcam (1:200), phalloidin 568, Invitrogen (1:25), phalloidin 647, Invitrogen (1:25) and Hoechst, Invitrogen (1:1000).

LysoTracker and Lysosensor staining

For acidotropic dye staining, no fixative was added and all dissections and staining were done in *Drosophila* Ringer's solution. Third instar larvae were transferred to Ringer solution and the larval brain and imaginal discs were dissected and collected. The brain was removed and the eye-antennal disc complex was directly added to a slide containing a drop of Ringer's solution. A drop of 150 nM LysoTracker Red DND-99 (Invitrogen) was added for 1 minute. The tissue was then washed twice with Ringer's solution. The slide was sealed with a coverslip and visualized immediately. Neither Vectashield nor glycerol were added. The time from dissection until visualization was less than 10 minutes. All images were taken using confocal microscopy and the parameters were kept constant for the experimental and control samples. Only 1 slide was prepared at a time to avoid long incubation periods. The same steps were followed for the Lysosensor yellow/blue dextran (invitrogen) except that the concentration used for the Lysosensor was 1 μM and the incubation was for 90 seconds. Quantification for the number and sizes of the LysoTracker punctae

was done using Imaris (Bitplane Scientific Software). A z-stack of images was taken at a 1 μm difference and punctae in all planes were counted.

Drosophila Ringer's solution composition

A solution containing 3 mM $CaCl_2 \cdot 2H_2O$, 182 mM KCl, 46 mM NaCl and 10 mM Tris base was prepared. The final pH was adjusted to 7.2 and the solution was autoclaved.

2.4 Western Blots

Protein was extracted from equal number of decapitated heads of adult flies. Briefly, the heads were added to PBS buffer (2 microliter per head) containing protease inhibitor (Roche) and phosphatase inhibitor (Roche). The heads were mechanically homogenized for 1 minute on ice and centrifuged at 13,000 rpm for 5 minutes. The supernatant was collected and was designated a 'PBS-soluble fraction'. The pellets were resuspended in 2% SDS-Lysis PBS buffer containing protease and phosphatase inhibitor. The Eppendorf tubes were centrifuged at 13,000 rpm for 5 minutes and the supernatant was collected and termed an 'SDS-soluble fraction'. The remaining pellets were resuspended in 2.0 M Urea along with a protease and phosphatase inhibitor and were sonicated at short intervals for 30 seconds on ice. Equal volume of SDS loading buffer with beta-mercaptoethanol (Laemmli, 1970) was added and the mixture was left overnight at room temperature. The supernatant was then collected and termed a

'urea-soluble fraction'. Before loading the gel, SDS loading buffer with beta-mercaptoethanol was added to the PBS-soluble and the SDS-soluble fractions. All three fractions were boiled for 10 minutes then placed immediately on ice before loading. 4-20% SDS-polyacrylamide precast gels from Bio-rad were used. 20 microliters were added in each lane. The molecular weights were determined according to the Precision Plus Protein Dual Color Standards (Bio-Rad). The voltage was set at 110 Volts for approximately 90 minutes. Proteins were transferred to a nitrocellulose membrane incubated in a transfer buffer (Bio-Rad) containing 20% methanol. Proteins were transferred at a constant current (200 mAmp) for 2 hours. The membranes were incubated for one hour at room temperature in a blocking solution containing 0.1% Tween and 5% nonfat milk in TBS. The primary antibodies diluted at the appropriate dilutions were added to a fresh blocking solution and incubated overnight at 4^0C. The membranes were then washed four times with agitation at room temperature for 1 hour with a TBS solution containing 0.1% Tween. Secondary antibodies; HRP-linked anti-mouse IgG or HRP-linked anti-rabbit IgG were added to a blocking solution at a dilution 1:1000 and the membrane was incubated for 1 hour at room temperature. The membranes were then washed four times with agitation at room temperature for 1 hour with a TBS solution containing 0.1% Tween. An ECL solution was added to the membrane for 4 minutes and the bands were detected using an X-ray film or digitally.

The following antibodies were used at the designated dilution: 4E-BP (rabbit) 1:500 (Abcam), phospho-p70 S6k (rabbit) 1:500 (Cell Signaling), blue cheese (rabbit) 1:2000 (gift from Dr. Kraut), anti-GFP (mouse) 1:250 (Abcam), tubulin (mouse) 1:1000 (DHSB), tau-5 (mouse) 1: 500, *Drosophila* Atg5 (rabbit) 1:500 (Abcam), HRP-linked anti-mouse IgG (1:1000) and HRP-linked anti-rabbit IgG (1:1000).

2.5 Electron microscopy

Heads of adult female flies, 3 to 5 days old, were prepared for electron microscopy as follows. Adult flies were anesthetized and decapitated. An incision was made near the proboscis and the proboscis was removed **(Figure 2.1)**. The heads were placed in a fixative solution containing 4% paraformaldehyde for 4 hours. They were then transferred to a fixative solution containing 1% tannic acid for 18 hours. Next, they were placed in 0.1 M sodium cacodylate (pH 7.4) and infiltrated by incubation in 2% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.4). These were followed by two washes in distilled water, 15 minutes each. A 2% uranyl acetate solution was then added for 18 hours. Dehydration was achieved by incubation in ethanol (30%, 50%, 70%, 90% and absolute ethanol), for 15 minutes each. After dehydration, the heads were embedded in the embedding material; acetonitrile for 5 minutes, 33% resin in acetonitrile for 2 hours, 66% resin in acetonitrile for 2 hours and 100% resin for 18 hours. The

heads were placed in molds containing the embedding material and were baked for two days at 70ºC until they were hardened.

Semi-thin sections were used for toluidine blue staining. Ultra-thin sections[3], 80 nanometers-thick, were placed on a grid and post-stained with 3% uranyl acetate for 15 minutes, rinsed with distilled water then dried at room temperature. The grids were then stained with 0.4% lead citrate for 5 minutes, rinsed with distilled water then dried at room temperature. Images were acquired using a transmission electron microscope (JEM 1400). 50 images from the retina of 6 different flies were analyzed for the presence of double membranous vesicles. Giant autophagic bodies were not present in the control and thus no quantification at the ultrastructure level was made.

2.6 High resolution imaging of the external retina

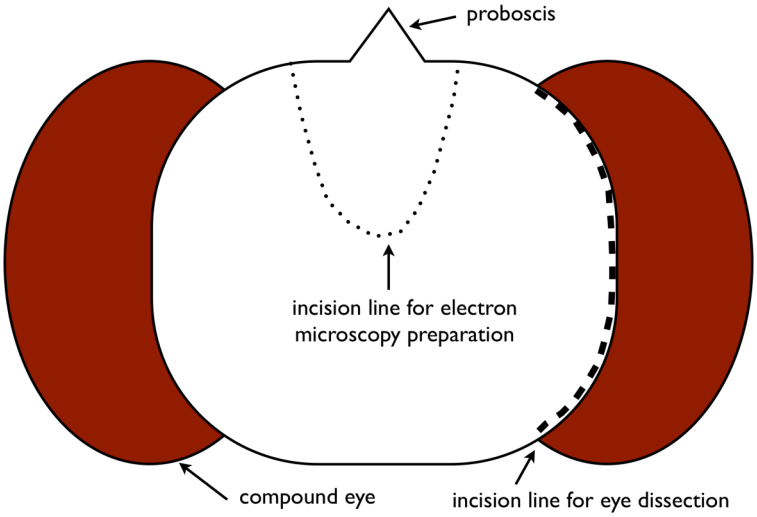
The rough eye phenotype was visualized using a high resolution Nikon EZ-100 upright light microscope. Flies were anesthetized and mounted on a slide using a drop of nail polish. The left eye was always visualized with its anterior side to the left. Serial high resolution images were taken at different planes covering the whole depth of the eye. An extended depth of focus analysis tool allows the production of a final image comprising the pixels in focus from each

[3] Ultrathin sections were prepared by Dr. Zhixia Ding from Dr. Susan Carlton's Laboratory, The University of Texas Medical Branch, Galveston, TX, USA.

plane to yield a single-plane high-resolution image. A fine brush was used to remove any food or debris if present on the cornea. Three to five day old female flies were used and those with collapsed eyes were omitted. Flies were selected randomly at low magnification to eliminate bias.

FIGURES

Figure 2.1. The compound eye dissection



The head of the fly was removed by holding it from the proboscis. For whole mount of the eye, an incision (dashed line) was made along the borders of the compound eye (red). For electron microscopy preparation, an incision was made (dotted line) and the proboscis was removed.

CHAPTER III

AUTOPHAGY: A GUARDIAN AGAINST HUMAN TAU-INDUCED TOXICITY IN A *DROSOPHILA* MODEL OF HUMAN TAUOPATHY

INTRODUCTION

3.1.1 Tau

The microtubule-associated protein tau was first identified as a protein that is associated with microtubules and stabilizes them *in vitro* (Weingarten et al., 1975). It is mainly expressed in the nervous system, both central and peripheral, as well as other non-neuronal systems such as the heart, skeletal muscles, lung and testis (Gu et al., 1996; Trojanowski et al., 1989). In the central nervous system, tau is expressed in neurons as well as oligodendrocytes (Klein et al., 2002). In the human brain, there are six isoforms of tau. They are classified based on the numbers of N-terminal exons (0N, 1N or 2N) and the number of microtubule binding repeats (3R or 4R) (Goedert et al., 1989).

Tau harbors many serine and threonine phosphorylation sites. It can also be acetylated (Cohen et al., 2011), glycated (Ledesma et al., 1994), transglutaminated (Wilhelmus et al., 2009), sumoylated (Dorval and Fraser, 2006), nitrated (Reyes et al., 2008) and ubiquitinated (Cripps et al., 2006), as well as be subject to other posttranslational modifications (Morris et al., 2011). This large spectrum of tau modifications suggests its many physiological roles.

Although it was first identified as a microtubule stabilizing factor, advances over the past decade have identified many other functions of tau.

3.1.2 Functions of Tau

Despite being studied extensively over the past decade, the exact role and function of tau are still not precisely known. Much of our knowledge about the functions of tau is derived from the analysis of tau knockout models (Morris et al., 2011). There are many proteins that have been shown to be binding partners for tau. Examples include cytoskeletal proteins (Fulga et al., 2007; Kar et al., 2003), protein kinases (Lee et al., 1998; Reynolds et al., 2008), lipid carriers (Fleming et al., 1996; Strittmatter et al., 1994) and signaling lipids (Flanagan et al., 1997; Surridge and Burns, 1994). The role of tau in microtubule stability has been among the earliest and most intensely studied properties of tau. Through its microtubule binding repeats, tau is believed to bind microtubules (Gustke et al., 1994) both *in vitro* and *in vivo*. However, its role in microtubule stability has been more controversial. Initial reports showed that tau promotes microtubule assembly *in vitro*. However, in cell cultures and primary neuronal culture, tau-bound microtubules have the highest turnover rates and are more sensitive to drug-induced depolymerization (Fanara et al., 2010; Kempf et al., 1996). Moreover, knockdown of tau did not alter microtubule polymerization (King et al., 2006). On the other hand, tau has been shown to protect the microtubules from being severed by katanin (Qiang et al., 2006). This suggests that the role of tau

in stabilizing microtubules cannot be simply attributed to tau alone, and the pathogenesis of microtubule instability in neurodegeneration cannot be solely attributed to loss of function of tau.

Another speculated role of tau is in regulating axonal transport. Tau has been shown to preferentially interfere with the binding of kinesin and dynein to the microtubules (Dixit et al., 2008; Ebneth et al., 1998). Although human tau overexpression in mammalian cells, rodents and flies has been shown to slow down axonal transport (Cowan et al., 2010b; Ebneth et al., 1998; Talmat-Amar et al., 2011), tau ablation did not negatively affect it (Vossel et al., 2010; Yuan et al., 2008). The mechanism by which tau would interfere with microtubule stability or axonal transport is not well defined. Recent discovery of multiple tau-binding partners has raised the possibility that it may act as a scaffolding molecule, thus altering different signaling pathways.

3.1.3 The autophagic machinery

Autophagy is derived from two Greek words meaning, “eating oneself”. It is a catabolic process by which cytosolic components are degraded. There are three types of autophagy: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. In CMA, which has only been described in higher eukaryotes, cytosolic proteins containing a KERFQ motif unfold by binding to cytosolic chaperones and which are transported into the lysosome through the lysosomal-associated membrane protein 2A (LAMP 2A). In

microautophagy, extensively studied in yeast, the lysosomal membrane engulfs cytosolic constituents and pinches off within the acidic lysosomal lumen to degrade the newly formed vesicle. Macroautophagy – hereinafter referred to as autophagy – is a catabolic pathway involving the lysosomal machinery. Its hallmark is the formation of a double membrane vesicle around the cargo to be digested. The resulting double membrane vesicle, referred to as an autophagosome, can then fuse to the endolysosomal vesicles to mature into an amphisome then autolysosome. Having a highly acidic lumen, autolysosomes are able to degrade the sequestered cargo and recycle the amino acids for further use by the cell. Thus the three main steps in autophagy are induction, elongation of the double membrane and clearance. The players involved in the autophagic machinery, over thirty in number (Xie and Klionsky, 2007), have been given a unified nomenclature in which genes and proteins are named *ATG* and Atg respectively (Klionsky et al., 2003).

3.1.4 The role of autophagy in development and cellular homeostasis

Autophagy plays a physiological role in the development of many model organisms including *Saccharomyces cerevisiae*, *Caenorhabditis elegans, Drosophila melanogaster and Mus musculus*. Genetic downregulation of autophagic proteins in mice has revealed that it is important for embryonic development. Atg5 has been shown to be required for the preimplantation phase

in mice (Tsukamoto et al., 2008). Its activity persists throughout development and until 24 to 48 hours after birth (Kuma et al., 2004). In flies, autophagic degradation has been shown to occur in larval tissues such as the fat bodies and salivary glands. Although metamorphosis can proceed normally with minimal or no autophagy, the pupal period is prolonged in the Atg7 knockout larvae (Juhász et al., 2007; Juhász and Neufeld, 2008).

Post-development, autophagy continues to play a role in cellular differentiation (Mizushima and Levine, 2010). Erythroblasts mature into erythrocytes, and in doing so, they release the nuclei and eliminate intracellular organelles. The process by which the cell loses the organelles is poorly understood, but autophagy is suspected to play a role based on electron microscopy studies (Gronowicz et al., 1984; Heynen et al., 1985; Kent et al., 1966; Takano-Ohmuro et al., 2000). Also, mice lacking ULK1, a mammalian homologue of Atg1, exhibit an increase in their reticulocyte count (Kundu et al., 2008). Proper lymophocyte differentiation has also been shown to require autophagy (Zhang et al., 2009).

In mature and differentiated cells autophagy plays an important role in cellular homeostasis and its quality control mechanism. Autophagy was first described in hepatic cells as a cellular response to glucagon administration (Ashford and Porter, 1962). Under nutrient-rich conditions, autophagy is inhibited by target of rapamycin (TOR). TOR mediates its effects on autophagy through the inhibition of Atg1, a kinase that is involved in the earliest steps of

autophagosome formation. Upon starvation, this brake on autophagy is released and degradation of intracellular cargo provides amino acids for further use by the cell (Chang et al., 2009; Jung et al., 2010; Neufeld, 2010). The nonselective bulk degradation is not the only mechanism by which autophagy degrades intracellular components. Recently, a more selective form of autophagy has been proposed, in which different substrates, such as mitochondria, peroxisomes, bacteria and protein aggregates, can be selectively targeted for degradation. Similar to the cytoplasm to vacuole targeting (Cvt) pathway described in yeast (Nair and Klionsky, 2005), selective autophagic degradation of protein aggregates involves receptor and adapter molecules such as p62, also known as sequestosome 1 (SQSTM1) (Pankiv et al., 2007), NBR1 (neighbor of BRCA1 gene 1) (Kirkin et al., 2009) and ALFY (Autophagy linked FYVE protein) (Filimonenko et al., 2010). Selective autophagy may play an important role in the homeostasis of the cell quality control machinery. The aforementioned proteins have been shown to be involved in the clearance of polyubiquitinated protein aggregates in mammalian cells. In *Drosophila*, blue cheese, the homologue of ALFY (Finley et al., 2003), has been shown to be dispensable for starvation-induced autophagy, further confirming its involvement in selective autophagy rather than nonselective starvation-induced autophagy.

3.1.5 Autophagy induction can be a protective intervention in neurodegeneration

Since many adult onset neurodegenerative diseases are characterized by the deposition of protein aggregates and inclusions, it has been suspected that autophagy activation may reduce aggregate burden on the neuron. Autophagy activation has thus been implicated as a potential therapeutic intervention. Rapamycin, a potent inducer of autophagy, proved beneficial in clearing the aggregate load in different neurodegeneration models (Bove et al., 2011). For example, in the PDAPP mice overexpressing a mutant human amyloid precursor protein (Games et al., 1995), rapamycin feeding suppressed the AD-like cognitive deficits and reduced the levels of Abeta-42 (Spilman et al., 2010). The amelioration of cognitive deficits was observed in triple transgenic AD mice as well. Also, the reduction in Abeta-42 was also observed in 7PA2 cells, which contains the familial APP 717(Val-Phe) mutation, upon rapamycin treatment. Soluble tau and Abeta levels were similarly reduced in triple transgenic mice feeding on rapamycin, and this reduction was not seen with 3-methyladenine, an inhibitor of autophagy (Caccamo et al., 2010). In PC12 cells, rapamycin reduced the levels of exogenous wild type and mutant alpha-synuclein (Webb et al., 2003). Rapamycin also suppressed neurodegeneration in a polyglutamine Atrophin (Nisoli et al., 2010), a polyglutamine-expanded huntingtin, and a tau *Drosophila* model (Berger et al., 2006).

RESULTS

3.2 Human tau localized to the microtubules and induced a rough eye phenotype

We utilized the previously developed human tauopathy fly eye model (Jackson et al., 2002) in which the full length, four repeat isoform of human tau is fused to an eye specific promoter – the glass multimer reporter (GMR) (Hay et al., 1994; Ollmann et al., 2000). In this model, human tau misexpression is first noted in the developing eye disc posterior to the morphogenetic furrow in third instar larvae **(Figure 3.1)**. Post-eclosion, the toxic effect of human tau is manifested as a dosage-sensitive rough eye phenotype. Expression of two copies of human tau resulted in a more severe phenotype as seen by the increased rough area and reduced the area of organized ommatidia **(Figure 3.2)**.

The mechanism by which human tau induces neurodegeneration in flies is still unclear. There have been conflicting results about the potential of human tau to affect fly microtubules. Mudher and colleagues showed that neuronal misexpression of human tau reduces the binding affinity of the fly endogenous tau to the microtubules by about 60% and consequently disrupts the microtubule-based axonal transport (Cowan et al., 2010a; Cowan et al., 2010b). Lecourtois and colleagues, however, showed that human tau, unlike *Drosophila* tau, has low affinity for *Drosophila* microtubules (Feuillette et al., 2010). We therefore sought to determine whether human tau would co-localize within the same subcellular compartment as the fly microtubules. Fortini and colleagues have previously

shown that the endogenous fly tau is co-localized within the same subcellular compartment as the fly microtubules (Heidary and Fortini, 2001). Using tubulin as a marker for microtubules, we found that human tau was localized to the same subcellular compartment as tubulin **(Figure 3.3)**. As a positive control, we sought to determine whether human tau would also colocalize with the cytoskeletal elements. Phalloidin, which is a marker for actin filaments, did not show colocalization with the human tau. Although we cannot conclude solely based on this finding that human tau and the endogenous fly microtubules or endogenous fly tau physically interact, we can assume based on this result that human tau is colocalized to the same subcellular compartment as the fly microtubules in our model.

3.3 Rapamycin feeding suppressed human tau-induced phenotype

Since autophagy activation has been suggested as a potential therapeutic intervention in various neurodegenerative models, we sought to determine whether a potent inducer of autophagy such as rapamycin would be able to exert a suppressive effect on human tau toxicity. As mentioned previously, rapamycin has been shown to be protective in mice, flies and cellular models of Huntington disease, Parkinson disease and AD (Berger et al., 2006; Rubinsztein, 2006). In view of the fact that the human tau-induced phenotype is a developmental one, flies were fed on rapamycin throughout their development. Larvae expressing human tau were grown on a medium containing either 0.45% DMSO or 5 μM

rapamycin in 0.45% DMSO. After eclosion, adult female flies were analyzed using high resolution microscopy to assess the extent of roughness in the eye. We noticed that the flies that were grown on rapamycin-rich food, had a less severe rough eye phenotype when compared with those grown on DMSO alone **(Figure 3.4A and 3.4B)**. This indicated that rapamycin feeding potentiated the suppression of human tau-induced rough eye phenotype. However, given that TOR inhibiton may also affect a myriad of other pathways such as translation and cell proliferation (Cardenas et al., 1999; Cutler et al., 1999; Thomas and Hall, 1997), the rapamycin effects are not confined to inducing autophagy. In the next few sections we demonstrated that autophagy activation through caloric restriction or by overexpressing key autophagic genes exerted the same effects.

3.4 Rapamycin feeding reduced the soluble levels of human tau

In most adult-onset neurodegenerative conditions, there is a progressive loss of neuronal function associated with cellular pathology. Clearly the tauopathy model we use exhibits a development-induced phenotype rather than an adult-onset one. The development of the fly eye is complete by the late pupal stage, (Ting and Lee, 2007) and thus, human tau expression mediates its neurotoxic effects during the larval and pupal stage and is manifested as a rough eye phenotype in adults. In order to assess whether rapamycin feeding could modulate human tau toxicity in the adult stage as well, we measured the levels of human tau in flies feeding on rapamycin. In a *Drosophila* model of human

tauopathy, Lecourtois and colleagues have shown that the soluble form of human tau is the more toxic form of human tau *in vivo* (Feuillette et al., 2010). Given the expected role of rapamycin in autophagy induction, we sought to determine the effect of rapamycin feeding on the levels of soluble human tau in adults. Files miesexpressing human tau were grown on regular medium then transferred to vials containing 5 µM rapamycin dissolved in 0.5% ethanol or 0.5% ethanol as a control. After feeding the glass-tau flies on rapamycin or the regular food for one week, we measured the levels of soluble human tau. After 1 week, adult flies feeding on rapamycin had a decrease in the soluble levels of human tau **(Figure 3.4C)**. First this indicated that the central nervous system in adult flies is amenable to the effects of rapamycin feeding. It also suggested that rapamycin likely induced autophagy and hence resulted in a reduction in the soluble levels of human tau. Collectively, these results show that rapamycin feeding was neuroprotective. Based on previous reports and our findings, rapamycin most likely mediated its neuroprotective effects via activation of autophagy and hence reduced the soluble levels of human tau. We also attempted to overexpress key autophagic genes to determine if they would result in the same outcome as rapamycin feeding.

3.5 Caloric restriction suppressed the toxic phenotype of human tau

Initially discovered as a cellular response to starvation (Yang and Klionsky, 2010), autophagy also occurs as a cellular response to dietary

restriction in higher eukaryotes (Martinet et al., 2006; Mizushima et al., 2004). Neuronal autophagy has also been shown to be amenable to short term fasting in mice (Alirezaei et al., 2010). We turned our attention to the effect of caloric restriction on human tau toxicity. Caloric and dietary restrictions are one of the reproducible anti-aging interventions in many organisms (Fontana et al., 2010; Hansen et al., 2008; Mair and Dillin, 2008; Thompson and Voss, 2009; Yu, 1996), and low caloric intake has been shown to be associated with lower incidence of AD (Gustafson et al., 2003; Luchsinger et al., 2002; Pasinetti et al., 2007). We thus sought to determine whether tauopathy flies growing on a caloric restricted diet exhibited amelioration in the human tau-induced phenotype. A caloric-restricted diet was achieved by diluting the food in agar, therefore reducing the caloric content to about 30%. We found that feeding larvae on a caloric restricted diet significantly suppressed the toxic eye phenotype of human tau in adults **(Figure 3.5)**. This is an example of an environmental intervention that can modulate the neurotoxicity of human tau in the fly eye.

3.6 Upregulation of the autophagy-lysosomal pathway suppressed the toxic phenotype of tau

The two previous interventions, rapamycin feeding and caloric restriction were both environmental interventions that have been previously shown to induce autophagy. In order to confirm the role of autophagy induction in suppressing human tau toxicity, we co-expressed autophagic and lysosomal

genes along with human tau. Genetic overexpression was achieved using the GAL4/UAS binary system, allowing for the eye-specific expression of these genes (Venken and Bellen, 2007). Flies carrying the GMR-GAL4 on the X chromosome in trans to glass-tau on the second chromosome were crossed with transgenic flies containing an upstream activating sequence (UAS) fused with the gene of interest, or with a P-element insertion containing an empty UAS inserted into the promoter of the endogenous gene (Bellen et al., 2004). We found that genetic upregulation of key autophagic and lysosomal genes suppressed the human tau-induced phenotype **(Figure 3.6)**. The positive modifiers were ribosomal S6 kinase (S6k), Atg2 and cathepsin D. S6k is a kinase downstream of TOR kinase participating in autophagy activation (Scott et al., 2004). In S6k knockout *Drosophila*, autophagy cannot be induced in the fat bodies under starvation, or upon TOR inactivation. Similarly, when TOR levels were reduced leading to a chronic state of autophagy induction, overexpression of S6k lead to an increase in autophagy levels (Scott et al., 2004). Atg2 conjugates with Atg9 and Atg18 and is involved in the early steps of vesicle nucleation (Baehrecke, 2003; Obara et al., 2008; Simonsen et al., 2007). Silencing of Atg2 in mammalian cells inhibitis autophagosome maturation leading to the formation of unclosed autophagic vacuoles (Velikkakath et al., 2012). Cathepsin D, which has been detected at high levels in AD brains, is a lysosomal aspartic protease (Cataldo et al., 1995). Feany and colleagues have previously shown that loss of cathepsin D potentiates the neurotoxic effects of human tau – decreased longevity and

increased vacuoles in the fly brain (Khurana et al., 2010). These modifiers confirm the role of autophagy in suppressing human tau toxicity.

Atg1 is a kinase involved in the first steps of autophagy induction. In flies, overexpression of Atg1 in the fat body or in imaginal discs has been shown to induce high levels of autophagy (Scott et al., 2007). When expressing Atg1 under a GMR driver we noticed that it induces a rough eye phenotype **(Figure 3.7)**. Unlike the other autophagy transgenes that did not cause a rough eye phenotype, Atg1 overexpression did. This is likely due to very high levels of autophagy induction and potential induction of apoptosis (Scott et al., 2007). Since Atg1 is involved in different pathways and induced a rough eye phenotype in the adult fly, we did not use it in our screen. The phenotype caused by Atg1 overexpression was similar to the phenotype caused by human tau misexpression, both causing an external rough eye phenotype. It would have then been challenging to dissociate the phenotypic effects of Atg1 from that of human tau.

3.7 3-methyladenine feeding enhanced the toxic phenotype of tau

Next we turned our attention to the role of autophagy inhibition on human tau toxicity. First we examined the effects of 3 methyladenine (3-MA), which has been traditionally known as an autophagy inhibitor (Blommaart et al., 1997), on human tau toxicity. It exerts its effects through inhibition of class 3 phophatidylinositol 3-kinase (Blommaart et al., 1997). Although 3-MA has since

been used as an inhibitor of autophagy, Shen and colleagues have recently shown that under nutrient rich conditions 3-MA enhances autophagy induction (Wu et al., 2010). They showed that prolonged treatment of mouse embryonic fibroblasts with 3-MA in nutrient rich conditions lead to the accumulation of LC3 punctae. Thus, the negative effects of 3-MA on autophagy are only prevalent under starvation conditions and are reversed under nutrient-rich conditions.

In order to circumvent the effects of food, we incubated human tau-expressing late third instar larvae in either DMSO or 3-MA in DMSO for 90 minutes. They were then transferred along with the incubating solution to a vial containing fresh food where they soon acquired a puparium. Once flies acquired a puparium they were not able to feed and were secluded from the outside environment. Thus the exposure to 3-MA was only limited to the time between when the late third instar larvae were incubated in 3-MA until they acquired a puparium. We examined the eye phenotype of the adult flies and found that flies which were incubated in 3-MA during their larval stage exhibited more retinal roughness than those which were incubated in DMSO only **(Figure 3.8)**. This further supported the role of autophagy in modulating human tau toxicity. Blocking autophagy by incubating larvae in 3-MA enhanced the toxic eye phenotype of human tau consistent with the effects of autophagy activation on human tau toxicity.

3.8 Endogenous autophagy is involved in suppressing human tau toxicity

Next we attempted to confirm the previous finding genetically. We examined the effect of genetic downregulation of key autophagic and lysosomal genes on human tau toxicity. Heterozygous loss of function of target genes was achieved by utilizing P-element insertions or EMS-induced mutant alleles. The mutant allele was put *in trans* to the glass-human tau transgene. We found that partial loss of function of Atg4 and Atg6 enhanced the human tau-induced phenotype **(Figure 3.9B and 3.9C)**. Atg4 is a cysteine protease that cleaves pro-Atg8 at its C-terminus, generating Atg8, an essential component of the autophagic vacuoles (Hemelaar et al., 2003; Obara et al., 2008). Atg6 (beclin 1) forms a complex with Vps15 and Vps34, both of which are involved in vesicle nucleation (Chang et al., 2009; Chang and Neufeld, 2009; Ravikumar et al., 2010).

Atg6 was previously identified as one of the human tauopathy modifiers by our laboratory (Ambegaokar and Jackson, 2011). Partial loss of function of either Atg4 or Atg6 did not cause a rough eye phenotype by itself. Next, we expressed a kinase-dead version of Atg1 to block the initial step of autophagy: its induction. Unlike in yeast where the role of the kinase domain in autophagy is uncertain (Abeliovich et al., 2003; Kamada et al., 2000), the role of the Atg1 kinase domain in *Drosophila* is more established (Scott et al., 2007). Neufeld and colleagues showed that the kinase domain was necessary to induce autophagy,

and expression of a kinase-dead form of Atg1 inhibited starvation-induced autophagy in the fat body (Scott et al., 2007). We utilized the kinase-dead Atg1 transgene (UAS-Atg1.KD) to inhibit autophagy in the eye. Overexpression of Atg1.KD using a GMR driver did not induce any phenotype in the eye. However, when co-expressed along with human tau, it enhanced the rough eye phenotype induced by human tau **(Figure 3.10)**. This showed that endogenous autophagy plays an important role in suppressing human tau toxicity since downregulation or inhibition of autophagy led to an enhancement of human tau-induced toxicity.

3.9 Genetic downregulation of components of the selective autophagic machinery enhanced human tau toxicity

There are different roles and types of autophagy. Autophagy has long been thought of as a nonselective bulk degradation pathway. A selective form of autophagy called the cytoplasm to vacuole targeting (Cvt) pathway has been well established in yeast but not in higher organisms. Recently, a selective form of autophagy has also been described in higher eukaryotes, including flies and mice. This form of autophagy is involved in degrading organelles such as mictochondria and peroxisomes, and in clearing pathogens and aggregates. It has distinct players that are dispensable for the nonselective, starvation-induced autophagy (Filimonenko et al., 2010). Since we showed that endogenous autophagy was able to partially protect against human tau toxicity, we sought to determine whether components of selective autophagy also participated in the

protection against human tau toxicity. Among these components is the phosphatidylinositol 3-phosphate-binding protein, autophagy-linked FYVE domain (ALFY). Through its WD-40 domain, ALFY interacts with Atg5 and recruits it to protein aggregates. It acts as a scaffold to bring components of the autophagic machinery to protein aggregates to be targeted for degradation. The fly homologue of ALFY is called Blue cheese. It is involved in clearing aggregates but is dispensable for the starvation induced, *non-specific*, autophagy in the larval fat body (Filimonenko et al., 2010). Overexpression of Blue cheese was protective in a fly eye model of polyQ toxicity and reduced the inclusions in a neuronal model of Huntington disease (Filimonenko et al., 2010). Mutations in the *blue cheese* gene, however, caused neural degeneration and extensive accumulation of ubiquitin-positive inclusions in the CNS (Finley et al., 2003; Simonsen et al., 2007). Given the role of Blue cheese in the selective autophagic machinery, we attempted to examine the effects of its partial downregulation on human tau toxicity. We found that heterozygous loss of function of Blue cheese enhanced the toxic phenotype of human tau **(Figure 3.9D)**. This suggested that the endogenous autophagy involved in partially suppressing human tau toxicity required adapter molecules that were necessary for selective autophagy.

Another player in selective autophagy is histone deacetylase 6 (HDAC6), a microtubule deacetylase that interacts with polyubiquitinated aggregates. HDAC6 has been suggested to play a role in the transport of aggregates into autophagosomes and aggresomes (Kawaguchi et al., 2003). We also examined

the effects of HDAC6 partial loss of function on human tau toxicity and found that it enhanced the rough eye phenotype **(Figure 3.9E)**.

3.10 Genetic downregulation of genes involved in lysosomal acidifcation enhanced human tau toxicity

Lastly, we turned our attention to the role of the lysosomal machinery in protecting against human tau toxicity. Acidification of autophagosomes is necessary for the degradation of cargo, and initiates the last step in autophagy: clearance. It is dependent upon fusion with lysosomes to form acidic autolysosomes. We showed previously that overexpression of cathepsin D suppressed human tau toxicity. Cathepsins are activated in the acidic milieu of lysosomes and autolysosomes. Acidification depends upon the proton pump vesicular adenosine triphosphatase (v-ATPase). It consists of two subunits: the membrane bound V0 sector and the cytosolic V1 sector (Nishi and Forgac, 2002). The *Drosophila* gene *vha-100-1 (v100)* encodes a subunit of the membrane-bound V0 sector and has been shown to be required for the acidification of late endosomes and autophagosomes (Williamson et al., 2010). We introduced a *vha* mutant allele in trans to glass-tau and found that partial loss of function of Vha enhanced human tau toxicity **(Figure 3.9F)**.

In order for the autolysosomes to acquire an acidic lumen, the v-ATPase V0 a1 subunit must be recruited to the lysosome. Presenilin has been found to be required for this step (Lee et al., 2010). Presenilin is part of the catalytic subunit

of the gamma secretase complex and is involved in amyloid precursor protein (APP) and Notch cleavage. Mutations in the *presenilin* gene have been associated with AD. Many of them were associated with increased production of the amyloidogenic Abeta fragment (Citron et al., 1997), yet a number of other *presenilin* mutations failed to show an increase in Abeta production (Shioi et al., 2007). This suggests that the neurotoxic effects of some of the *presenilin* mutations may be independent of the amyloidogenic cleavage. The new suggested role of Presenilin in lysosomal acidification could explain other means by which *presenilin* mutations could lead to neurodegeneration. We sought to determine whether partial loss of function of Presenilin could enhance human tau toxicity. Introducing a mutant allele of *presenilin*, Psn^{B3}, in trans to the glass human tau transgene resulted in an enhancement of the rough eye phenotype **(Figure 3.9G)**. In our model, human tau expression by itself led to a rough eye phenotype, while a *presenilin* mutation alone did not alter the eye morphology. Thus the effects of *presenilin* mutation were more likely due to a defect in lysosomal acidification rather than aberrant amyloidogenic cleavage. These results collectively suggest that properly acidified lysosomes were necessary for suppressing the toxic effects of human tau.

DISCUSSION

3.11.1 Autophagy induction was neuroprotective

Autophagy activation has been shown to alleviate neurotoxicity in many neurodegenerative disease models. Here we showed that autophagy induction was protective in the fly model of human tauopathy. Early activation of autophagy, through genetic overexpression of autophagic and lysosomal genes, suppressed the rough eye phenotype induced by human tau. Rapamycin feeding suppressed the toxic human tau-induced phenotype as well. We suspected that the mechanism through which rapamycin mediates its neuroprotective effects is autophagy. We measured the soluble levels of human tau in response to rapamycin feeding. We found that rapamycin feeding reduced the amount of soluble human tau. A reduction in the soluble levels of human tau implies that it is either being degraded or sequestered possibly into autophagosomes. Whether it is only sequestered or completely degraded will be the focus of the next chapter.

Regardless of the fate of human tau, a reduction in its soluble levels was associated with the neuroprotective effects observed. This is consistent with previous reports demonstrating that the soluble form of human tau is the major toxic entity in the fly tauopathy model (Feuillette et al., 2010). Although protein aggregates are a hallmark of neurodegeneration, and their presence correlates with the disease severity, they may not be the primary neurotoxic factor. In cellular and mice model, soluble forms of tau have recently been shown to be pathogenic (Brunden et al., 2008; Lasagna-Reeves et al., 2011). This suggests

that development of new therapies directed against the pre-fibrillar forms of tau may be more, or equally, effective than the ones aimed at clearing aggregates. The reduction in the soluble levels of human tau in response to rapamycin was observed in adult tauopathy flies feeding on rapamycin as well. This demonstrates that autophagy activation through rapamycin was not only beneficial in the developing *Drosophila* but in fully developed adult flies as well. Since adult-onset neurodegeneration is usually not due to a developmental pathology, showing that rapamycin was able to exert its effects in adult flies renders the model more relevant to studying the disease.

These findings suggest that autophagy can play an important neuroprotective role in neurodegeneration. However, the role of autophagy in reversing the pathology was not examined. In a fly model of Abeta pathology, early activation of autophagy alleviated Abeta42-induced neurotoxicity, which is consistent with our findings. However, late activation of autophagy, through Atg1 overexpression late during the *Drosophila* adult stage, enhanced the toxicity of Abeta42 (Ling and Salvaterra, 2011). This underscores the importance of timing in autophagy induction. In our model of human tauopathy, autophagy was genetically induced as early as when human tau was expressed. The autophagic and lysosomal genes were expressed under the GMR driver, as was human tau. In *Drosophila,* eye development is largely complete by 70% pupal development (Ting and Lee, 2007). Therefore, the adult-onset expression of autophagic and lysosomal genes, even when they continue to be expressed post-eclosion,

cannot reverse the rough eye phenotype. Thus, this model of human tauopathy cannot be used to test the capacity of autophagy induction in reversing the neurotoxic phenotype of human tau.

3.11.2 Caloric restriction was neuroprotective

One of the reproducible anti-aging interventions in many organisms is caloric restriction (Fontana et al., 2010; Hansen et al., 2008; Mair and Dillin, 2008; Melendez et al., 2003). In flies, dietary restriction increases lifespan and reduces mortality (Bauer et al., 2009; Mair et al., 2003; Rogina and Helfand, 2004). We found that feeding larvae on a caloric restricted diet resulted in the suppression of the adult rough eye phenotype. This finding was also observed in other neurodegenerative models. Caloric restriction and intermittent fasting have both be shown to ameliorate the cognitive decline observed in AD mice models (Halagappa et al., 2007; Wu et al., 2008).

Our findings are consistent with other reports showing that early autophagy induction may be used an early intervention to prevent the progression of neurotoxicity in various neurodegeneration models (Berger et al., 2006; Bove et al., 2011; Nisoli et al., 2010; Spilman et al., 2010). However, pharmacologic interventions aimed at inducing autophagy to treat neurodegeneration in humans have not been very fruitful (Bove et al., 2011; Mendelsohn and Larrick, 2011; Menzies and Rubinsztein, 2010; Santos et al., 2011). Until recently, there were no data demonstrating that starvation and

caloric restriction can induce autophagy in the mammalian brain (Mizushima et al., 2004). However, Kiosses and colleagues showed that intermittent starvation induces profound neuronal autophagy in the mouse brain (Alirezaei et al., 2010). Although we cannot conclude that autophagy activation or caloric restriction could exert the same neuroprotective effects in humans, there are epidemiological data linking high caloric intake and the risk for AD development (Gustafson et al., 2003; Luchsinger et al., 2002; Pasinetti et al., 2011). Even if these measures do not result in curing AD, they may delay its progression. Delaying the onset of AD by few years could delay the incidence of the disease by more than 50% (Brookmeyer et al., 1998).

3.11.3 Basal autophagy participated in suppressing human tau toxicity

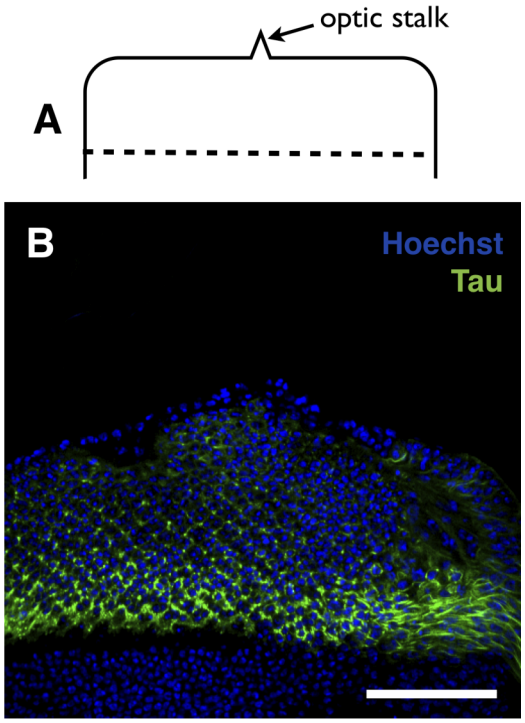
After establishing that ectopic induction of autophagy is neuroprotective, we sought to determine whether the cell is able to make use of the autophagic/lysosomal pathway in reducing human tau toxicity. Exposing the larvae to 3-methyladenine, a known inhibitor of autophagy, enhanced the human tau-induced rough eye phenotype. Genetic downregulation of key autophagic and lysosomal genes resulted in a similar outcome. Interestingly, among the modifiers of human tau toxicity was Blue cheese, the fly homologue of ALFY, a key component in selective autophagy. This suggests that the neuron was able to utilize autophagy to limit the neurotoxic effects of human tau. The role of the

lysosomal machinery in modulating human tau toxicity in flies has also been previously described. Feany and colleagues demonstrated that loss of cathepsin D enhanced the toxicity of human tau in *Drosophila* (Khurana et al., 2010). This is consistent with our findings that overexpression of cathepsin D suppressed human tau-induced toxicity. Partial loss of function of other proteins such as Presenilin and Vha100 also confirmed the importance of the lysosomal compartment in protecting against human tau toxicity.

Collectively, these findings supported the idea that endogenous levels of autophagy can limit human tau toxicity. However, the fact that ectopic activation of autophagy was able to further suppress the phenotype implied that the neuron was either not able to fully activate autophagy or that the autophagic flux did not proceed properly. This will be addressed in the next chapter, which will focus on the impact of human tau on the autophagic machinery.

FIGURES

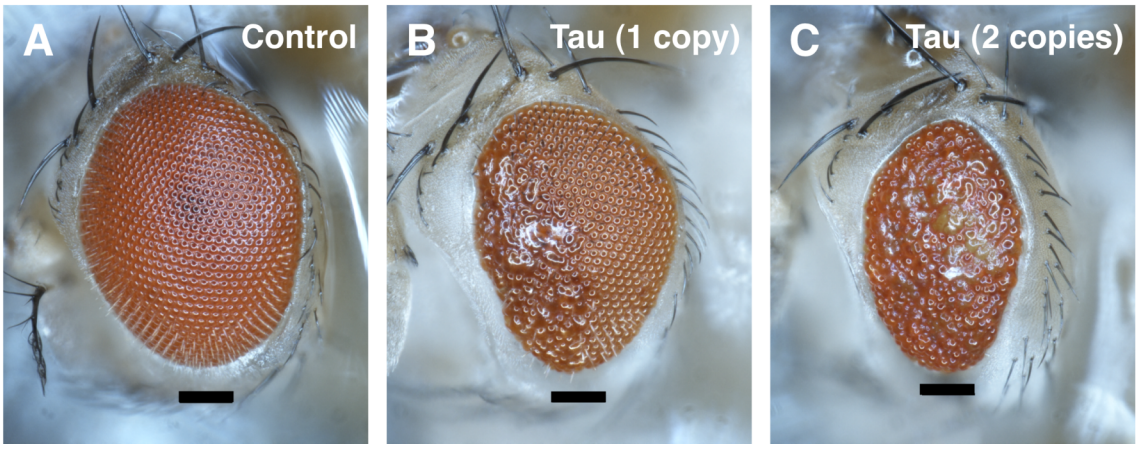
Figure 3.1. Human tau is expressed posterior to the morphogenetic furrow in the eye-antennal disc



A) A schematic showing the eye discs of third-instar larvae. The dashed line marks the morphogenetic furrow. **B)** Human tau (green) is expressed between the morphogenetic furrow and the eye disc. The eye disc is positioned at the same orientation as in A. Hoechst staining is shown in blue. *Scale bar is 50* μm*.*

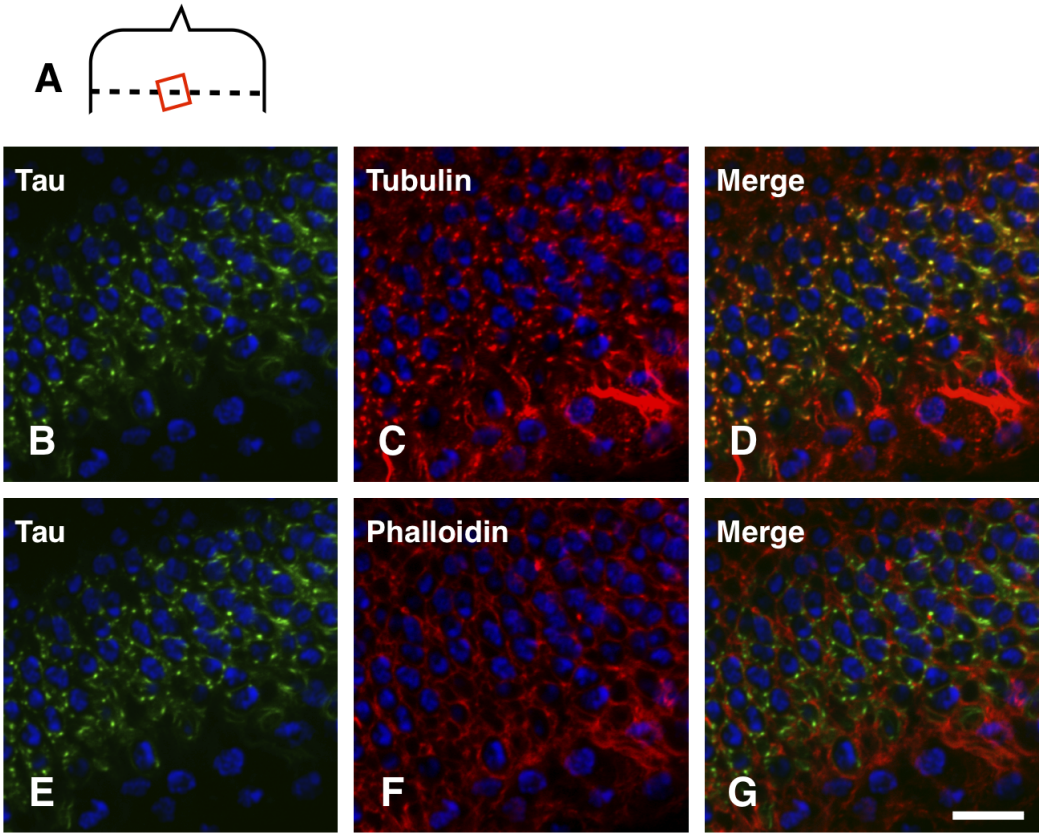
Genotype: w^{1118}; glass-tau$^{1.1}$.eGFP.

Figure 3.2. Human tau misexpression resulted in a dosage-dependent rough eye phenotype



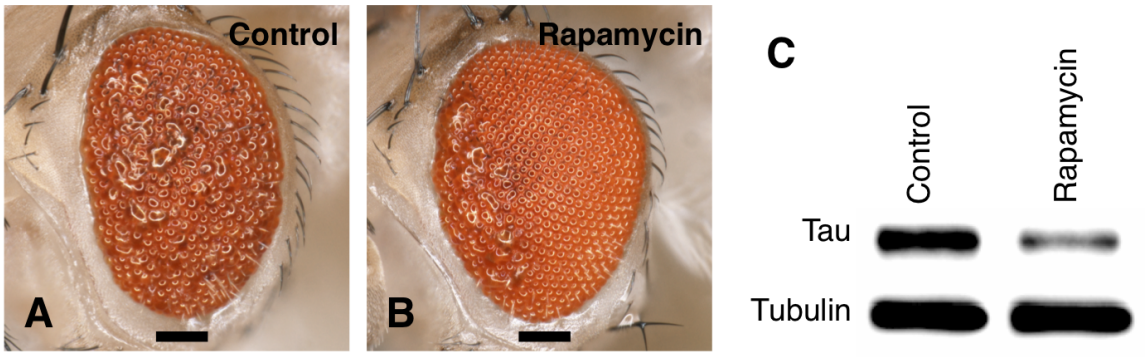
A) Wild type flies (Canton S). **B)** Flies expressing one copy of glass-human tau. C) flies expressing two copies of glass-human tau. Scale bar is 50 µm. *Genotypes:* ***A)*** *Canton S,* ***B)*** w^{1118}*; glass-tau*$^{1.1}$*/+ and* ***C)*** w^{1118}*; glass-tau*$^{1.1}$*.*

Figure 3.3. Human tau was localized to the same subcellular compartment as *Drosophila* endogenous microtubules



A) A schematic showing the eye disc of third instar larvae. The location of the section used in immunostaining is outlined in red, and the morphogenetic furrow is marked by a dashed line. **B - D)** Human tau subcellular localization resembles that of endogenous fly microtubules. **B)** Human tau (green), **C)** tubulin (red) and **D)** merge. **E - G)** Human tau is not localized to the same subcellular localization as the actin cytoskeleton. **E)** Human tau (green), **F)** Phalloidin (actin) (Red) and **G)** merge. Hoechst staining is shown in blue. Scale bar is 10 µm. *Genotype: w^{1118}; glass-tau$^{1.1}$.eGFP.*

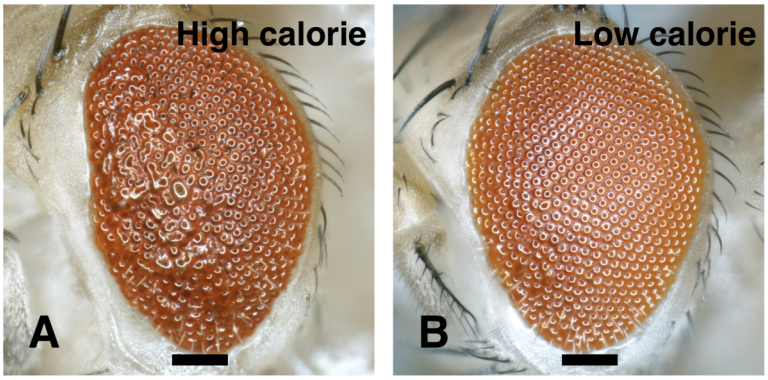
Figure 3.4. Rapamycin feeding suppressed the toxic phenotype of human tau



The rough eye phenotype caused by htau misexpression was suppressed in flies growing on a diet containing 5 μM rapamycin **(B)** than those growing on DMSO alone **(A)**. **C)** Adult glass-tau flies feeding on rapamycin had reduced amount of htau in the soluble fraction as shown by the human tau-5 monoclonal antibody reactivity. Control: glass-tau flies feeding on 0.48% ethanol for 1 week. Rapamycin: glass-tau flies feeding on 5 μM rapamycin in 0.48% ethanol for 1 week. Scale bar is 50 µm.

Genotypes: ***A & B)*** *w^{1118}; glass-tau$^{1.1}$/+ and* ***C)*** *w^{1118}; glass-tau$^{1.1}$.*

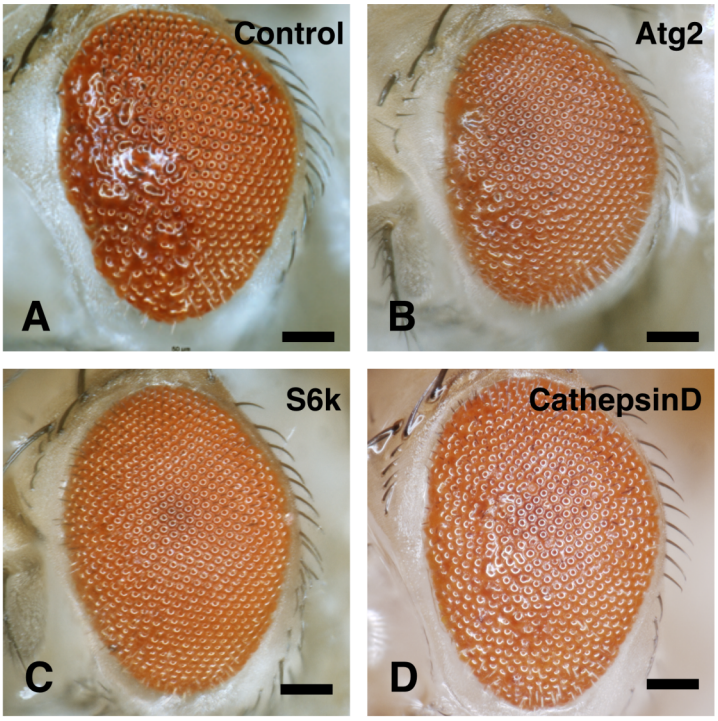
Figure 3.5. Caloric restriction suppressed the toxic phenotype of tau



The rough eye phenotype caused by human tau misexpression was suppressed in flies growing on a diet containing a third of the caloric content **(B)** versus flies growing on a full caloric diet **(A)**.

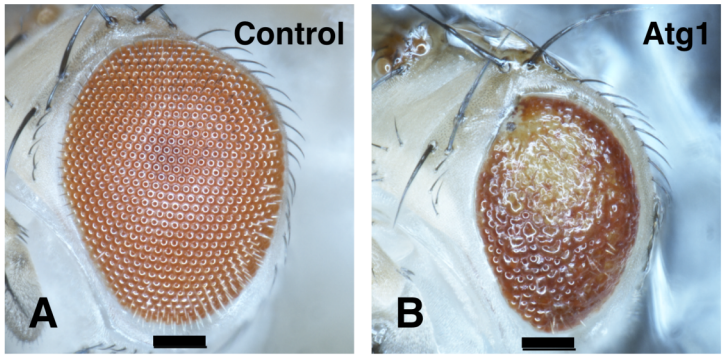
Genotype: w^{1118}; glass-tau$^{1.1}$/+. Scale bar is 50 µm.

Figure 3.6. Genetic upregulation of autophagic and lysosomal genes suppressed the toxic phenotype of human tau



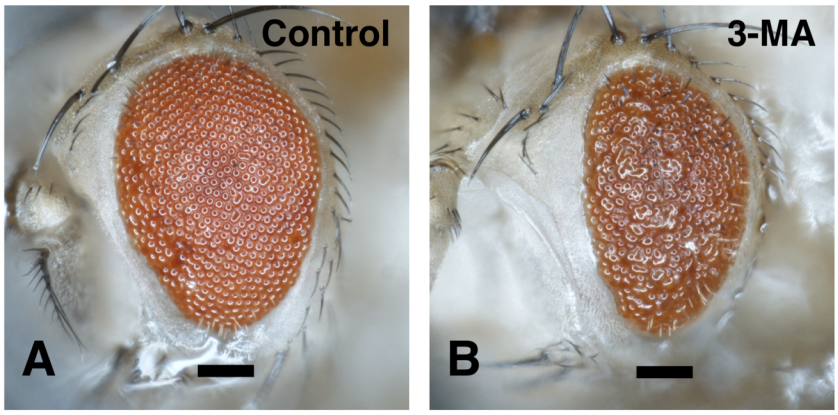
The rough eye phenotype caused by human tau misexpression was suppressed in flies that overexpressed autophagic and lysosomal genes. Eye specific overexpression was achieved using the GMR-GAL4/UAS binary system. **A)** Control, **B)** UAS-Atg2, **C)** UAS-S6k and **D)** UAS-Cathepsin D. *Genotypes:* ***A)*** *w^{1118},GMR-GAL4/+; glass-tau/+,* ***B)*** *w^{1118},GMR-GAL4/w^{1118};glass-tau/UAS-Atg2^{EP3697},* ***C)*** *w^{1118},GMR-GAL4/w^{1118};glass-tau/UAS-S6k,* ***D)*** *w^{1118},GMR-GAL4/w^{1118};glass-tau/UAS-CathepsinD.* Scale bar is 50 μm.

Figure 3.7. Flies overexpressing Atg1 exhibited a rough eye phenotype



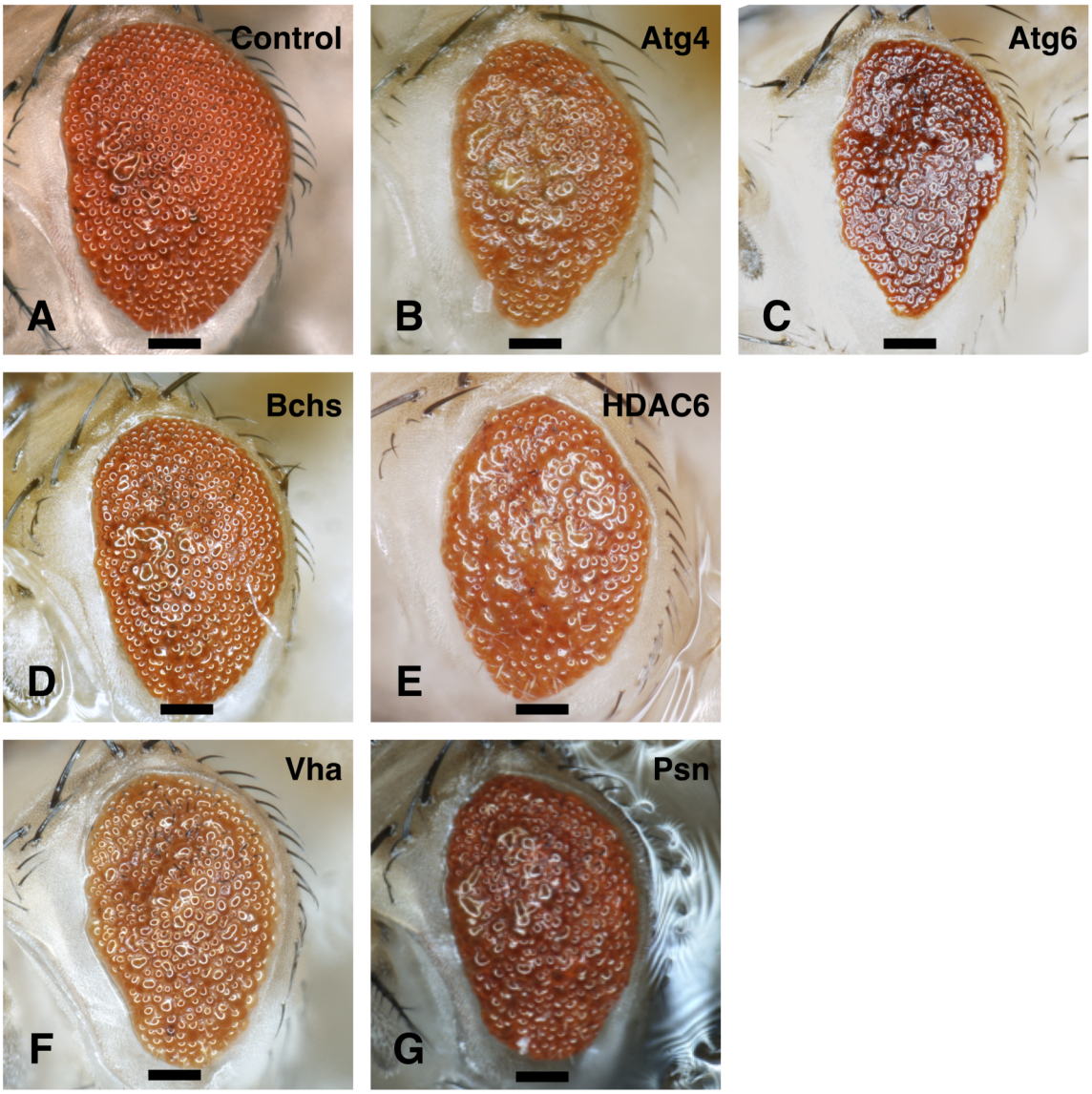
Overexpression of Atg1 under a GMR driver induces a rough-eye phenotype and the eye was reduced in size. **A)** w,GMR-GAL4/+ and **B)** w,GMR-GAL4/+;; UAS-Atg1/+. Scale bar is 50 µm.

Figure 3.8. Exposure to 3-methyladenine enhanced human tau toxicity



The rough eye phenotype caused by human tau misexpression was enhanced in flies exposed to 3-methyladenine (3-MA) during their late larval stage **(B)** versus tauopathy flies exposed to DMSO alone **(A)**. Scale bar is 50 µm. *Genotype: w^{1118}; glass-tau$^{1.1}$/+.*

Figure 3.9. Genetic downregulation of autophagic and lysosomal genes enhanced the toxic phenotype of human tau

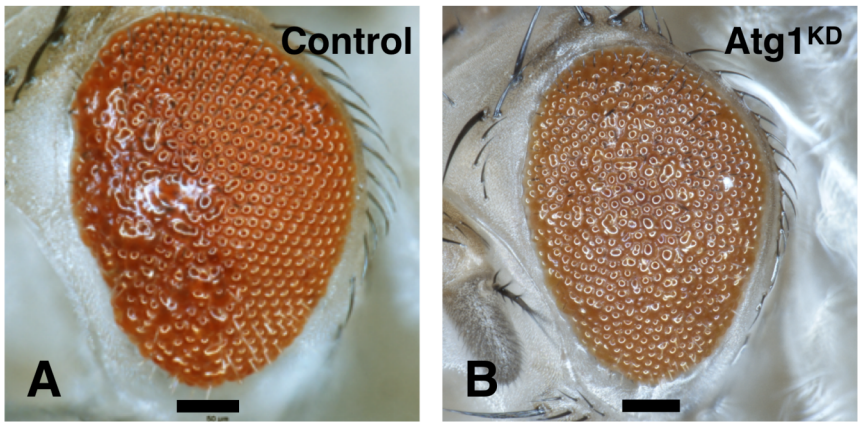


The rough eye phenotype caused by human tau misexpression was enhanced in flies with partial loss of function of autophagic and lysosomal genes. Partial loss of these genes without human tau misexpression did not result in a rough eye phenotype. **A)** Control, **B)** Atg4, **C)** Atg6, **D)** BCHS, **E)** HDAC6, **F)** vha^{100} and **E)** Psn^{B3}. *Genotypes: A) w^{1118};glass-tau/+, B) w^{1118};glass-tau/Mi[ET]Atg4^{MB3551}, C)*

w^{1118};glass-tau/P[PZ]Atg6^{96}, D) w^{1118};glass-tau/bchs^{17},

E) w^{1118}/w^{1118},Mi[ET1]HDAC6^{MB6564};glass-tau/+, F) w^{1118};glass-tau/vha^{100} and G) w^{1118};glass-tau/+; Psn^{B3}/+. Scale bar is 50 μm.

Figure 3.10. Co-expression of a kinase dead Atg1 enhanced the toxic phenotype of human tau



The rough eye phenotype caused by human tau misexpression was enhanced in flies co-expressing a kinase dead version of Atg1. **A)** Control, **B)** Atg1^{KD}. *Genotypes:* ***A)*** *w^{1118},GMR-GAL4/+;glass-tau/+,* ***B)*** *w^{1118},GMR-GAL4/w^{1118};glass-tau/UAS-Atg1^{KD}.* Scale bar is 50 μm.

CHAPTER IV

THE IMPACT OF HUMAN TAU MISEXPRESSION ON THE AUTOPHAGIC MACHINERY

INTRODUCTION

4.1.1 Tauopathies

Tauopathies are group of neurodegenerative diseases characterized by the presence of aggregates containing the microtubule associated protein tau. A subgroup of these disorders, frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) has been linked to mutations in the tau gene (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998), whereas sporadic tauopathies such as AD, Pick disease, corticobasal degeneration and progressive supranuclear palsy have not been associated with any tau mutations (Ballatore et al., 2007). In both groups, modifications of tau such as phosphorylation, truncation, and conformational changes have been observed. It has been widely believed that these modifications reduce tau binding affinity to the microtubules, thus depriving them of their main stabilizing protein, tau (Iqbal et al., 2009; Weingarten et al., 1975). However in recent years, this hypothesis has been brought into question by *in vivo* data from animal and cellular and fly models as will be discussed later.

4.1.2 Tau pathology in AD

One of the most leading worldwide causes of dementia is AD. It affects about 45% of people over the age of 85. In 2012 there are about 5.4 million people diagnosed with the disease[4]. Neurofibrillary tangle pathology in AD was described by Dr. Alois Alzheimer over a century ago (Graeber et al., 1997). Mainly composed of tau aggregates, the neurofibrillary tangles along with the extracellular amyloid plaques containing Abeta aggregates have been thought of as the hallmarks of this disease (Grundke-Iqbal et al., 1986a; Kosik et al., 1986; Wood et al., 1986). Braak and Braak relied on the analysis of plaques and tangles load to stage the progression of the disease (Braak and Braak, 1991). Pathological changes were classified into 6 different stages, with stage VI being the most advanced stage of the disease pathology. The Braak and Braak neuropathological staging has been shown to be useful in predicting the cognitive decline in AD patients; memory function declined from stages II to III, while the mental status was associated with stages III to IV (Grober et al., 1999). Since then, many attempts have been made to unravel the contribution of each of the plaques and tangles in disease pathogenesis. Although amyloid deposits appeared early in the course of progression of the disease, they were of low utility in staging the neuropathological changes. Tangles on the other hand were more closely associated with the clinical manifestation of the disease (Bennett et al., 2004). Animal models have also contributed to the knowledge in this area.

[4] 2012 Alzheimer's Disease Facts and Figures
<http://www.alz.org/downloads/facts_figures_2012.pdf >

Cultured hippocampal neurons from wild type mice degenerated in the presence of Abeta only when tau was present; in the tau knockout mice no signs of degeneration were noted (Rapoport et al., 2002). The mechanistic link between the amyloid plaques and neurofibrillary tangles in mediating the disease is still a subject of debate (Duyckaerts et al., 2009; Gotz et al., 2011; LaFerla, 2010). However, animal models have proved beneficial in studying the neurotoxic effect and the developed pathology of each of the proteins separately as will be demonstrated here.

4.1.3 The Drosophila homolog of tau

Drosophila tau shares high homology with its mammalian counterparts. It has 46% identity and 66% similarity with the human tau protein. There are five microtubule-binding repeats present in the fly protein, but the N-terminal inserts are absent. *Drosophila* tau is widely expressed in the developing and adult nervous system of the fly and exhibits high localization with the microtubules (Heidary and Fortini, 2001). Overexpression of fly tau in mushroom body neurons compromised associate olfactory learning and memory (Mershin et al., 2004). In the eye, overexpression of the fly tau results in a rough eye phenotype similar to that observed with human tau overexpression (Chen et al., 2007). In larvae, *Drosophila* tau overexpression impaired axonal transport, altered neuromuscular junction morphology and had a negative impact on locomotion (Ubhi et al., 2007). However, since the major interest is in elucidating the pathogenesis of the human

disease, most of the tauopathy models in flies have been generated using the human tau.

4.1.4 Human tau pathogenesis in *Drosophila* - phosphorylation

Drosophila has been widely used to model a number of neurodegenerative diseases including tauopathies[5] (Jackson et al., 2002; Kosmidis et al., 2010; Williams et al., 2000; Wittmann et al., 2001). The mechanism by which human tau misexpression induces a toxic phenotype is not fully understood. Fly models have, however, elucidated many cellular pathways as being potentiators of tau neurotoxicity. Given the popularity of the hypotheses linking tau phosphorylation or its microtubules binding ability with disease pathogenesis, these two properties have been widely investigated in Drosophila.

Paired helical filaments, the hallmark of tauopathies, have been shown to contain hyperphosphorylated forms of tau (Grundke-Iqbal et al., 1986b; Kosik et al., 1986). Tau contains 79 putative serine or threonine phosphorylation sites (Buee et al., 2000; Hanger et al., 2009). Additionally, several kinases and phosphatases have been shown to directly affect tau phosphorylation. Examples include glycogen synthase kinase-3Beta (GSK-3Beta) (Hanger et al., 1992; Mandelkow et al., 1992), cyclin-dependent kinases (Baumann et al., 1993; Kobayashi et al., 1993), the extracellular regulated kinase (Drewes et al., 1992;

[5] A brief overview of these models was outlined in the introductory chapter

Goedert et al., 1992), the microtubule-associated regulating kinase (MARK) (Drewes et al., 1997), p38 (Reynolds et al., 1997), and protein phosphatases 1, 2A and 2B (Buee et al., 2000). This wealth of knowledge has facilitated the study of the role of human tau phosphorylation in mediating neurodegeneration in fly models. Kinases and phosphatases have been identified as modifiers of tau toxicity in *Drosophila* (Shulman and Feany, 2003), lending credence to the assertion that tau phosphorylation plays an important role in mediating its toxicity. This assertion is further supported by the finding that a phosphorylation-incompetent form of tau, obtained by mutating all the kinase sites to alanine, has reduced neurotoxicity as compared to wild type tau (Steinhilb et al., 2007). On the other hand, pseudo-hyperphosphorylated tau, produced by substituting all serines and threonines with glutamate, has increased toxicity (Fulga et al., 2007). Jackson and colleagues, however, found that a mutant tau resistant to GSK-3β (S11A) retained substantial toxicity, perhaps because of an increased affinity for microtubules (Chatterjee et al., 2009).

Fly models investigating human tau phosphorylation are not limited to the eye. Skoulakis and colleagues recently investigated the role of tau phosphorylation in the CNS. Wild type tau overexpression pan-neuronally causes developmental mushroom body ablation in the fly (Kosmidis et al., 2010). Human tau phosphorylation at Ser^{238} and Thr^{245} was necessary for mediating its toxicity. As expected, these flies exhibited severe impairment of associative learning and memory. Expression of human tau in the dorsal mesothorax (notum) of

Drosophila under the notum driver Eq-GAL4, caused loss of mechanosensory bristles on the dorsal surface of the fly. The toxic phenotype manifested by loss of bristles was enhanced by tau phosphorylation and was suppressed by a reduced dosage of shaggy (Yeh et al., 2010).

These results indicate that a phosphorylated form of human tau is more neurotoxic to the fly than a wild type or a pseudophosphorylated version. However, the role of human tau phsophorylation in mediating its toxicity appears to be one of many other pathways that can mediate human tau neurotoxicity in flies. Jackson and colleagues performed a genome-wide screen to identify modifiers of human tau toxicity (Ambegaokar and Jackson, 2011). Besides those affecting human tau phosphorylation, other genes identified were linked to autophagy, cell cycle regulators, RNA-binding proteins and microtubule-based transport. Surprisingly, the majority of the modifiers identified failed to show a strong association with the level of human tau phosphorylation using the AT8, 12E8 and AT270 epitopes, which detect phosphorylation at S202/T205 (Biernat et al., 1992), S262/S356 (Seubert et al., 1995) and T181 (Goedert et al., 1994), respectively. Also, the modifiers identified were equally successful in modulating the phosphorylation-resistant version of human tau (S11A), pointing to the fact that human tau phosphorylation is not essential in mediating its toxic effects. Collectively, these results point to the fact that there are many facets to human tau neurotoxicity. Tau phosphorylation is one of them but is not a common factor to all pathways affecting human tau toxicity.

4.1.5 Human tau pathogenesis in *Drosophila* – microtubule-based transport

Besides its phsophorylation, tau binding to microtubules also plays an important role in disease pathogenesis. When first discovered, tau was identified as a microtubule stabilizing factor *in vitro* (Weingarten et al., 1975). Tau is thought to bind microtubules and regulate axonal transport (Gustke et al., 1994). Phosphorylation can also modulate how strongly tau binds to microtubule. Phosphorylated tau at AD-linked epitopes has lower affinity for microtubules (Alonso et al., 1994; Lovestone et al., 1996; Mandelkow et al., 1995). Linking tau hyperphosphorylation to its microtubule binding properties has been used to explain the defective microtubule assembly observed in AD brains (Iqbal et al., 1986). The effect of human tau misexpression on the endogenous fly microtubules and the microtubule-based transport such as axonal transport has thus been thoroughly investigated in fly models. However, the uncertainty of the involvement of microtubules stability in mediating the pathology in the human disease is also paralleled in fly models; the potential of human tau to directly impact endogenous *Drosophila* microtubules has been controversial.

In flies, endogenous tau has been shown to have the same subcellular localization as microtubules (Heidary and Fortini, 2001). Although there is a high homology between fly tau and its mammalian counterparts (46% identity and 66% similarity) (Heidary and Fortini, 2001), the effect of human tau on

endogenous fly microtubules is not well defined. Lecourtois and colleagues have shown that human tau, unlike *Drosophila* tau, has low affinity for *Drosophila* microtubules. Most of human tau was recovered in the soluble fraction as hyperphosphorylated species. Knockout of *Drosophila* tau did not alter human-tau induced toxicity in the eye, nor did it affect human tau binding to microtubules (Feuillette et al., 2010). Mudher and coworkers reached similar results demonstrating that hyperphosphorylated human tau has very low affinity for the endogenous microtubules. They showed that only 10% of human tau is bound to fly microtubules. When treating larvae expressing human tau with lithium chloride, which inhibits tau phosphorylation by GSK-3beta (Lovestone et al., 1999; Mudher et al., 2004; Stambolic et al., 1996), the amount of human tau bound to microtubules increased to more than 20% (Cowan et al., 2010a). However, they showed a mechanistic link between the ectopic human and the endogenous fly tau in regulating their binding to microtubules. In wild type flies, the percentage of endogenous tau bound to microtubules was about 50%. However in the presence of human tau, the fraction of endogenous tau bound to microtubules was only 20% and increased to more than 30% in the absence of hyperphosphorylated human tau (Cowan et al., 2010a). In summary, although the binding of human tau to fly microtubules is not very well established, human tau misexpression has been shown to negatively impact the fly tau binding properties to the endogenous microtubules.

Regardless of how human tau interacts with the fly microtubules, its expression has been shown to negatively impact microtubule-based transport. Electron micrographs of peripheral nerves of *Drosophila* larvae revealed disorganized and misaligned microtubules upon human tau misexpression (Cowan et al., 2010a). It also caused axonal transport and synaptic defects (Cowan et al., 2010b). Paramentier and colleagues, on the other hand, reached a different conclusion regarding the impact human tau phosphorylation on endogenous microtubule integrity. They found that a hypophosphorylatable version of human tau (tau^{AP}), with 14 serine or threonine residues mutated to alanine mutations, had more detrimental effects on the microtubules and axonal transport than wild type human tau or a pseudohyperphosphorylated version (tau^{E14}) (Talmat-Amar et al., 2011). Although there are conflicting data explaining the mechanism by which human tau impacts microtubules, it has been clearly shown that human tau misexpression negatively impact axonal transport in neurons and causes synaptic defects. Different versions of human tau seem to be more preferentially toxic than others.

4.1.6 Human tau pathogenesis in *Drosophila* – cell death

Since neurodegeneration is characterized by the selective loss of specific neuronal subpopulations, programmed cell death has been suspected to play a role in the development of pathology. In tauopathies, this is supported by histological evidence from autopsy cases. DNA damage and activated caspase-3

immunoreactivity is observed in brains from frontotemporal dementia cases (Su et al., 2000). In AD brains, DNA fragmentation and signs of apoptotic cell death are noted (Cotman and Anderson, 1995; Jellinger and Stadelmann, 2000; Lassmann et al., 1995; Roth, 2001; Su et al., 1994). In cellular models, overexpression of a mutant human tau has been shown to be pro-apoptotic (Furukawa et al., 2000). Mice expressing wild type human tau supported these findings; they exhibited cell death with DNA fragmentation (Andorfer et al., 2005).

In flies, signs of apoptotic cell death have been shown in response to human tau misexpression. In the larval eye disc, human tau misexpression induced apoptotic cell death evident by accumulation of lamin (Jackson et al., 2002). Lamin accumulation is a sign of nuclear envelope breakdown, a cardinal feature of apoptotic cell death (Lazebnik et al., 1995; Oberhammer et al., 1994). Feany and colleagues have demonstrated that wild type as well as mutant human tau induced apoptotic neurodegeneration in *Drosophila*. Ectopic cell cycle activation accompanied tau-induced apoptotic cell death and was necessary for apoptosis to occur (Khurana et al., 2006). Genetic modifiers of human tauopathy in flies have also highlighted the role of apoptosis in neurodegeneration. Inhibitors of apoptosis suppressed human tau toxicity while activators of apoptosis enhanced it (Shulman and Feany, 2003). However, a large functional genomic screen in flies covering 1900 genes identified 40 genes as modifiers of human tau toxicity. None of them had any anti-apoptotic effects (Ambegaokar and Jackson, 2011). It is noteworthy however that, in this study, antiapoptotic

effects was measured by the ability of the modifier stock to suppress the effects of a proapototic gene, hid. Hid misexpression is in the eye results in an ablation of the eye. Hid effects may thus be too strong to be suppressed in this system.

Apoptosis is not the only type of cell death that has been observed in tauopathies. Pathologies indicative of necrotic and autophagic cell death have been described in autopsy specimens from diseased brains and animal models (Lim et al., 2001; Lucassen et al., 1997; Ma et al., 2010; Nixon et al., 2000; Stadelmann et al., 1998). A genome-wide screen from the Jackson laboratory identified Atg6, the beclin-1 homologue, as a modifier of human tau toxicity (Ambegaokar and Jackson, 2011). Atg6 is involved in the vesicle nucleation, one of the earliest steps in autophagy initiation as well as autophagosome maturation (Chang et al., 2009). The role of autophagy in mediating human tau-induced pathology in fly models has not been well investigated. Here we attempted to study the impact of human tau on the autophagic machinery in *Drosophila*.

4.1.7 Neuronal autophagy – a process highly dependent upon microtubule-based transport

In most cells, cellular debris can be diluted during subsequent cellular divisions. This is not the case in post-mitotic cells such as neurons. In these cells, the catabolic machinery is important in maintaining the quality control system; defective organelles and proteins can be targeted to degradation through

the ubiquitin-proteasome system or autophagy[6]. Large aggregates and organelles that cannot be easily degraded by the proteasome are more accessible to the autophagic machinery (Kanki et al., 2009; Klionsky, 2005; Okamoto et al., 2009). Autophagy may play an important role in maintaining neuronal homeostasis. However, most of what we know about autophagy is derived from non-neuronal experimental models. The autophagic machinery has been thoroughly studied utilizing the fat body in *Drosophila* (Chang and Neufeld, 2010; Rusten et al., 2004; Scott et al., 2004; Zirin and Perrimon, 2010), liver tissue in rodents (Novikoff et al., 1964; Yang and Klionsky, 2010) as well as mammalian cellular models and yeast (Klionsky et al., 2008). Recently neuronal autophagy has been shown occur in the nervous system (Yue et al., 2009; Yue et al., 2008). Autophagy has also been shown to occur in neurons in response to starvation (Alirezaei et al., 2010). But since this is not consistent with previous findings (Mizushima et al., 2004), further investigations are necessary to confirm the effect of starvation on brain tissue in mice.

Although there is no evidence showing that neuronal autophagy requires specific adapter molecules different than those described in other cell types, one would expect that autophagy in neurons requires an intact microtubule system. This stems from the fact that most neurons have long axons; thus microtubule-based transport linking the distal parts of the axon with the soma plays an

[6] An overview of the autophagic machinery has been described in the third chapter

important role in neuronal homeostasis. The initial step of the autophagic pathway, which is autophagosomal biogenesis, can occur anywhere along the axon (Jahreiss et al., 2008). But in order for autophagosomes to fuse with the lysosomes, they must travel towards the centrioles where the lysosomes are abundant (Abou-Sleiman et al.; Jahreiss et al., 2008; Kimura et al., 2008; Seglen et al., 1996). Disruption of microtubule-based transport with vinblastine, a microtubule depolymerizing agent, causes the accumulation of autophagosomes and prevents their clearance (Kovacs et al., 1982; Xie et al., 2010). In PC12 neurites, autolysosomes move in both an anterograde and retrograde directions, but upon starvation their net movement is biased towards the nucleus (Yang et al., 2011). This fast microtubule-based movement was inhibited by applying nocodazole, which binds tubulin and prevents microtubule polymerization. The transport of autolysosomes was equally affected by selectively inhibiting kinesin or dynein motor proteins, thus disrupting anterograde or retrograde transport (Yang et al., 2011). The role of microtubules in the transport of autophagic intermediates has also been shown in primary dorsal root ganglion neurons, in which autophagosomes mature distally and undergo a bidirectional transport followed by uni-directional transport towards the peri-nuclear area that is driven by dynein. Thus the retrograde transport of autophagosomes towards the soma is necessary for clearance (Maday et al., 2012). The role of microtubule elements, such as histone deacteylase 6 (HDAC6), in autophagy has also been demonstrated in different systems (Batlevi et al., 2010; Cai and Sheng, 2011;

Cardoso et al., 2009; Xie et al., 2011; Xie et al., 2010; Yamamoto et al., 2010; Yang et al., 2011).

Since microtubule integrity and axonal transport have both been shown to be compromised in various neurodegeneration models, one would expect that a process heavily dependent upon axonal transport such as autophagy could easily be involved in the development of neurodegenerative pathology.

4.1.8 Autophagy failure in neurodegeneration

Given its role as a quality control system, autophagy dysfunction may contribute to the development of neurodegenerative pathology. Mice lacking Atg5 or Atg7, key autophagic genes, in the central nervous system exhibit behavioral and motor deficits and die very early. They also accumulate protein aggregates and polyubiquitinated inclusion bodies that increase in number and size with aging (Hara et al., 2006; Komatsu et al., 2006). Although an Atg5/Atg7-independent form of macroautophagy has been described (Nishida et al., 2009), loss of Atg5 or Atg7 significantly reduces levels of autophagy. The findings described in the previous chapter showed that loss of autophagy exacerbated human tau-induced toxicity, which is consistent with these aforementioned reports highlighting the importance of protein degradation pathways including autophagy in maintaining neuronal homeostasis.

Pathologies indicative of autophagic dysfunction have been demonstrated in various neurodegenerative conditions such as AD (Nixon et al., 2001; Nixon et

al., 2005), PD (Anglade et al., 1997; Cuervo et al., 2004; Meredith et al., 2002; Pan et al., 2008; Vila et al., 2011), HD (Kegel et al., 2000; Ravikumar et al., 2002; Rudnicki et al., 2008) and amyotrophic lateral sclerosis (Martinez-Vicente and Cuervo, 2007; Rubinsztein, 2006; Rubinsztein et al., 2005; Wong and Cuervo, 2010). Brain specimens from AD cases show extensive involvement of the autophagic machinery in diseased neurons. Ultrastructural studies show that autophagic vacuoles, which are scarce in normal brains, are abundant in AD brains. In dystrophic neurites, autophagosomes, multivesicular bodies (MVBs), and lysosomes accumulate in large numbers (Nixon et al., 2005). Although the presence of these structures may suggest an increased rate of autophagy in diseased neurons, the accumulation of immature autophagic vacuoles equally suggests an inability of the autophagosome to mature and fuse to the lysosome. Thus the formation of the autolysosome, which is dependent on axonal transport as well as acidifcation by lysosomes, may be impaired. In AD brain, autophagic components are abundant in dystrophic neurites containing tau, neurofibrillary tangles and Abeta plaques (Ma et al., 2010). Recently, granulovacuolar degeneration bodies, one of the large aggregates that form in AD brains (Ball, 1978) and other tauopathies (Lagalwar et al., 2007), have been associated with late-stage autophagic markers (Funk et al., 2011).

Modeling neurodegeneration in cells, rodents, and flies, among other systems, has contributed to our knowledge about autophagic dysfunction. Neurons deprived of nerve growth factor degenerate and accumulate autophagic

intermediates in neuritic beadings (Yang et al., 2007). In primary cotical neurons, disruption of the late steps in autophagy such as the fusion between autophagosomes and lysosomes, or interfering with the catabolic pathway within lysosomes, caused a marked increase in the number of aberrant autophagic intermediates. These vesicles with undigested cargo were similar to the autophagic pathology observed in dystrophic neurites in the AD brain and and AD mouse model (Boland et al., 2008). Neurons from mice harboring an AD-linked *presenilin* mutation, with defects in lysosomal proteolysis, accumulated aberrant autophagic intermediates (Lee et al., 2010). These abnormalities were a common factor to other neurodegenerative models; they were noted in PD mouse models (Dehay et al., 2010; Vila et al., 2011), PC12 cells expressing A53T mutant alpha-synuclein (Stefanis et al., 2001), SOD1 mutant mice (Li et al., 2008; Morimoto et al., 2007), dentatorubral-pallidoluysian atropht (DRPLA) model in *Drosophila* (Nisoli et al., 2010), and others (Wong and Cuervo, 2010). Neurons rely heavily on autophagy to maintain cellular homeostasis, and autophagic intermediates do not normally accumulate even under high levels of autophagy. In flies and primary cortical neurons, induction of high levels of autophagy by rapamycin does not result in the accumulation of autophagic intermediates in large numbers (Boland et al., 2008; Ling et al., 2009), indicating that the accumulation of aberrant autophagic intermediates may be a sign of an aberrant rather than an increased autophagic flux.

4.2 Phosphorylation of TOR substrates 4E-BP and p-70 S6k did not change significantly in tauopathy

In the previous chapter, we showed that endogenous autophagy was involved in partially suppressing the human tau toxic phenotype which raised the possibility that autophagy might be induced in response to human tau expression. We therefore sought to examine the effect of human tau misexpression on basal autophagy levels. As an initial indirect method of measuring autophagy levels *in vivo*, we measured the phosphorylation levels of proteins that are downstream of *target of rapamycin* (TOR). TOR is a serine/threonine protein kinase that activates anabolic processes such as protein synthesis and inhibits catabolic pathways such as autophagy (Diaz-Troya et al., 2008). TOR activation results in the phosphorylation of the eIF4E-binding proteins, 4E-BPs and the AGC serine/threonine protein S6 kinase (S6k). Thus, low phosphorylation levels of these substrates is widely used as an indication for low TOR activity and thus high levels of autophagy. 4EBP1 is phosphorylated by the TOR complex at residues Thr37 and Thr46, and p70 S6k is phosphorylated at residue Thr 398. Using phospho-specific antibodies, we sought to determine whether the endogenous levels of these proteins were decreased in tauopathy. Protein extracts from glass-tau flies exhibited a slight reduction in the levels of phospho-4EBP1 when compared with wild type flies **(Figure 4.1A)**. Quantification of the western data revealed that the normalized band intensity of

the glass-tau extract was about 2.0 +/- 0.9, whereas that of the control was 4.9 +/- 0.6 (p value was 0.06, n = 3). This indicated that in the tauopathy models, TOR activity may be slightly suppressed, correlating with higher levels of autophagy induction. On the other hand, the levels of phospho-p70 S6k in human tau-expressing flies were comparable with those in control flies **(Figure 4.1A)** Quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 3.3 +/- 1.0, whereas that of the control was 3.0 +/- 0.4 (p value was 0.76, n = 3). The trend toward a small decrease in the levels of phospho-4E-BP but not of phospho-p70 S6k can have different interpretations. One explanation is that human tau was not able to induce high levels of autophagy. Consequently, this would imply that TOR activity was not suppressed, hence the low or negligible change of phosphorylation in 4E-BP and p70 S6k. Alternatively, It can be interpreted as a sign of chronic autophagy. Acute starvation reduces the phosphorylation levels of TOR substrates p70 S6k and 4E-BP, but after prolonged starvation, their levels are increased to normal or higher than normal levels (Yu et al., 2010). Therefore one cannot conclude solely based on these data whether or not autophagy was induced in tauopathy. A more direct assay of autophagy was thus needed to measure the levels of accumulated autophagic intermediates in tauopathy.

4.3 The inducible tauopathy system was leaky and was not used to measure autophagy induction

In order to test whether autophagy might be chronically induced in tauopathy, we attempted to measure the phosphorylation level of TOR substrates in response to an acute increase in human tau expression. Obtaining an acute expression of human tau can be achieved by utilizing the binary GAL4/UAS system[7] along with a temperature sensitive GAL80 repressor. At a low temperature (18ºC), $GAL80^{ts}$ effectively binds to the UAS thus preventing the binding of GAL4, and consequently the transcription of the target transgene is halted. At a higher temperature, however (e.g., 29ºC), $GAL80^{ts}$ is inactivated and the GAL4 can freely bind the UAS, and hence the target transgene can be expressed. We coexpressed both GAL4 under a GMR promoter and GAL80 ubiquitously using a tubulin promoter, both *in trans* to human tau fused to a UAS. A stable line carrying all three transgenes was generated (w^{1118}; GMR-GAL4, UAS-tau/CyO; $GAL80^{ts}$) and its phenotype was analyzed. If grown and maintained at 18ºC, the eye appeared normal and retained its crystalline ommatidial architecture **(Figure 4.2B)**. This is in contrast to flies carrying the identical transgenes except for the $GAL80^{ts}$ repressor, which exhibit a robust rough eye phenotype **(Figure 4.2A)**. Since the human tau induced phenotype is a developmental one, shifting the adult flies from 18ºC to 29ºC post-eclosion did not alter the external architecture of the eye **(Figure 4.2C)**.

[7] The binary yeast GAL4/UAS system was described in the introductory chapter

Next we tested whether the levels of phospho-4E-BP and phospho-p70 S6k changed in response to human tau misexpression. After transferring the GMR-GAL4/GAL80/UAS-TAU which grew at 18ºC to 29ºC, we did not note any changes in the levels of phospho-p70 S6k or phospho-4E-BP **(FIGURE 4.2D)**. In order to confirm that human tau was acutely expressed in response to changing the incubation temperature, we measured the levels of human tau at both temperatures: 18ºC and 29ºC. We noted that at 18ºC human tau was still expressed despite the presence of $GAL80^{ts}$. When shifted to a higher temperature (e.g., 29ºC), the change in human tau levels was not significant **(FIGURE 4.2E)**. The GMR promoter drives the expression of many eye-specific genes and results in stronger expression during the developmental stages and lesser expression during the adult stage (*data not shown*). This may explain why a temperature-shift did not result in a net change in expression since GMR-GAL4 expression during the adult stage is lower. We could not then rely on the GMR-GAL4/GAL80/UAS-TAU inducible system to measure autophagy levels since it was leaky at lower temperatures and did not results in a robust expression at higher temperatures.

4.4 Human tau expression was accompanied by a robust increase in the lysosomal compartment.

In order to obtain *in vivo* evidence of autophagy induction, we analyzed the eye discs of third instar larvae using confocal microscopy. Under the GMR

driver, human tau is first expressed in eye discs of third instar larvae posterior to the morphogenetic furrow. In **Figure 3.1** we show that human tau is expressed in the larval eye disc posterior to the morphogenetic furrow and extends into the optic stalk. The imaginal discs of the fly are ideal for studying *in vivo* processes in that they are transparent, are a few cellular layers in thickness and can remain alive for about an hour in an isotonic and physiologic solution. In order to assess the level of autophagy induction, we attempted to measure the levels of late autophagic intermediates that had an acidic content. We utilized an acidotropic live dye in order to determine whether human tau misexpression was coupled with an increase in the lysosomal compartment. The acidotropic dye, LysoTracker DND-99, is a live pH indicator dye that is taken up by endocytosis, thus staining acidic vesicles. Staining the eye discs with LysoTracker revealed that there was an increase in the number of acidic punctae in the presence of glass-tau.GFP **(Figure 4.3A and 4.3B)**. Quantification of these punctae revealed a tenfold increase in the number of acidic punctae in glass-tau (540 +/- 75.7 in human tau-expressing larvae vs 47 +/- 18.5 in control, $p < 0.05$). **(Figure 4.3C)**. This suggested that there was an upregulation of the autophagic-lysosomal system in our tauopathy model.

LysoTracker is taken up by all acidic vesicles via endocytosis regardless of their nature or their degree of acidity. It does not discriminate between lysosomes, autolysosomes or late endosomes. In order to determine whether some of these punctae were autophagic intermediates, we coexpressed human

tau with an autophagy-specific marker, Atg5. It is part of the Atg12-Atg5-Atg16 conjugation system that localizes to the phagophore assembly site and binds to the outer side of the phagophore (Mizushima et al., 2003; Suzuki et al., 2001). Its association with the phagophore persists until the complete formation of the autophagosome **(Figure 4.4)**. We used an Atg5-GFP fusion in order to be able to visualize Atg5 subcellular localization in real time – LysoTracker staining necessitates that the cells be alive. LysoTracker staining of eye discs expressing human tau revealed a partial localization between the LysoTracker-positive punctae and the green Atg5-GFP signal **(Figure 4.3D)**. This indicated that a portion the LysoTracker punctae were autophagic intermediates. The GFP signal was low in intensity and the necessity to use a live dye precluded the use of fixative solutions or antibodies to amplify the fluorescent signal. However, the presence of partial localization was sufficient to conclude that some of these acidic punctae were autophagic intermediates. In the coming sections we will show a more robust staining of Atg5 punctae in the retina of glass-tau flies coupled with ultrastructure analysis in order to confirm the presence of autophagic intermediates in tauopathy.

We also attempted to measure the levels of lysosomal-associated membrane protein 1 (LAMP-1) for which there is a commercially available fly-specific antibody. LAMP-1 is a glycoprotein that shares 37% homology with another lysosomal membrane protein called LAMP-2 (Eskelinen et al., 2003). Although they diverged early in evolution, they have redundant functions; loss of

LAMP-1 led to a compensatory increase in LAMP-2 (Andrejewski et al., 1999). LAMP proteins have been shown to be required for the correct fusion between the autophagosome and lysosome (Binker et al., 2007; Penas et al., 2011; Saftig et al., 2008). In AD brains, there is an upregulation of the mRNA and protein levels of LAMP-1 (Barrachina et al., 2006). Given the robust increase in the lysosomal compartment, which is evident by the LysoTracker staining, we sought to establish whether LAMP-1 levels were increased in tauopathy. **Figure 4.3E** shows that the levels of LAMP-1 were upregulated in flies expressing human tau. Quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 1.51 +/- 0.19, whereas that of the control was 0.8 +/- 0.15 (p value was 0.001, n = 4). This supported our earlier findings and demonstrated that the lysosomal compartment is upregulated in tauopathy.

4.5 Autophagic intermediates accumulated in the retina of adult human tau-expressing flies

Earlier in **figure 4.1A**, we demonstrated that there was a small decrease in the phosphorylation levels of TOR substrates, 4E-BP and p70 S6k in glass-tau flies, yet in glass-tau larvae there was a robust LysoTracker staining in the eye disc. One possible explanation we proposed to explain this discrepancy is that autophagy was chronically induced. Alternatively, autophagy could only be induced in the larval stage and not the adult fly stage. In order to determine whether the accumulation of autophagic intermediates persisted post-

development, we examined the distribution of Atg5-GFP when coexpressed with human tau in the retina of adult fly. As we mentioned earlier, Atg5 is localized to the phagophore and its association with the autophagic membrane persists until the maturation of autophagosomes. In control flies, the Atg5-GFP expression pattern was ubiquitous and diffuse **(Figure 4.5B)**. On the contrary, human tau expression led to the accumulation of Atg5-GFP punctae **(Figure 4.5E)**. This demonstrated that autophagy was also induced in the adult glass-tau fly. Thus, the explanation of a chronic autophagy status leading to a mild or negligeable decrease in TOR substrates seemed a plausible interpretation of the mild decrease in the TOR substrates. This also showed that autophagy which was induced in response to human tau expression in the larval eye disc was still induced in the adult stage.

4.6 The autophagic intermediates in tauopathy were immunoreactive for Blue cheese, a marker of the selective autophagic machinery

As we mentioned previously, autophagy can have different physiological roles in the cell. Initially it was described as a survival response to starvation, and it has also been shown to be involved in development. One of the recently identified roles of autophagy is its involvement in the quality control of the cell; in the clearance of long-lived organelles and protein aggregates. In contrary to nonselective starvation-induced autophagy, autophagy involved in the clearance of aggregates can be more selective and requires specific adapter proteins that

are dispensable for starvation-induced autophagy. One such protein is Blue cheese (ALFY homologue). Blue cheese has been identified in the pilot screen as a modifier of the rough eye phenotype induced by human tau **(figure 3.9)**. Earlier, we demonstrated that partial loss of function of autophagy enhanced human tau toxicity. Blue cheese exerted the same effect, suggesting that the endogenous autophagy induced in response to human tau was a selective autophagy – the nonselective autophagy can proceed in the absence of Blue cheese.

Therefore, we sought to determine whether the autophagic intermediates that accumulated in tauopathy were also immunoreactive for Blue cheese. Through its many domains, ALFY acts as a scaffold that brings the E3-like ligase, Atg5-Atg12-Atg16L and LC3 to the proteins destined for degradation. Using a polyclonal antibody against Blue cheese we found that the punctae formed were also immunoreactive for Blue cheese **(Figure 4.5F and 4.5G)**. Along with the phenotypic data, this confirms the involvement of selective autophagy as a cellular response to human tau misexpression in flies.

Next we wanted to measure the protein levels of Atg5 and Blue cheese in response to human tau misexpression. We found that the levels of either Atg5 or Blue cheese in the protein extracts from tauopathy did not differ from their levels in the control **(Figure 4.6)**. For BCHS, quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 1.1 +/- 0.04, whereas that of the control was 1.2 +/- 0.05 (p value was 0.08, n = 3). For Atg5,

quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 0.50 +/- 0.05, whereas that of the control was 0.48 +/- 0.04 (p value was 0.77, n = 3). Collectively, these western blots results along with the immunohistochemical ones indicate that the levels of these proteins did not change but their subcellular distributions did; the diffuse staining changed into more discrete punctae.

4.7 Ultrastructural analysis of human tau-expressing flies identified autophagic intermediates

In order to further investigate the involvement of the autophagic machinery in tauopathy, we used transmission electron microscopy. Human tau misexpression resulted in the disorganization of the internal architecture of the retina. The rhabdomeres in the photoreceptor neurons were disorganized and signs of neurodegeneration were noted throughout the retina **(Figure 4.7B)**. Autophagic intermediates were also more abundant in glass-tau flies; vesicles with double membranes were present in glass-tau flies but were scarce in control flies **(Figure 4.7A and 4.7C)**. In order to quantify their relative numbers, we analyzed 130 electron micrographs from glass-tau flies and controls and counted the frequency of double-membrane vesicles **(Figure 4.7D)**. The frequency of autophagic intermediates, characterized by their enclosing double membrane and their less than 1 μm diameter, increased fourfold in tauopathy. Their frequency was 6.28 +/- 1.60 in tauopathy versus 1.54 +/- 0.78 in control, with a p-

value of 3.32 * 10^{-14}. Given their size (less than 1 μm in diameter on average) and their enclosing double membrane, we identified these vesicles as autophagic vacuoles. This confirmed our previous findings that demonstrated that autophagic intermediates accumulate in human tauopathy. Collectively the results acquired from live imaging, confocal microscopy and electron micrographs supported the hypothesis that there was an upregulation of autophagic intermediates in tauopathy.

4.8 Giant autophagic bodies (GAB) accumulated in the retina of tauopathy flies

The previous findings suggested that autophagic intermediates accumulated in tauopathy. The increase in their number was more significant than the small change in autophagy induction, as measured by the levels of phospho-4EBP and phospho-p70 S6k, two kinases downstream of TOR. Signs of low autophagy induction may be due to failure of human tau to induce autophagy efficiently, or may indicate a chronic state of autophagy. We could not test the effect of acute human tau expression on TOR substrates since the inducible system (GMR-GAL4/GAL80/UAS-TAU) was leaky. The high levels of autophagic intermediates indicated that high autophagy induction by itself could not account for the accumulation of these vacuoles. Autophagy is a highly efficient process; ectopic induction of autophagy in flies through rapamycin does not result in the strong accumulation of autophagic intermediates (Boland et al., 2008; Ling et al.,

2009). Therefore, the accumulation of autophagic intermediates might be indicative of an obstructed rather than an increased autophagic flux, a state previously described as autophagic stress (Chu, 2006). A defect in the later steps of autophagy, impeding the clearance of autolysosomes, could in turn lead to the accumulation of earlier autophagic intermediates. A portion of these autophagic intermediates would consequently be aberrant autophagic intermediates, i.e. containing undigested cargo and large in volume. In order to test this hypothesis, we examined the intracellular ultrastructure of tauopathy flies, looking for aberrant forms of autophagic intermediates. In human tau-expressing flies, we noted the formation of giant bodies with an average diameter of 3 to 10 μm **(Figure 4.8)**. Ultrastructure analysis identified large spheres that contained a mixture of what seems to be digested and undigested material as evident by the electron dense material. They were enclosed by a double membrane with the inner membrane frequently undulating **(Figure 4.8A)**. The presence of multilamellar membrane in some of them **(Figure 4.8B)** might be indicative of their high phospholipid content. Longitudinal plastic sections of the eye were stained with toluidine blue in order to determine their relative location. Visualization at low magnification showed they were mostly present in the retina **(Figure 4.8D)** and were not present in control flies **(Figure 4.8C)**.

The presence of undigested material may be indicative of a failure in their degradative capacity. Most of them had a double membrane, but we could not classify them as autophagosomes because of their large diameter. In order to

further investigate the nature of these spheres, we tested their immunoreactivity for autophagic markers. They were immunoreactive for the early autophagic marker Atg5, LC3 and the selective autophagy adapter blue cheese **(Figure 4.9A, 4.9B and 4.9C)** and were observed in the pupal eye as well **(Figure 4.9D)**. Next, we wanted to confirm that they were not a developmental pathology. We utilized the inducible system of human tauopathy, GMR-GAL4/GAL80/UAS-TAU, to determine whether these structures can form post-eclosion. The GMR-GAL4/GAL80/UAS-TAU flies were grown at 18ºC and after eclosion they were transferred to 29ºC. We noted that these spherical bodies were present, yet at very low levels **(Figure 4.9E)**. They were not seen in the control flies. This suggested that these structures did not only form during development but that they were forming during adulthood as well.

Based on their morphology and their immunoreactivity to autophagic markers, we could safely claim that they were related to the autophagic machinery. Their large size, however, prevented us from classifying them as regular autophagic intermediates. Due to their content, morphology and size we named them giant autophagic bodies (GAB). This nomenclature was solely based on our observation and does not indicate whether they were functional or not. In the subsequent experiments, we attempted to get a better characterization of their content and their involvement in the autophagic machinery.

4.9 Large autophagic intermediates appeared early in the course of progression of human tau pathology

Large autophagic intermediates can indicate failing autophagy. Disrupting microtubule-based transport (Munafo and Colombo, 2001; Yu et al., 2010), blocking the fusion between autophagososmes or lysosomes (Gutierrez et al., 2004; Marzella et al., 1982) or interfering with the acidification of lysosomes (Rong et al., 2011) all result in the generation of large autophagic intermediates before the blockage. Similarly, the large autophagic bodies we noted in tauopathy might be indicative of a blockage in the autophagic flux. First, we sought to determine whether similar pathology was present during the early stages of the disease. We noted that large autophagic bodies were present as early as when human tau was first expressed in the eye discs of third instar larvae. In tauopathy, the LysoTracker DND-99 punctae not only increased in number but in size as well **(Figure 4.10B to 4.10D)**. In control larvae, the average area of the LysoTracker punctae was about 5.60 +/- 0.92 μm^2, whereas in the presence of human tau they had an average area of about 10.67 +/- 0.70 μm^2, p-value was 0.005. Detailed analysis of these particles revealed that in tauopathy 65.1% +/- 1.2% of the punctae had a surface area less than 10.0 μm^2, in contrast with 82.4% +/- 2.1% of the punctae in control, with a p-value of 0.0003. The distribution of larger particles had an opposite trend; in tauopathy 18.4% +/- 1.0% of the punctae had a surface area between 10.0 μm^2 and 20.0 μm^2, as opposed to only 12.3% +/- 2.0 of the punctae in control: p-value was 0.04. For particles

with an area between 20 μm^2 and 30 μm^2, the distribution was 8.3% +/- 0.6% in tauopathy vs 3.8% +/- 1.8% in control: p-value was 0.08. For particles with an area above between 30 μm^2 and 40 μm^2, the distribution was 4.0% +/- 0.5% in tauopathy vs 1.5% +/- 1.5% in control: p-value was 0.2. For particles with an area above 40 μm^2, the distribution was 4.3% +/- 1.4% in tauopathy vs 0% +/- 0% in control: p-value was 0.02. Pie charts summarizing theses results for control and tauopathy are shown in **figure 4.10E and 4.10F**. These results confirmed that the acidic punctae in tauopathy were much larger in size than the punctae forming in the control flies.

4.10 GAB are poorly acidified autophagic intermediates

Although the size of autophagosomes can differ depending on the tissue (Mizushima et al., 2004), large autophagic intermediates have been described in other models, and they are sometimes indicative of autophagic dysfunction. In rat hepatocytes, disrupting the fusion between autophagosomes and lysosomes results in the formation of large autophagic vacuoles that were three times larger in volume than an average autophagosome (Marzella et al., 1982). This was also observed in Chinese hamster ovary cells treated with vinblastine (Munafo and Colombo, 2001). Lenardo and colleagues have also shown that disruption of microtubules with nocodazole led to the formation of giant autolysosomes in starved normal rat kidney cells (Yu et al., 2010). Similarly, knockout of genes responsible for the maturation and acidification of autophagosomes results in the

formation of large autophagic vacuoles. A dominant negative form of Rab7 (Rab7.T22N) results in a decrease in the number of autophagic vacuoles but their volume increases by a threefold difference on average (Gutierrez et al., 2004). Rab7 plays an important role in the fusion between autophagosomes and lysosomes. The generation of large autophagic vacuoles can also result from a disruption distal to the autophagosome and lysosome fusion. Genetic knockout of spinster results in the accumulation of large autolysosomes that cannot degrade their content. Spinster is a lysosomal efflux permease suspected to play a role in the export of degraded cargo from the autolysosomes lumen (Rong et al., 2011). In flies it is localized to the lysosomes and late endosomes and nerve terminals from spinster knockout accumulate large autolysosomes (Sweeney and Davis, 2002). The presence of large autolysosomes in tauopathy could then be indicative of autophagic failure. Regardless of where along the pathway disruption occurs, the accumulation of large autophagic intermediates is indicative of impairment in their clearance mechanism. This last step of the autophagic flux requires properly acidified autolysosomes and is dependent upon the retrograde transport of autophagosomes (Maday et al., 2012; Munafo and Colombo, 2001; Yang et al., 2011; Yu et al., 2010), their fusion with lysosomes (Gutierrez et al., 2004; Marzella et al., 1982), and their acidification (Rong et al., 2011). Thus, a blockage at any of these sites would yield large autophagic intermediates that are not as acidic as the small mature autolysosomes. We

hypothesized that the large autophagic intermediates were less acidic than the small mature autolysosomes.

In order to test whether these large autophagic bodies were properly acidified, we used the ratiometric dual color yellow/green Lysosensor dye to compare the relative acidity of the larger punctae with the smaller ones. This could be appreciated by utilizing the tandem fluorescence property of the Lysosensor probe. Upon its excitation at a 352 nm wavelength, the probe emits a fluorescent signal at wavelengths 450 nm and 540 nm in neutral and basic environments. However, in more acidic environments it fluoresces primarily at 540 nm. In order to appreciate the difference in acidity among the different punctae, we used the green filter to show fluorescence at wavelength 450 nm and the red filter to show fluorescence at wavelength 540 nm. We found that in the eye discs of third instar larvae expressing human tau, the larger punctae were yellow in color, whereas the smaller ones were red[8]. This indicated that the larger punctae were less acidic than the smaller punctae **(Figure 4.11A)**. This in turn suggested that there was a blockage in the maturation of autophagic intermediates that impeded their acidification.

Next we sought to confirm that the large LysoTracker-positive punctae were autophagic intermediates. We used the tandem fluorescence reporter Atg8.mCherry.GFP. Atg8 is localized to the autophagic intermediates, both

[8] A threshold was adjusted to ensure that none of the fluorescent signals were saturated.

autophagosomes and autolysosomes. The double fluorescent tag emits signals at both colors at a neutral pH, however the GFP fluorescence is quenched in an acidic environment. This is not the case for the red fluorescent signal (mCherry); it persists at low pH. In other words, the autophagosomes immunoreactive for Atg8 should emit both green and red signal while the autolysosomes should only emit a red fluorescence signal (Kimura et al., 2007; Nezis et al., 2010). Upon coexpression of Atg8.mCherry.GFP with human tau in the eye discs, we were able to determine the relative acidity of small punctae versus larger ones. We noticed that while many small punctae (~1 μm in diameter) fluoresced in red only, larger punctae (~5 μm in diameter) fluoresced both in green and red **(Figure 4.11C to 4.11C")**. This suggested that the large autophagic intermediates were not able to fully mature or acquire a highly acidified lumen. This was consistent with the results obtained with the Lysosensor staining, both demonstrating that large autophagic intermediates were less acidic than mature autolysosomes. They were immunoreactive for Atg8, and this suggested that they were autophagic intermediates that failed to properly acidify.

4.11 Large LysoTracker punctae were preferentially clustered towards the soma

Microtubule-based transport is important for the maturation of autophagic intermediates in neurons. Autophagosomes can form anywhere along the axon, even in the distal parts, but in order for them to fuse with lysosomes, they move

in a retrograde direction toward the soma, where lysosomes are abundant. We then sought to determine the relative position of the GAB along the axon, whether they were evenly distributed along the axons or not. We utilized the eye disc made up of few cellular layers in thickness to get an approximate location of the large LysoTracker punctae along the axon. As shown in **Figure 1.3**, the axons of the developing photoreceptors neurons (in blue) project to the brain lobes through the optic stalk. Human tau is expressed around the nucleus and along the axons of the developing neurons. The nuclei of the axons are not present in the optic stalk, and this allowed us to determine the relative positions of the LysoTracker punctae along the axon. After staining the eye discs from larvae expressing human tau with LysoTracker, we found that small punctae could be found along the axon but the larger ones were preferentially clustered at a level proximal to the soma **(Figure 4.12A and 4.12B)**. This suggested that large autophagic intermediates were not sequestered at the distal parts of the axon and preferentially accumulated at a site near the soma.

In order to confirm this finding, we examined different planes taken at a 1 µm interval, to look for the distribution of the large LysoTracker punctae in the eye disc. The nuclei of the photoreceptor neurons are clustered at the caudal side of the eye disc. We stained the eye disc with LysoTracker, and the punctae were color-coded based on their approximate location. The proximal ones (closer to the plane containing the nuclei) were given a color on the left end (red) of the spectrum, whereas the more distal ones were given a color on the right end

(blue) of the spectrum. Stacking of all the planes revealed that the larger punctae were clustered around a plane closer to the soma **(Figure 4.12D)**. To confirm this finding and account for any small punctae that could have been masked by a larger one, we repeated the same reconstruction from the opposite (rear) side. In **Figure 4.12E** the colors were reversed, with the blue color denoting a distal plane, that is closer to the nuclei. In this rear view, larger punctae appeared mostly in blue or green denoting that they were clustered distally, i.e. closer to the nuclei. The small punctae were distributed along the different planes, though with a preference towards the nuclear plane. On the other hand, larger punctae appeared mostly proximal to the soma, confirming our previous observations at the optic stalk; that they formed at the proximal ends of the axon. The small LysoTracker punctae could be functional late autophagic intermediates, and thus can be found throughout the axon but with a preference towards the soma, while GAB appeared to be stalled at a later stage during the autophagic flux and accumulated near the soma.

4.12 Autophagy downregulation reduced GAB formation in the eye

The observations provided so far support the notion that GAB might be large nonfunctional autophagic intermediates. As mentioned previously, cargo targeted to degradation by autophagy is transported towards the soma where it accumulates forming aggresomes. Most (Iwata et al., 2005; Johansen and Lamark, 2011; Kopito, 2000; Wong et al., 2008) but not all aggresomes (Wong et

al., 2008) have been shown to be cleared by autophagy. We then sought to determine whether GAB were structures in transit waiting to be degraded by autophagy, or whether they were stalled autophagic intermediates that failed to be properly acidified. Because of their low acidity and high phospholipid content, we hypothesized that GAB might be autophagic intermediates that failed to mature and degrade their cargo. A blockage in the autophagic flux preventing the proper maturation of autophagic intermediates and the recycling of their content may thus be responsible for the generation of this pathology. In order to test this hypothesis, we attempted to reduce basal autophagy levels and measure the number of GAB. If they were structures in transit to be degraded by autophagy, then their numbers should have increased when autophagy was reduced. If on the other hand they were stalled autophagic intermediates, then reducing the input, i.e. reducing the numbers of early autophagic intermediates, would limit the number of autophagic intermediates stalled at the later stages. This in turn would lead to a reduction in the number of GAB. We thus attempted to count the number of GAB in response to lowering autophagy levels. GAB could easily be counted in the eye of the adult fly due to their large size, and they are easily visible under a light microscope using Nomarski optics. First, we attempted to measure the levels of GAB in a heterozygous null Atg1 background. Atg1 is a kinase conserved from yeast to flies that is responsible for the early steps in the autophagic flux, that is induction. In flies, activation of Atg1 is essential for induction of autophagy (Scott et al., 2007). We found that the numbers of GAB in

tauopathy were reduced to about 50% (2.2 +/- 0.9 GAB in tauopathy with a heterozygous loss of function of Atg1 vs 6.4 +/- 1.4 GAB in tauopathy with an Atg1 wild type background, p-value was 0.04) **(Figure 4.13A)**. For the control we used otherwise genetically identical tauopathy flies with normal endogenous levels of Atg1. This confirmed our hypothesis that GAB were not a pathology resulting from human tau aggregation but that they formed due to the accumulation of dysfunctional autophagic intermediates resulting from a blockage in the autophagy/lysosomal system.

Rabs play an important role in autophagy (Funderburk et al., 2010). The small GTPase Rab5 has been shown to interact with Beclin1 and Vps34, both of which are part of the first step of autophagosome formation (Ravikumar et al., 2008). Rab 7 was shown to localize to the autophagic membrane and is required for the normal progression of autophagy possibly by mediating the fusion between autophagosomes and lysosomes (Gutierrez et al., 2004; Jager et al., 2004). We used kinase dead transgenes of Rab5 (S34N) and Rab 7 (T22N) in order to block induction and maturation of autophagosomes respectively. The kinase domains are necessary for proper Rab functions. A kinase dead version of a Rab would then act like a dominant negative mutation and significantly impede Rab-mediated functions. We found that decreasing the autophagosome biogenesis by expressing the kinase dead Rab5 (S34N) caused a sixfold decrease in the level of GAB (4.9 +/- 1.1 GAB in tauopathy with a co-expressed kinase-dead mutant of Rab5 (S34N) vs 30.4 +/- 2.8 GAB in tauopathy with a wild

type background, p-value was $7.9*10^{-11}$) **(Figure 4.13B)**. Similarly, expression of a kinase-dead form of Rab7 (T22N) resulted in a decrease in the number of GAB but to a lesser extent (12.4 +/- 2.3 GAB in tauopathy with a co-expressed kinase-dead mutant of Rab7 (T22N) vs 30.4 +/- 2.8 GAB in tauopathy with a wild type background, p-value was $1.3*10^{-4}$) **(Figure 4.13B)**. We used genetically identical tauopathy flies with normal levels of Rab5 and Rab7 as controls. This confirms the previous data showing that reducing autophagosome biogenesis results in a reduction of GAB. Since Rabs are also involved in the endosomal/lysosomal pathway, we tested whether introducing a kinase dead Rab14 (S49N) would affect the levels of GAB. Rab 14 is part of the early endosomes (Proikas-Cezanne et al., 2006) and is involved in the trafficking between the Golgi complex and endosomes (Junutula et al., 2004). Expression of a kinase dead Rab14 (S49N) did not result in any significant changes in the levels of GAB (27.8 +/- 4.7 GAB in tauopathy with a co-expressed kinase dead mutant of Rab14 (S49N) vs 30.4 +/- 2.8 GAB in tauopathy with a wild type background, p-value was 0.65) **(Figure 4.13B)**. This suggested that the changes observed with introducing dominant negative versions of Rab5 and Rab7 were probably due to impeding autophagosome biogenesis and maturation, respectively.

4.13 Autophagy induction exacerbated the autophagy gridlock in tauopathy

After establishing that downregulation of autophagosome biogenesis and maturation reduces the number of GAB, we sought to determine whether the opposite is true as well. If there is a blockage in the autophagic flux leading to the accumulation of improperly acidified autophagic intermediates, then an increase in the number of autophagosomes should increase the number of the aberrant autophagic intermediates and eventually large autophagic intermediates. We utilized rapamycin, a potent inducer of autophagy, to increase autophagosome biogenesis in the eye discs. Larvae were reared in vials containing 5 μM rapamycin and were then incubated in Drosophila Ringer solution with 10 μM rapamycin for 1 hour. The eye discs were dissected and left in the solution for another 15 minutes. We used larvae coexpressing both human tau and the tandem fluorescent Atg8.mCherry.GFP driver[9]. This reporter allowed us to identify large and poorly acidified autophagic intermediates and to stage autophagic intermediates. We noticed an increase in the level of Atg8 in larvae feeding on rapamycin that was accompanied by an increase in the poorly acidified large autophagic intermediates (**Figure 4.14A and 4.14B)**. These were identified by their large diameter, and they fluoresced in both red and green signifying low acidic content. This evidence further supported the aforementioned

[9] The use of this driver was outlined in the previous chapter.

findings, all pointing to a blockage in the autophagic flux leading to the accumulation of GAB.

Due to their large size, we suspected that large autophagic bodies could further worsen the blockage in the autophagic flux. This could be analogous to a traffic gridlock where additional vehicles can worsen an existing jam. In order to test this hypothesis, we tested whether upon induction of autophagy the levels of mature autolysosomes would decrease. Since GAB were not properly acidified, we used the acidic Atg8 immunoreactive punctae as an indicator for mature autolysosomes. Using the tandem fluorescence reporter, Atg8.mCherry.GFP, mature autolysosomes were identified as punctae that fluoresced in red but not green (red only in **figure 4.14A" and 4.14B"**). After subtracting the green from the red fluorescence, we found that upon rapamycin feeding the levels of red-only Atg8 punctae decreased **(Figure 4.14C and 4.14D)**. This meant that there was a decrease in the final number of mature autolysosome in contrast with the significant increase in GAB levels upon autophagy induction. Collectively, these results indicated that in tauopathy there was a gridlock leading to the formation of large autophagic intermediates that was enhanced by autophagy induction.

4.14 Rapamycin feeding increased urea-soluble human tau levels

We have shown in chapter 3 that autophagy activation suppressed human tau toxicity, and its downregulation enhanced the rough eye phenotype. On the other hand, the effect of autophagy modulation on the internal pathology

produced the opposite effect. In this chapter, we showed that autophagy activation resulted in the formation of large and poorly acidified autophagic intermediates, whereas a downregulation in autophagy increased GAB levels. We attributed this behavior to a gridlock in the distal steps of autophagy. Upon induction of autophagy by rapamycin, the sequestration of human tau seemed to proceed normally and the soluble levels of human tau were decreased. Soluble human tau seems to be the more toxic entity in *Drosophila* models of human tauopathy (Feuillette et al., 2010), and this may explain the reason that human tau-induced toxicity was suppressed with rapamycin feeding. A sequestration of human tau does not necessarily mean that it has been completely degraded. Since we showed that rapamycin enhanced autophagy gridlock, increased the levels of GAB and resulted in a decrease in the levels of mature autolysosomes, we sought to determine whether human tau was totally degraded upon rapamycin modulation. Given the observations consistent with an autophagy gridlock, one would expect that human tau was mainly sequestered in the stalled autophagic intermediates. We looked at the levels of human tau upon rapamycin feeding and found that they did not change in the SDS-soluble fraction but increased in the urea-soluble fraction **(Figure 4.15A and 4.15B)**. A possible interpretation of these results is that human tau was sequestered into an insoluble fraction, however, because of the gridlock, autophagy did not proceed to completion, and human tau was not completely degraded but accumulated in the insoluble fraction.

DISCUSSION

We have shown in the previous chapter that autophagy was protective in our human tauopathy model in flies. In this chapter, we investigated the impact of human tauopathy on the autophagic machinery. We showed that human tau expression resulted in autophagy gridlock, coupled with the formation of GAB.

4.15.1 Accumulation of autophagic intermediates in tauopathy with little TOR inactivation

Autophagy can be induced in response to misexpression of aggregation-prone proteins in neurodegeneration models. Examples in *Drosophila* include misexpression of expanded polyglutamine atrophin (Nisoli et al., 2010), and Aβ-42 (Ling et al., 2009), both of which are coupled with increased autophagosome accumulation. We thus investigated whether autophagic intermediates accumulate in our model of human tauopathy. In the developing and adult fly eye, accumulated autophagic intermediates were much higher in tauopathy as shown by the acidic punctae accumulation, the Atg5 immunoreactive punctae and the ultrastructure analysis confirming the presence of autophagic intermediates. The robust accumulation of these intermediates was indicative of low rather than a high autophagic flux. By definition, autophagic flux is the turnover of cargo protein from sequestration until clearance **(Figure 4.4)**. Impeding induction or clearance

will reduce the autophagic flux, but only impeding clearance will result in the accumulation of autophagic intermediates. High levels of autophagy induction can, in principle, increase the accumulation of autophagic intermediates. However in flies, induction of high levels of autophagy by rapamycin does not cause a robust accumulation of autophagic intermediates (Boland et al., 2008; Ling et al., 2009). Thus, accumulation of autophagic intermediates is usually more indicative of impaired autophagic flux, especially if aberrant autophagic vacuoles are present.

We sought to establish whether human tau misexpression had the ability to induce high levels of autophagy. We found that the change in the phosphorylation levels of 4E-BP were slightly reduced in the presence of human tau, while those of p70 S6k did not change. Although low phosphorylation levels of these proteins have been widely used as an indication of increased autophagy induction, the interpretation of these results can be more complex. Rapamycin and starvation-induced autophagy have both been shown to be coupled with a decrease in the phosphorylation levels of TOR substrates (Scott et al., 2004). However, after prolonged starvation (6 hours) the levels of phosphorylated p70 S6k and 4E-BP were restored to normal levels despite ongoing starvation (Yu et al., 2010). This indicates that in chronic states of autophagy, TOR activity may be restored to normal.

We also found that the autophagic intermediates were immunoreactive for a component of selective autophagy, blue cheese (ALFY homologue). Through

its WD-40 domain, ALFY recruits Atg5 to protein aggregates. In other words, it acts as a scaffold to bring components of the autophagic machinery to protein aggregates to be targeted for degradation. In flies, Blue cheese is involved in clearing aggregates but is dispensable for starvation-induced autophagy in the larval fat body (Filimonenko et al., 2010). This supports the findings described in the previous chapter showing that Blue cheese is part of the endogenous autophagy involved in suppressing human tau toxicity. We showed that partial loss of function of Blue cheese enhanced the toxic phenotype of human tau. To my knowledge this is the first evidence showing that ALFY (or Blue cheese) is involved in human tauopathy.

4.15.2 GAB pathology in tauopathy

Given the robust increase in the accumulation of autophagic intermediates, in combination with a modest decrease in TOR inactivation, we suspected that the autophagic flux could be blocked at later stages. Blocking clearance either by interfering with the transport of autophagosomes or by preventing acidification results in the accumulation of aberrant autophagic intermediates characterized by their large size, their undigested content, and sometimes their higher than usual pH. Large autophagic vacuoles were formed by disrupting the fusion between autophagosomes and lysosomes in rat hepatocytes (Marzella et al., 1982) and chinese hamster ovary cells (Munafo and Colombo, 2001), as well as starved NRK cells (Yu et al., 2010). Similar pathology

was also observed when the acidification of lysosomes was impaired (Gutierrez et al., 2004; Rong et al., 2011) or when cathepsin-mediated proteolysis was impaired (Boland et al., 2008). We sought to determine whether large autophagic vacuoles were present in the *Drosophila* model of human tauopathy. Ultrastructure analysis revealed that there were large spherical bodies accumulating in the retina of human tau expressing flies. Their size ranged from just above few microns to more than 10 μm in diameter. They were often enclosed by a double membrane, but some had multilamellar membranes, indicative of their high phospholipid content. Since they were immunoreactive for autophagic markers, we termed them GAB. They exhibited certain features that suggested they were dysfunctional autophagic intermediates. First, they contained a mixture of digested and undigested material. Second, they were much larger than the average autophagosomes, and lastly, they were less acidic than the mature autolysosomes. They were also heterogeneous in size, and a few of them seemed to be fusing together, suggesting that smaller ones may coalesce together and form large GAB.

What caused the autophagic intermediates to stall and accumulate is not precisely known, but Holzbaur and colleagues have shown that in dorsal root ganglion neurons, the retrograde movement of autophagosomes is necessary for their acidification and hence their turnover. Disruption of microtubule-based transport or their associated motor proteins blocks the autophagic flux (Batlevi et al., 2010; Cai and Sheng, 2011; Cardoso et al., 2009; Xie et al., 2011; Xie et al.,

2010; Yamamoto et al., 2010; Yang et al., 2011). In *Drosophila*, misexpression of human tau in neurons induces clog formation which disrupts axonal transport, reduces the number of moving vesicles and increases their pause time (Talmat-Amar et al., 2011). Axonal blebbing is a feature in other models of human tauopathy, as well (Hall et al., 2000; Lee et al., 2009; Mandelkow et al., 2003). Axonal disruption and clogging in tauopathy may in turn lead to poorly acidified autolysosomes. Additionally, impairing autophagosome acidification by loss of function of spinster, also known as benchwarmer, a lysosomal efflux permease, results in the formation of large autolysosomes that were not able to fully degrade their cargo (Rong et al., 2011). In our model, the GAB we observed were less acidic than the smaller punctae, as evident by using the ratiometric acidotropic dye, yellow/blue Lysosensor. We also utilized the tandem fluorescence reporter Atg8.mCherry.GFP to show that GAB were poorly acidified autophagic intermediates. This autophagy fluorescence reporter is useful in staging autophagic intermediates since the green fluorescence is quenched at low pH while the cherry signal is not, thus distinguishing early from late autophagic intermediates. Collectively, these results suggest that there was a blockage in autophagic flux leading to the accumulation of large and improperly acidified autophagic bodies. This may explain the discrepancies between the significant increase in the number of autophagic intermediates, coupled with the small increase in autophagy induction indicated by the small decrease in phosphorylation of proteins downstream of TOR. This is consistent with previous

reports that TOR activity is not suppressed if the later steps of autophagy are inhibited (Boland et al., 2008).

4.15.3 An autophagy gridlock

Since GAB formed in proximity to the soma, it was important to determine whether these structures were aggresomes or autophagic intermediates. Aggresomes refer to the accumulation of proteins in large ubiquitin-rich inclusions in the perinuclear region (Johnston et al., 1998). Small aggregates forming at the periphery are transported retrogradely to the microtubule-organizing center (MTOC) to form the large inclusions termed aggresomes (Garcia-Mata et al., 1999). Since their number increases when the proteosome is inhibited, it has been suspected that aggresome formation may be a way for the cell to sequester unwanted proteins, which overwhelms the proteosome capacity, until they are degraded by the autophagic/lysosomal pathway (Iwata et al., 2005; Johansen and Lamark, 2011; Kopito, 2000; Wong et al., 2008). However, the term aggresomes has been used indiscriminately to describe any perinuclear inclusions that are immunoreactive for components of the ubiquitin-proteasome system (Wigley et al., 1999) and the intermediate filament vimentin (Kopito, 2000). This broad classification has resulted in a large spectrum of inclusions that behave differently; while many aggresomes are amenable to clearance via autophagy, some exhibit resistance to the autophagic machinery (Wong et al., 2008).

We utilized genetic manipulation to show that GAB were stalled autophagic intermediates rather than pre-autophagosomal inclusions. Impairing autophagosome biogenesis by expressing human tau in an Atg1 partial loss of function background reduced the number of GAB by about 50%. If GAB were pre-autophagosomal structures, then inhibiting autophagy should have increased their numbers; yet it decreased, indicating that GAB were formed at a stage after autophagy induction. We also utilized Rabs, which have been recently shown to play a role in interfering with the autophagic pathway (Funderburk et al., 2010). Rab5 has been shown to interact with Beclin1 and Vps34, both of which are part of the first step of autophagosome formation (Ravikumar et al., 2008). Rab7 localizes to the autophagic membrane and may mediate the fusion between autophagosomes and lysosomes (Gutierrez et al., 2004; Jager et al., 2004). Introducing a dominant negative version of Rab5 or Rab7 into the glass-tau background resulted in a dramatic decrease in the number of GAB (80% in the case of Rab5.DN and 50% in the case of Rab7).

GAB pathology may have thus resulted from an autophagy blockage located downstream of autophagosome formation but upstream of their maturation. Since GAB were large in diameter, we suspected that they might worsen the blockage causing a gridlock. In order to determine whether an increase in GAB was coupled with a decrease in the maturation of autolysosomes, we fed larvae expressing human tau on rapamycin and measured the relative number of autolysosomes. Induction of autophagy led to

an increase in GAB numbers, yet the number of mature autolysosomes decreased. This is analogous to a traffic gridlock; introducing additional vehicles to an already stalled highway makes the traffic even slower and creates additional gridlock, resulting in a reduced outflow. In tauopathy, impaired axonal transport and improper acidification of autophagic intermediates might lead to the accumulation of large autophagic bodies causing a gridlock in autophagy. Adding new autophagosomes will further worsen the gridlock and result in a decrease in the number of mature autolysosomes. A schematic of this model is shown in **figure 4.16.** In the healthy neuron, the autophagic flux can proceed normally with the maturation of autophagic intermediates. However, in tauopathy, a gridlock in autophagy may lead to the accumulation of autophagic intermediates and formation of GAB.

4.15.4 Dissociation between external phenotype and internal pathology

GAB did not seem to play a major role in producing the human tau rough eye phenotype since increasing autophagy exerted a suppressive effect on the phenotype yet increased the number of GAB. Decreasing autophagy had the opposite effect. Rapamycin feeding reduced the amount of soluble human tau but increased human tau levels in the pellet fraction. This suggested that human tau was being sequestered but not completely degraded by autolysosomes. Rapamycin feeding increased the levels of early autophagic intermediates yet

decreased the levels of mature lysosomes. This is consistent with a previous report pointing to the soluble form of human tau as the toxic entity in the fly tauopathy model (Feuillette et al., 2010). Although protein aggregates are a hallmark of neurodegeneration, and their presence correlates with disease severity, they may not be the primary neurotoxic factor. In cellular and mouse models, soluble forms of tau such as oligomers have recently been shown to be pathogenic and have been suggested to be more pathogenic than filamentous forms (Brunden et al., 2008; Lasagna-Reeves et al., 2011). In our model, human tau sequestration into the insoluble fraction, without being degraded, may be sufficient to exert a suppressive phenotype on its toxic phenotype. On the other hand, autophagy induction may increase the number of GAB and thus worsen the internal pathology.

4.15.5 Aggregates are protective in other neurodegenerative models

The contribution of aggregates to neurotoxicity is very controversial. Since aggregate formation is usually absent in brains from young and healthy individuals, aggregates were always thought of as detrimental to neurons. However, over the past decade, a more careful analysis using animal and cellular models has shown that aggregates may actually be neuroprotective. This observation has been demonstrated in various neurodegenerative models (Mizushima et al., 2004). Huntington disease is characterized by the formation of inclusion bodies composed mainly of huntingtin protein aggregates (Davies et al.,

1997; DiFiglia et al., 1997). While initial reports have demonstrated a link between the density of inclusion bodies and disease progression (Becher et al., 1998; Ordway et al., 1997), these observations have been challenged by others claiming that there was no correlation between inclusion bodies formation and neurodegeneration (Cummings et al., 1999; Saudou et al., 1998; Sisodia, 1998). Using automated microscopy, Finkbeiner and colleagues demonstrated that neuronal death is dependent upon the dose of huntingtin protein and can occur without the formation of inclusion bodies (Saudou et al., 1998). On the contrary, they showed that increased formation of inclusion bodies predicted survival and were associated with decreased levels of soluble mutant huntingtin (Arrasate et al., 2004). Interestingly, the *Drosophila* polyglutamine model (Jackson et al., 1998; Marsh et al., 2000) harbored in their retina structures that were similar in size and morphology to the GAB we described here (Sang et al., 2005)[10]. Although it has not been shown in fly models, these results are consistent with our observations that increased aggregates may not be causative of neurotoxicity. In a cellular model of parkinson disease models, similar findings are noted. Lewy bodies, which are pathognomonic of parkinson disease and Lewy body dementia (Hashimoto and Masliah, 1999), have been shown to be similar to aggresomes and were cytoprotective (Tanaka et al., 2004). This is consistent with the finding that neurons with Lewy body pathology are healthier than neighboring neurons with reduced numbers of Lewy bodies (Tompkins and

[10] For a picture of this structure refer to figure 1E in the referenced manuscript.

Hill, 1997). The finding that a subset of aggregates shared similarities with aggresomes has been shown in several neurodegenerative models (Ardley et al., 2004; Muqit et al., 2004; Olanow et al., 2004; Waelter et al., 2001; Wong et al., 2008). As mentioned previously, aggresomes refer to a large subset of perinuclear, ubiquitin-rich inclusions that are also rich in the intermediate filament vimentin (Garcia-Mata et al., 1999; Johnston et al., 1998; Kopito, 2000; Wigley et al., 1999). This broad classification has resulted in a large spectrum of inclusions that behave differently; while many aggresomes are amenable to clearance via autophagy, some exhibited resistance to the autophagic machinery (Wong et al., 2008).

Here we propose that a subset of aggregates we observe in our fly model of human tauopathy, GAB, may be due to failed autophagy. GAB did not always correlate with toxicity and exhibited an inverse correlation pawith the toxic phenotype when autophagy was up- or down-regulated. This suggests that even if they were not neuroprotective, they do not participate in the pathway leading to neurotoxicity. An alternative explanation may be due to different cell specificity to human tau toxicity. The fly development is dependent upon highly orchestrated developmental steps involving many cell types including photoreceptor neurons, pigment cells and cone cells. The latter type, the lens-secreting cone cells, play an important role in forming the crystalline architecture of the eye (Shamloula et al., 2002; Wech and Nagel, 2005; Wolff and Ready, 1991). Therefore human tau

induced rough eye phenotype may largely be a cone cell phenotype while the development of GAB pathology may occur in other cellular substypes.

4.15.6 GAB pathology in human tauopathies?

In AD brain, autophagic dysfunction manifested by the accumulation of aberrant autophagic intermediates has been demonstrated. Autophagic components were abundant in dystrophic neurites containing tau, neurofibrillary tangles and Abeta plaques (Ma et al., 2010). Among the tau-related pathologies that have been shown to be immunoreactive for autophagic markers are granulovacuolar degeneration (GVD) bodies. GVD bodies were first described over a century ago and have been well characterized since then: nevertheless, the mechanism by which they form has not been elucidated (Ball, 1978; Okamoto et al., 1991). Autophagic dysfunction has been suspected to play a role in the GVD body formation for over four decades with no concrete model describing how they originate or their role in disease pathogenesis. Based on their morphology and contents, we propose that GAB in flies share similar features with (GVD) bodies in AD brain based on previous ultastructural analysis[11]

[11] Figure 9 in the study titled 'Reexamination of graulovacuolar degeneration' shows an electron micrograph of GVD bodies that share similar features with the GAB we observed in tauopathy. *(Okamoto, K., Hirai, S., Iizuka, T., Yanagisawa, T., and Watanabe, M. (1991). Reexamination of granulovacuolar degeneration.*

(Okamoto et al., 1991). Both GAB and GVD bodies are similar in size, with a diameter ranging from 3 to 7 μm. Both share similar morphological features; they contain electron dense granules along with flocculent material in their core and are surrounded by a double membrane. The inner membrane is sometimes undulated. Both are immunoreactive for autophagic markers Atg5 and Atg8/LC3. GVD bodies are not only present in AD brains but in other tauopathies, as well, including Pick disease, non-Alzheimer, non-Pick dementia with Fahr's syndrome (NANPDF), Guam amyotrophic lateral sclerosis/parkinsonism dementia complex, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and frontotemporal dementia (Lagalwar et al., 2007; Okamoto et al., 1991; Schwab et al., 2000; Xu et al., 1992). This points to tau pathology as a common factor between all these conditions and underscores the relevance of our fly model of human tauopathy in examining similar pathologies.

We also found that there was an upregulation in the levels of LAMP-1 in flies expressing human tau. LAMP proteins play a role in the correct fusion between the autophagosome and lysosome (Binker et al., 2007; Penas et al., 2011; Saftig et al., 2008). Mice carrying the APP harboring the Sweedish and London mutations (APPSL) had an increase in the levels of LAMP-1 (Hashimoto et al., 2010). Moreover, in AD brains, there is an upregulation of the mRNA and protein levels of LAMP-1 (Barrachina et al., 2006). This was coupled with an

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Acta Neuropathol 82, 340-345. Refer to **Figure 4.8 in this chapter** for an electron micrograph of GAB.

increase in the LAMP-1 staining. Interestingly, LAMP-1 immunoreactivity was most robust in neurons with GVD bodies and had little correlation with NFT deposits.

As we mentioned earlier, our classification of GAB is very broad and may include a heterogeneous group of pathologies. Although most of the GAB were enclosed by double membrane like GVD bodies, a subset of them had multilamellar membranes. This has also been documented in specimens from AD brains (Nixon et al., 2005). Multilamellar bodies are not confined to neurodegeneration, but are also found in different cell types and neurodegenerative pathologies (Schmitz and Muller, 1991) and have been found to be autophagic in origin (Hariri et al., 2000). Multilamellar bodies accumulate in lysosomal galactosidase and salidase deficiencies indicating that they may form due to a failure in the degradation of glycoproteins or glycolipids (Allegranza et al., 1989; Alroy et al., 1985; Amano et al., 1983; Hariri et al., 2000; Ohshima et al., 1997).

4.15.7 Autophagy and cell death

The hallmark of neurodegeneration is cell death, yet the mechanism by which the neuron dies is still unknown. Apoptosis has been suggested to play an integral role in neurodegeneration, and signs of apoptotic cell death have been shown in brains from frontotemporal dementia cases (Su et al., 2000) and in AD brains (Cotman and Anderson, 1995; Jellinger and Stadelmann, 2000; Lassmann

et al., 1995; Roth, 2001; Su et al., 1994). In cellular and rodent models, overexpression of mutant and wild type human tau has been shown to be pro-apoptotic (Andorfer et al., 2005; Furukawa et al., 2000). In flies, apoptosis occurs in response to human tau misexpression (Jackson et al., 2002; Khurana et al., 2006). However, a large functional genomic screen in flies covering 1900 genes identified 40 genes as modifiers of human tau toxicity. None of them had any anti-apoptotic effects (Ambegaokar and Jackson, 2011). This suggests that apoptosis is the not the only means by which human tau may exert its toxic effects.

Apoptosis is not the only type of cell death that has been observed in tauopathies. Pathologies indicative of necrotic and autophagic cell death have also been described in autopsy specimens from diseased brains and animal models (Lim et al., 2001; Lucassen et al., 1997; Ma et al., 2010; Nixon et al., 2000; Stadelmann et al., 1998). Failure of autophagy may explain why apoptosis occurs (Boya et al., 2005). This may explain why in our fly model of human tauopathy, signs of both autophagic and apoptotic cell death have been described. Misexpression of human tau may impair autophagy as we have decribed and this may lead to apoptotic cell death.

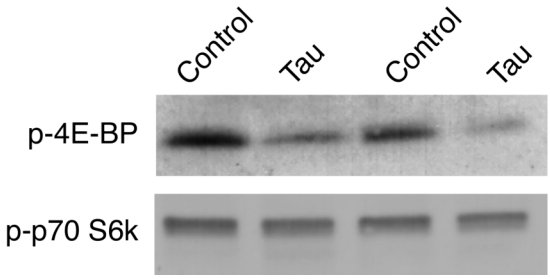
CONCLUDING REMARKS

In summary, our findings show that human tau both impairs and is itself regulated by autophagy. Early activation of autpophagy suppressed tauopathy,

likely through sequestration of soluble human tau; however, tauopathy also appeared to be associated with a gridlock in autophagic flux leading to the formation of GAB. The pathology we observed in the fly model may be analogous to similar autophagic pathology previously described in human cases. Ultrastructure analysis and high resolution imaging allowed us to gain insight about how these pathologies may be related to autophagic dysfunction. This demonstrates the power and beauty of utilizing fly models in studying human tau pathogenesis and its impact on a vital cellular pathway such as the autophagic machinery.

FIGURES

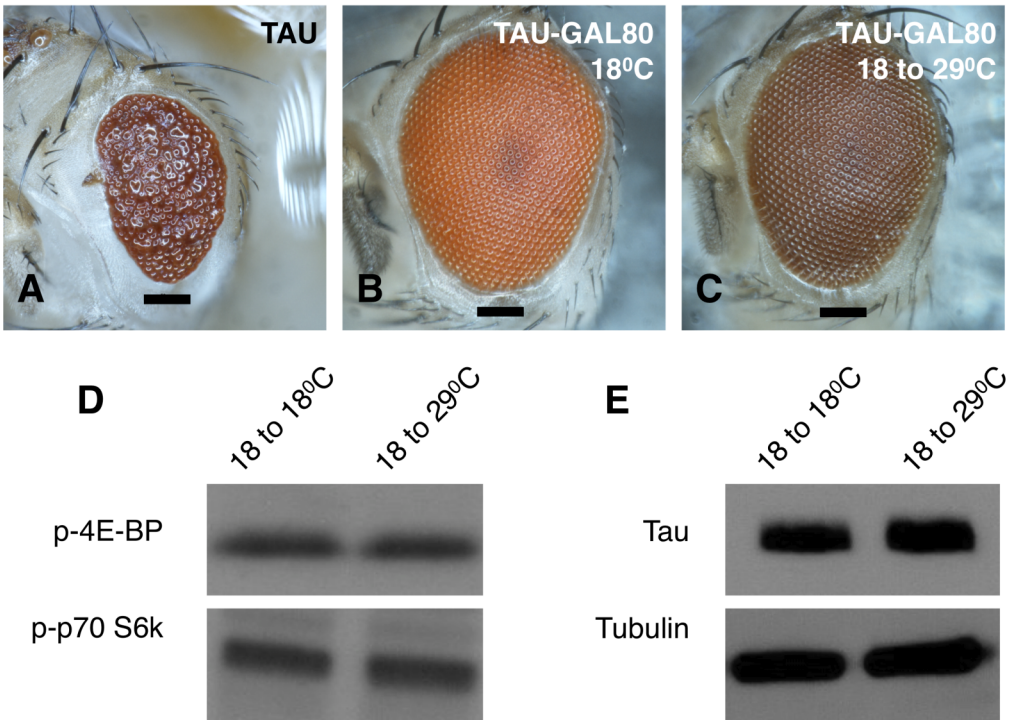
Figure 4.1. Phosphorylation of TOR substrates 4E-BP and p70 S6K were not significantly reduced in tauopathy



The levels of phospho-4E-BP, and phospho-p70 S6k, downstream of TOR were not significantly reduced in flies expressing human tau. For phospho-4E-BP, quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 2.0 +/- 0.9, whereas that of the control was 4.9 +/- 0.6 (p value was 0.06, n = 3). For phospho-p70 S6K, quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 3.3 +/- 1.0, whereas that of the control was 3.0 +/- 0.4 (p value was 0.76, n = 3).

Genotypes: ***A)*** *Control: Canton S, tau:* w^{1118}*;glass-tau.*

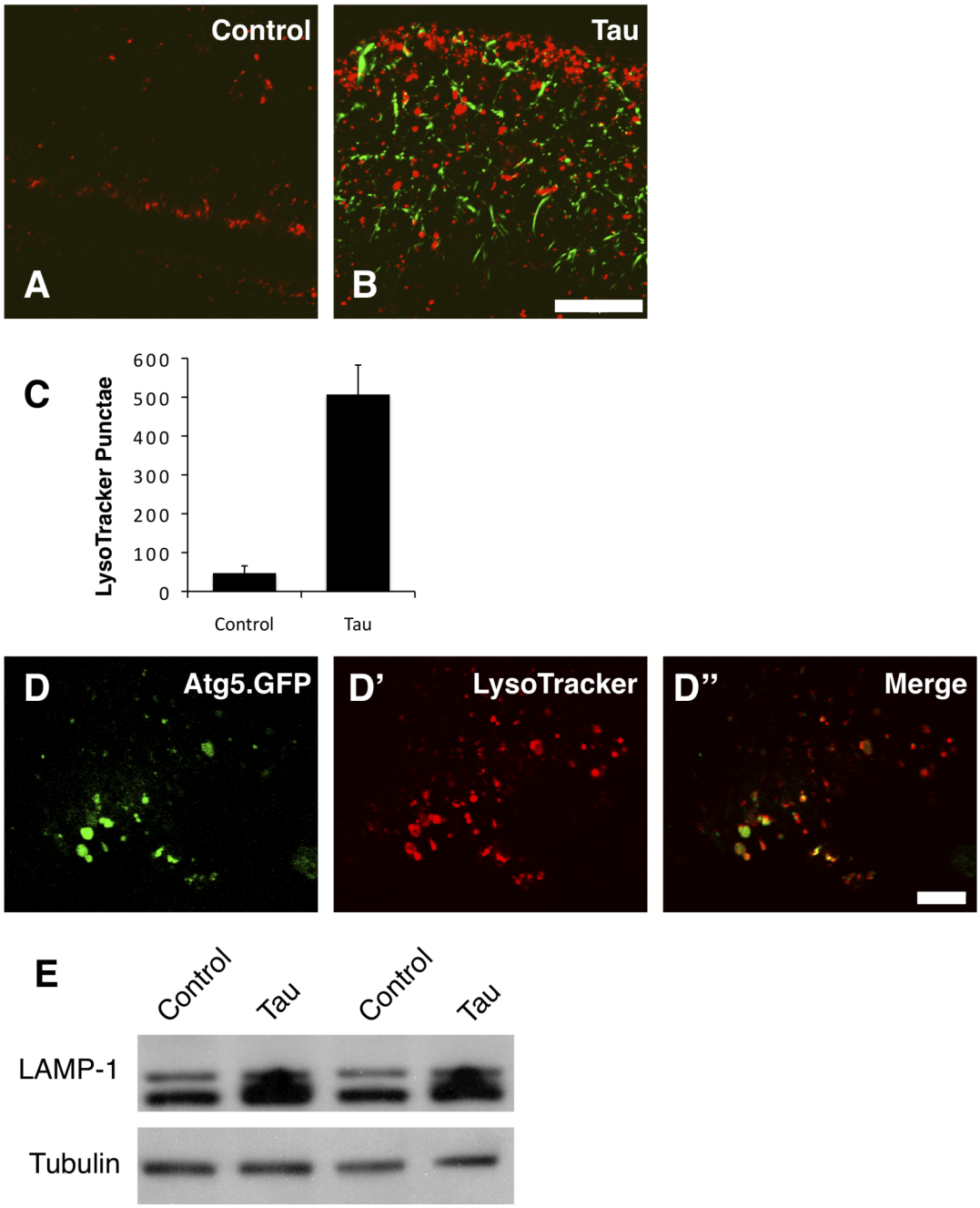
Figure 4.2. The inducible system of human tauopathy showed that human tau exerted its toxic effects during development



A) Expressing human tau (UAS-human tau) under GMR-GAL4 induced a rough eye phenotype. **B)** Introducing a GAL80 suppressed the phenotype, flies shown are GMR-GAL4/GAL80^{ts}/UAS-TAU reared at 18^{0}C and exhibited normal eye architecture, **C)** The human tau-induced phenotype is formed during development since GMR-GAL4/GAL80^{ts}/UAS-TAU reared at 18^{0}C and transferred to 29^{0}C post-eclosion for 1 week exhibited no change in the eye morphology. Scale bars are 50 μm. **D)** No changes in the phosphorylation levels of TOR substrates 4E-BP or p70 S6K were observed upon heat induction. **E)** Human tau levels did not change either upon transferring flies from 18^{0}C to 20^{0}C.

Genotypes: ***A)*** *w^{1118};GMR-GAL4, UAS-tau/+,* ***B*** *and* ***C)*** *w^{1118};GMR-GAL4, UAS-tau/+;tub-GAL80^{ts}/+.*

Figure 4.3. Human tau misexpression caused a robust increase in the lysosomal compartment

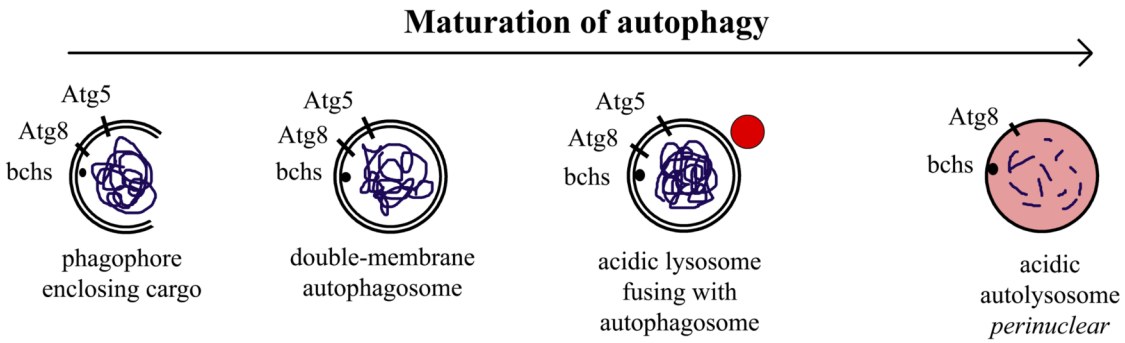


In the eye discs of third instar larvae, human tau (green) misexpression increased the LysoTracker (red) punctae by about ten fold. Control is shown in **A** and tauopathy is shown in **B**. Average number of punctae per eye disc is shown

in **C**, $p<0.05$. **D, D' and D")** The LysoTracker punctae (red) induced by human tau were immunoreactive for the autophagic marker, Atg5 (green). Green channel is shown in **D**, red channel is shown in **D'** and the channels are merged in **D"**. **E)** Flies expressing human tau had an increase in the levels of LAMP-1. Quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 1.51 +/- 0.19, whereas that of the control was 0.8 +/- 0.15 (p value was 0.001, n = 4). Scale bars are for **A and B)** 20 μm and for **D, D' and D")** 10 μm.

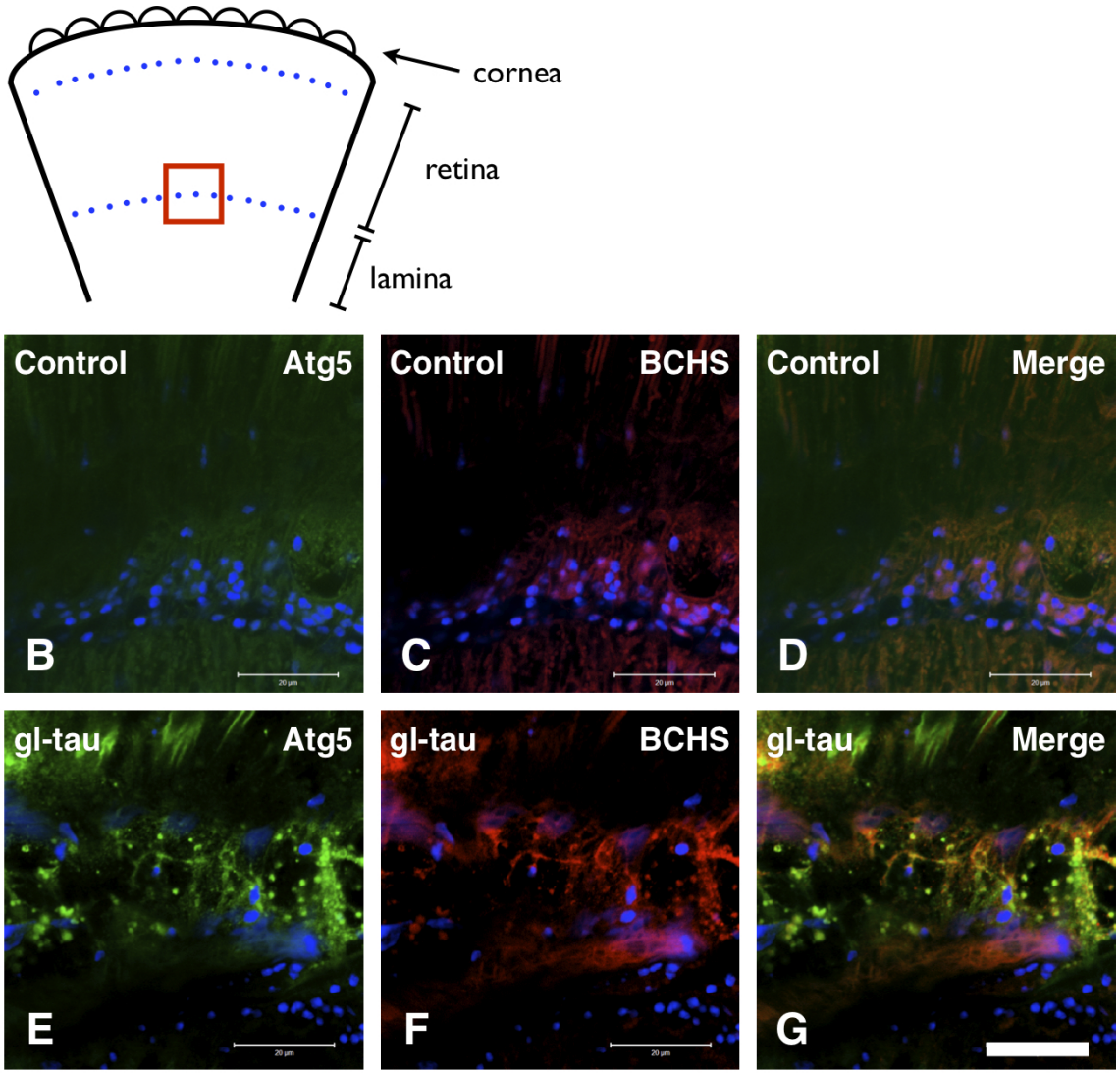
Genotypes: ***A)*** *w^{1118},* ***B)*** *w^{1118}; glass-tau$^{1.1}$.eGFP,* ***D to D")*** *w^{1118},GMR-GAL4/w^{1118}; glass-tau/+; UAS-Atg5.GFP/+,* ***C*** *and* ***E)*** *Control: Canton S, glass-tau: w^{1118}; glass-tau$^{1.1}$*

Figure 4.4. A schematic of the autophagic flux



There are three main steps in autophagy; vesicle nucleation, elongation and maturation. First, the cargo to be degraded is enclosed by a double membrane called an isolation membrane to form the phagophore. Upon closure it is called the autophagosome. Autophagosomes mature by fusing to an acidic lysosome (red) to form an autolysosome (pink). In the highly acidic lumen, the cargo is degraded and the amino acids are recycled. In neurons, maturation is dependent upon microtubule-based transport, and mature autolysosomes are usually perinuclear. Certain autophagy markers have been used to stage the progress of the autophagic intermediates. Atg5 and Atg8 associate with the isolation membrane, but only Atg8 continues to be attached after fusion with lysosomes. Blue cheese (Bchs) acts as a scaffold to bring aggregates to Atg5 and Atg8. *Not drawn to scale.*

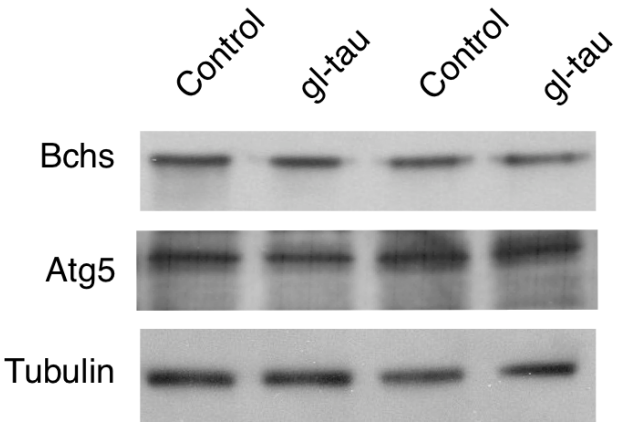
Figure 4.5. Human tau-induced autophagic punctae immunoreactive for Blue cheese were present in adults



A) A schematic showing a longitudinal section of the retina. The sections used were placed at the level of the red square at the base of the retina proper. Nuclei are shown in blue. **B and E)** Human tau misexpression induces formation of Atg5-punctae. **(B - D)** Control flies expressing Atg5.GFP, **(E - G)** glass-tau flies expressing Atg5.GFP. The Atg5 was visualized using an anti-GFP antibody, Bchs

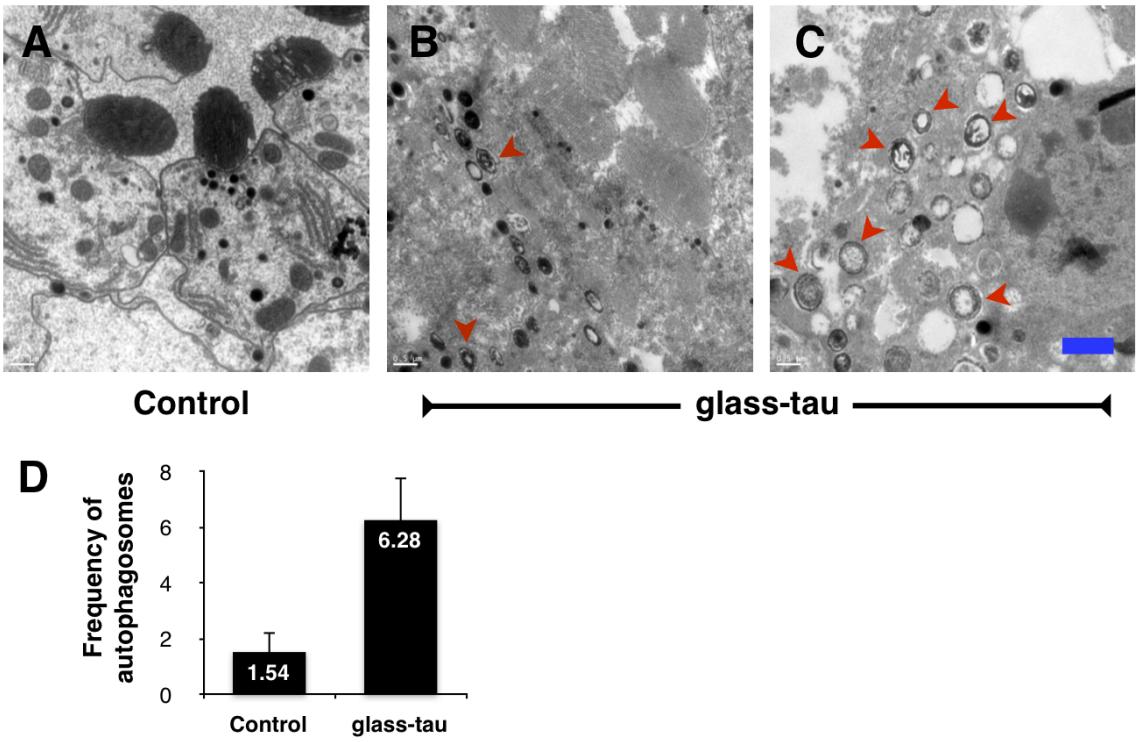
was visualized using anti-BCHS antibody. Hoechst staining is shown in blue. Atg5 and Bchs are colocalized as shown in **G**. Scale bar is 20 µm. *Genotypes:* ***B - D)*** *w^{1118},GMR-GAL4/w^{1118} ;; UAS-Atg5.GFP/+ and* ***E - G)*** *w^{1118},GMR-GAL4/w^{1118}; glass-$tau^{1.1}$/+; UAS-Atg5.GFP/+.*

Figure 4.6. Atg5 and Blue cheese protein levels did not change in tauopathy



The levels of Atg5 and Blue cheese did not change in tauopathy. For BCHS, quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 1.1 +/- 0.04, whereas that of the control was 1.2 +/- 0.05 (p value was 0.08, n = 3). For Atg5, quantification of the western data revealed that the normalized band intensity of glass-tau extract was about 0.50 +/- 0.05, whereas that of the control was 0.48 +/- 0.04 (p value was 0.77, n = 3). *Genotypes: Control: Canton S, Tau: w^{1118}; glass-$tau^{1.1}$.*

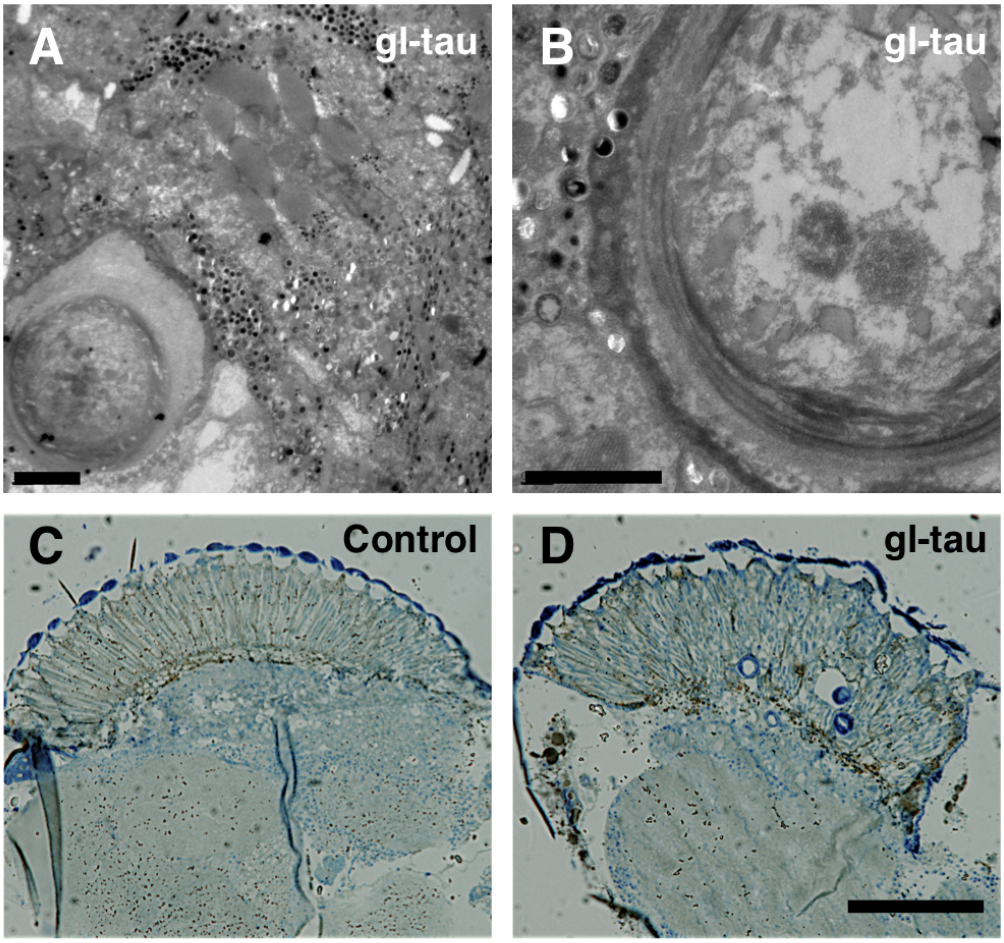
Figure 4.7. Ultrastructural analysis confirmed the presence autophagic intermediates in tauopathy



Human tau misexpression caused a disorganization of the rhabdomeres in the photoreceptor neurons **(B)**. The properly organized internal architecture of the retina in wild type Canton S flies **(A)** was lost in glass-tau flies **(B)**. Also, autophagic intermediates (red arrowheads) which were scarce in control flies **(A)** accumulated in tauopathy **(B and C)**. **D)** Quantification of the frequency of autophagosomes in control and tauopathy, p-value is $3.32 * 10^{-14}$. *Scale bar 1 µm.*

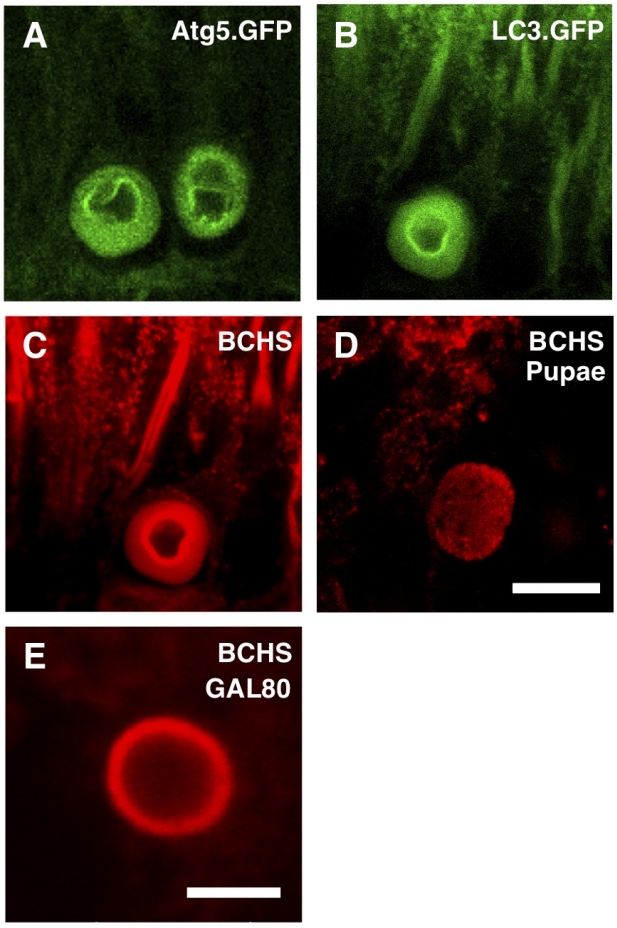
*Genotypes: **A and D)** Control: Canton S, **B - D)** glass-tau: w^{1118}/+; glass-tau/+.*

Figure 4.8. Giant bodies were observed in the retina of human tau-expressing flies



A) Electron micrographs identified giant bodies with double membranes, the inner membrane frequently undulating **(A)**. They contained a mixture of digested and undigested material. Some had multilamellar membrane **(B)**.Plastic sections stained with toluidine blue showed that these bodies were not present in control flies **(C)** and in tauopathy they were scattered in the retina **(D)**. Scale bars for **A)** 3 *μm*, **B)** 2 *μm*, **C and D)** 100 *μm*. *Genotypes **A, B and D)** w^{1118}/+; glass-tau/+, **C)** Canton S.*

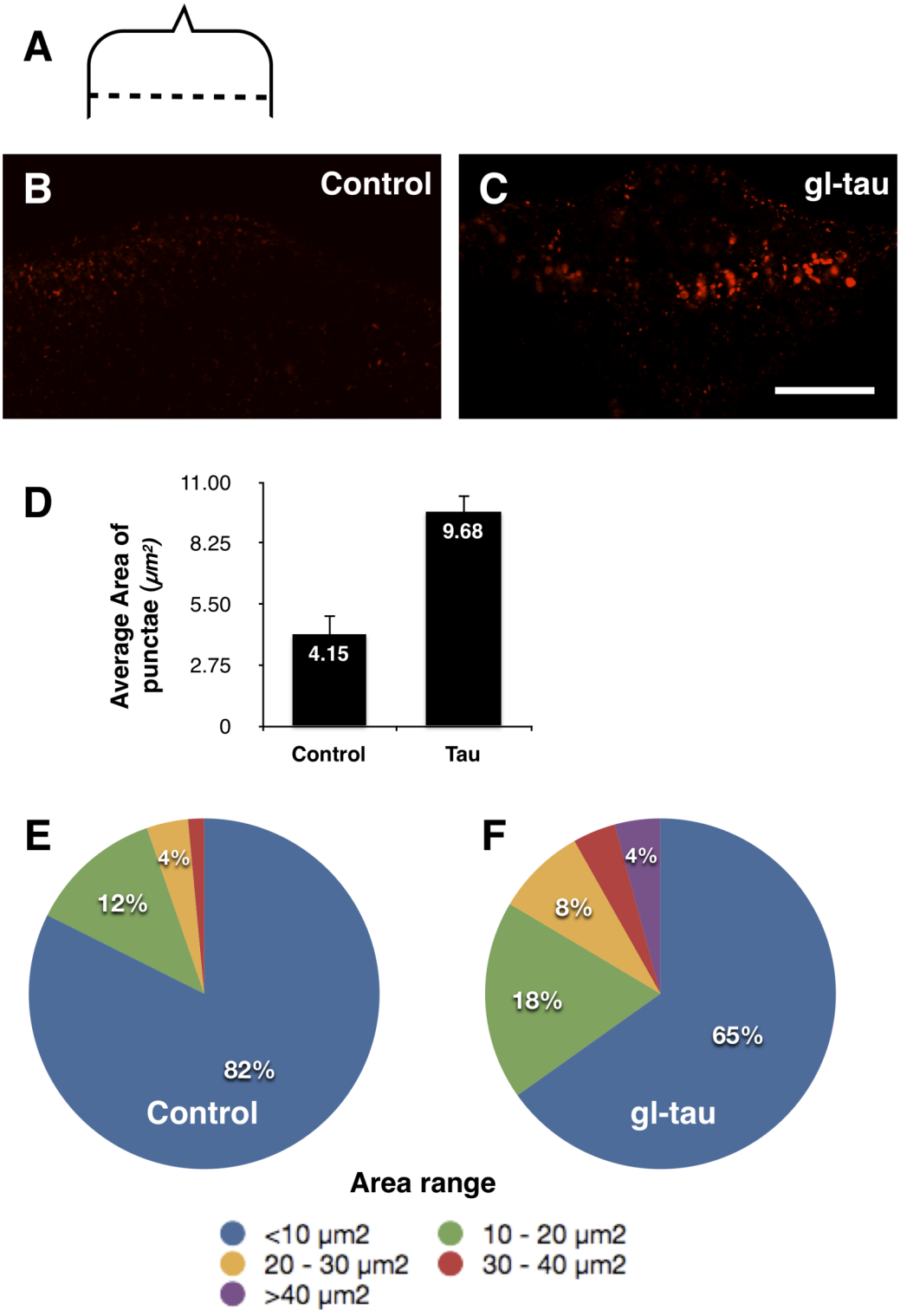
Figure 4.9. Giant Autophagic Bodies (GAB) were immunoreactive for autophagic markers



GAB in the retina of glass-tau flies were immunoreactive for Atg5 **(A)**, LC3 **(A)** and Blue cheese **(A)**, and were also present in the retina of the pupae **(D)** and following post-eclosion induction of human tau for 1 week **(E)**. A GFP tag was used to visualize Atg5 and LC3, whereas a Blue cheese antibody was used for immunostaining. Scale bars are **A-D)** 10 μm and **E)** 3 μm.

Genotypes: ***A and B)*** *w^{1118},GMR-GAL4/w^{1118}; glass-tau$^{1.1}$/+; UAS-Atg5.GFP/+,* ***C and D)*** *w^{1118}; glass-tau$^{1.1}$,* ***E)*** *w^{1118}; GMR-GAL4,UAS-tau/+; tub-GAL80^{ts}/+.*

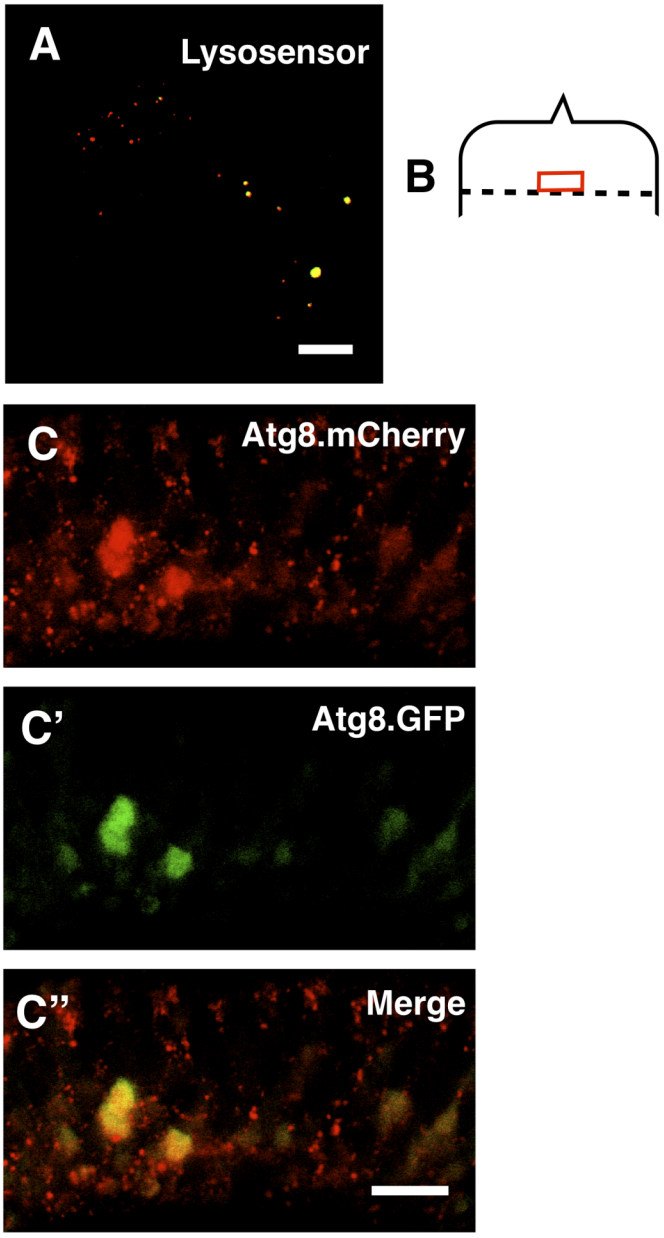
Figure 4.10. Accumulation of large acidic punctae in tauopathy



A) A schematic showing how the eye discs in **B** and **C** are positioned. LysoTracker punctae in eye discs of third instar larvae expressing human tau were larger **(C)** than those in controls **(B)**. The average area of punctae for control and tau is shown in **D**; 5.60 +/- 0.92 μm^2 in tauopathy vs 10.67 +/- 0.70 μm^2 in control, p-value was 0.005. Measurements were acquired from 50 planes of 9 eye discs from different larvae. Detailed analysis of the area distribution is shown in pie charts **E** (control) and **F** (glass-tau). The distribution of punctae with an area less than 10.0 μm^2 was 65.1% +/- 1.2% in tauopathy and 82.4% +/- 2.1% in control, with a p value of 0.0003. The distribution of punctae with an area between 10.0 μm^2 and 20.0 μm^2 was 18.4% +/- 1.0% in tauopathy and 12.3% +/- 2.0 in control, with a p value of 0.04. The distribution of punctae with an area between 20.0 μm^2 and 30.0 μm^2 was 8.3% +/- 0.6% in tauopathy and 3.8% +/- 1.8% in control, with a p value of 0.08. The distribution of punctae with an area between 30.0 μm^2 and 40.0 μm^2 was 4.0% +/- 0.5% in tauopathy and 1.5% +/- 1.5% in control, with a p value of 0.2. The distribution of punctae with an area above 40.0 μm^2 was 4.3% +/- 1.4% in tauopathy and 0% +/- 0% in control, with a p value of 0.02. Scale bar is 30 μm.

Genotypes: ***B, E and control in D)*** *w^{1118},* ***C, F and gl-tau in D)*** *w^{1118}; glass-tau$^{1.1}$.*

Figure 4.11. In tauopathy, large autophagic intermediates in the eye discs were poorly acidified

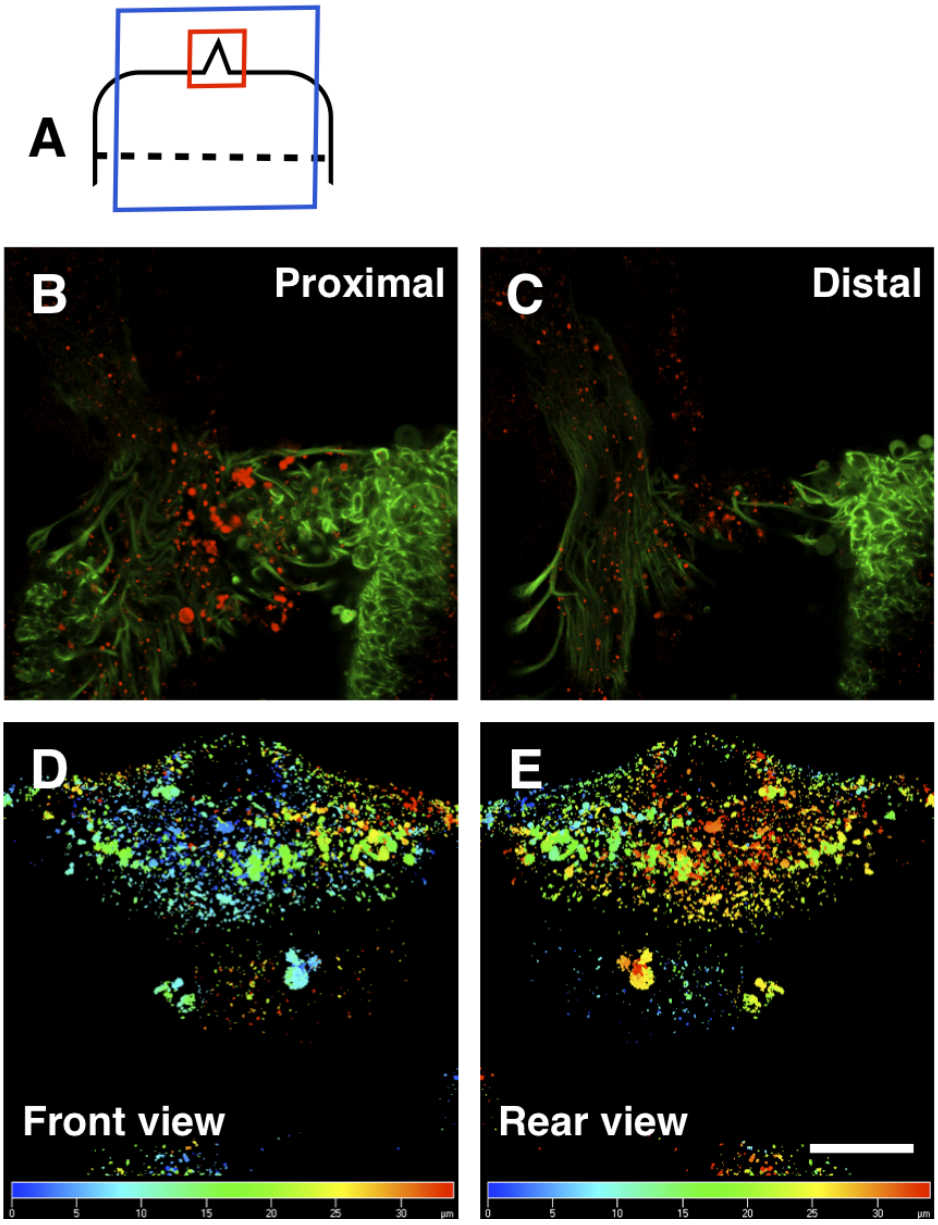


A) Eye disc from larvae expressing human tau were stained with the ratiometric Lysosensor probe which produces green fluoresence at neutral pH and changes to red at low pH. Larger punctae were less acidic (yellow) than the smaller punctae (red). **B)** A schematic diagram showing how the eye discs in **A** and **C**

were positioned. **C to C")** The autophagic marker Atg8.mCherry.GFP was coexpressed with human tau in the eye discs. At neutral pH, both the GFP and the Cherry signal fluoresce, but at low pH, the GFP signal is quenched and only red fluorescence is detected. Autolysosomes with low pH were small and only fluoresced red, whereas larger autophagic intermediates produced both green and red fluorescence indicating higher pH content. The mCherry signal is shown in **C**, The GFP signal is shown in **C'** and the merged channels are shown in **C"**. Scale bars are **A)** 20 μm, **C to C")** 10 μm.

Genotypes: ***A)*** *w^{1118}; glass-$tau^{1.1}$ and* ***C - C")*** *w^{1118}, GMR-GAL4/w^{1118}; glass-$tau^{1.1}$/UAS-Atg8.mCherry.GFP.*

Figure 4.12. Large autophagic intermediates were preferentially clustered close to the soma

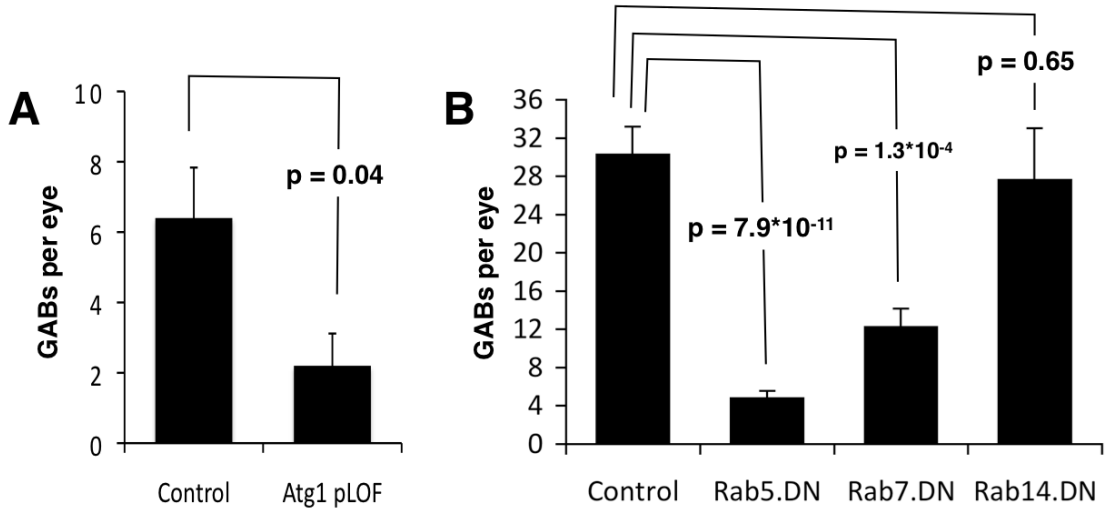


A) A schematic diagram showing how the eye discs in **B** and **C** (red square) and **D** and **E** (blue square) were positioned. Eye discs from tauopathy larvae were stained with the live dye LysoTracker. At the optic stalk, a proximal view, i.e. closer to the soma, and a distal view, i.e. along the axon is shown in **B** and **C**

respectively. GAB were more abundant in a level proximal to the nuclear layer **(B)** than distally (**C)**, (human tau (green) and LysoTracker (red). **D and E)** A z-stack compilation of all the planes from tauopathy eye discs stained with LysoTracker is shown. The punctae were color-coded according to a blue/red spectrum based on their relative position along the z-plane. In **D**, a front view was constructed; the blue end of the spectrum indicates a level closer to the soma while the red end indicates a more distal end. A rear view is shown in **E**, and the colors are reversed to ensure that smaller punctae are not hidden under the bigger ones. Both panels show that the larger punctae are located at a level closer to the soma. Scale bar is 30 μm,

Genotype: w^{1118}; glass-tau$^{1.1}$.GFP.

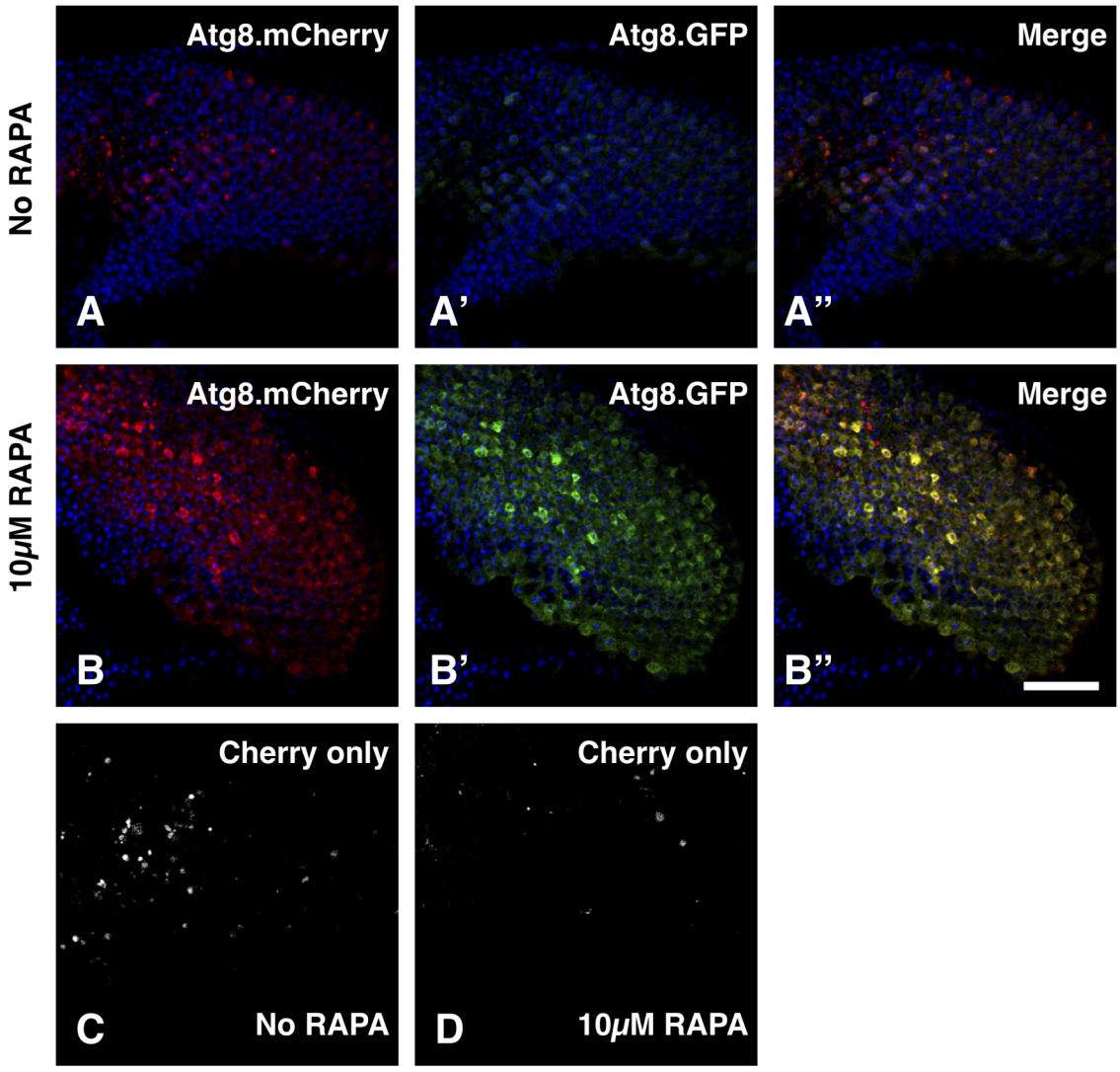
Figure 4.13. Autophagy downregulation enhanced GAB formation in the eye



Genetic downregulation of autophagy reduced the number of GAB in the adult flies. This was attained using Atg1 heterozygous loss of function **(A)** or using a dominant negative form of Rab5 and Rab7 **(B)**. Co-expression of Rab14 did not cause a significant decrease in the number of GAB **(B)**. GAB quantification was as follows: **A)** Control: 6.4 +/- 1.4 GAB and Atg1 pLOF:2.2 +/- 0.9 GAB (p-value = 0.04). **B)** Control: 30.4 +/- 2.8 GAB, *Rab5.DN:* 4.9 +/- 1.1 GAB (p-value = $7.9*10^{-11}$), *Rab7.DN:* 12.4 +/- 2.3 GAB (p-value = $1.3*10^{-4}$) *and Rab14.DN:* 27.8 +/- 4.7 GAB (p-value = 0.65).

Genotypes: ***A) control:*** *w^{1118};glass-tau/+,* ***Atg1 pLOF:*** *w^{1118};glass-tau/+;$Atg1^{d30}$/+,* ***B) control:*** *$w^{1118}/y^{1}w^{1118}$;glass-tau/+,* ***Rab5.DN:*** *$w^{1118}/y^{1}w^{1118}$;glass-tau/UAS-Rab5.S43N,* ***Rab7.DN:*** *$w^{1118}/y^{1}w^{1118}$;glass-tau/+;UAS-Rab7.T22N/+,* ***Rab14.DN:*** *$w^{1118}/y^{1}w^{1118}$;glass-tau/UAS-Rab14.S49N.*

Figure 4.14. Rapamycin enhanced a gridlock in tauopathy

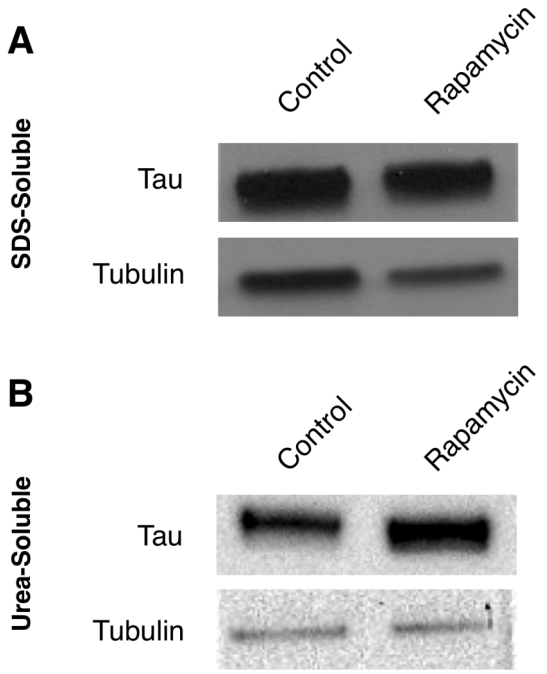


Third instar larvae expressing human tau were feeding on ethanol **(A to A" and C)** or 10 μM rapamycin **(B to B" and D)**. Those feeding on rapamycin exhibited higher levels of early autophagic markers identified by the tandem fluorescence of green and red of the autophagic marker UAS-Atg8.mCherry.GFP. However this was coupled by a decrease in the mature autolysosomes identified by subtracting the green from the red channel (shown in **C** and **D**). Note the weak signal in **A** to **A"** was due to reduced threshold to ensure that there was no

saturation in **B** to **B"**. Scale bar is 30 *μm*.

Genotype: w^{1118}; glass-$tau^{1.1}$.GFP.

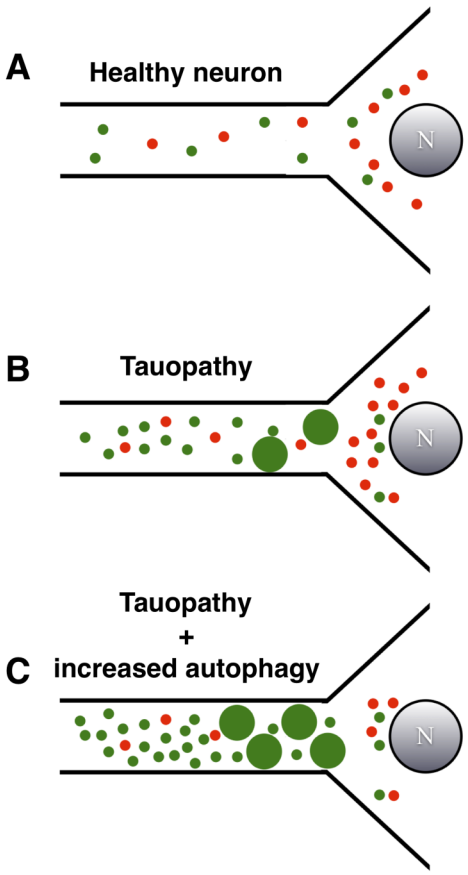
Figure 4.15. Rapamycin increased human tau levels in the insoluble fraction



Flies were fed on 10 μM rapamycin for 1 week and the levels of human tau in the pellet were measured. The levels of human tau in the SDS-soluble fraction **(A)** did not change upon feeding flies on rapamycin but their levels in the urea-soluble fraction **(B)** increased.

Genotype: w^{1118}; glass-$tau^{1.1}$.GFP.

Figure 4.16. A model for autophagy gridlock and GAB generation in tauopathy



In the healthy neuron **(A)**, autophagosomes (green) can mature to lysosomes (red) and acquire an acidic lumen. However, in tauopathy **(B)** there is a gridlock which leads to the formation of GAB (large green circles). This pathology is enhanced by increasing autophagy induction and leads to a decrease in the level of mature autolysosomes **(C)**. Small green circles (early autophagic intermediates), small red circles (mature acidic autolysosomes), big green circles (GAB) and N is nucleus. *Not drawn to scale.*

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