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Investigating the Effect of Neuronal Tau Expression on Aggression in a *Drosophila melanogaster* Model of Alzheimer's Disease

by

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This paper was prepared

under the direction of

Kenneth J. Colodner

for eight credits.

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For my grandfather, Segundo Javier Wilson Marin López.

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the aggregation and hyperphosphorylation of the microtubule-associated protein, tau. It is characterized by a variety of cognitive and behavioral symptoms, including aggression. However, the extent to which tau pathology underlies aggression remains to be determined. This line of research explores how aggression is affected by the expression of tau in neurons in a *Drosophila melanogaster* model of AD. We hypothesize that the expression of human wild-type tau in adult *Drosophila* will result in altered aggression levels in adult, male flies. In this study, we utilized the GAL4-UAS system to exclusively express human tau protein in *Drosophila* neurons, and assayed aggression in fighting pairs of tau-expressing flies or control flies, without tau. A standard *Drosophila* aggression assay was used to record fights and quantify parameters of aggressive behavior in 2-day and 5-day old flies. In 2-day old flies, we observed no significant difference in aggressive behavior. In 5-day old flies, we observed a significant decrease in aggressive behavior in tau-expressing flies compared to control flies. These results suggest that age-dependent increases of tau protein expression in neurons alters aggression in *Drosophila*, but it is unclear how this effect is mediated. Future experiments utilizing cell-type specific expression of tau and other behavioral assays should further help define the relationship between tau expression and aggression in this *Drosophila* model of AD.

INTRODUCTION

1. Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cortical loss in neural pathways that are important for memory and cognitive functions. This disease was first discovered by the German neuropathologist, Alois Alzheimer, in 1906 (Norfray and Provenzale, 2004). AD is the sixth-leading cause of death among Americans and there are no preventative measures, cures, or interventions to stop the progression of the disease (Beharry et al., 2014). While substantial research has been conducted, the cause and progression of AD is largely unknown (Wang et al., 2015). In 2016, 5.4 million people in the United States were diagnosed with AD or other dementias, and 5.2 million of those individuals are 65 years of age or older. Furthermore, the incidence of those diagnosed with AD will triple to 13.8 million individuals by 2050 (Hoglund et al., 2015). With so many lives affected by AD and no sustainable or effective treatments in existence, research aimed to finding sophisticated, long-term solutions to treat or prevent the disease are imperative.

The progression of AD can be divided into three stages: preclinical, mild to moderate, and severe. Most individuals are diagnosed at the mild to moderate stage and these symptoms include: memory loss, confusion, poor judgment, loss of spontaneity, mood/personality changes, and increased anxiety and aggression (National Institute on Aging, Alzheimer's Disease Fact Sheet, 2016). Aggression,

depression, overactivity, and psychoses are common symptoms classified as behavioral and psychological symptoms of dementia (BPSD) in AD patients (Matthews et al., 2002). Aggression is one of the most common behavioral manifestations of AD, observed in 12% to 70% of patients (National Institute on Aging, Alzheimer's Disease Fact Sheet, 2016). Aggressive behavior is believed to develop as a result of interactions between neuropathological and environmental factors, but the extent to which AD pathology underlies this behavioral symptom remains to be determined. In order to better understand how BPSD develop in AD patients, one must examine the known neuropathogenesis of AD.

AD is characterized by the shrinking of several regions of the brain, including the hippocampus and the cortex and enlargement of the ventricles (Figure 1; Fernandez-Funez et al., 2015). The cerebral cortex controls high-order executive functions, involving thought, awareness, memory, and language. Thus, AD patients exhibit deficits in language, cognition, and memory as the disease progresses (Wang et al., 2015). Patients are diagnosed with the sporadic or familial form of AD. While the cause of the disease is unknown in sporadic cases, it is proposed that AD emerges from interactions between genetic and environmental factors (Tatarnikova, Orlov, and Bobkova, 2015). In more rare familial cases, AD is hereditary where autosomal dominant mutations in the *amyloid precursor protein (APP)* gene or in the *presenilin (PSEN)* gene have been identified (Wang et al., 2015).

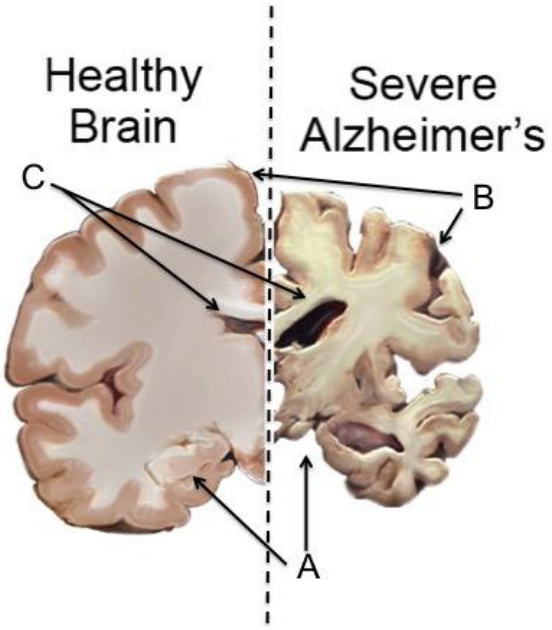


Figure 1. Cross section diagram of human brain comparing regions affected by Alzheimer's disease. A) The hippocampus, which is important for memory function, shrinks in an AD brain. **B)** In an AD brain, the cortices, which control high-order functions, also shrink. **C)** The ventricles, which hold cerebrospinal fluid, become enlarged in AD (Adapted from National Institute on Aging, Alzheimer's Disease Fact Sheet, 2016).

1.1 Role of Amyloid Beta

AD is neuropathologically characterized by the presence and spreading of two abnormal protein structures: intracellular neurofibrillary tangles (NFTs) composed of tau protein and extracellular plaques composed of amyloid beta (Aβ) (Figure 2; Hoglund et al., 2015; Hasegawa, 2016). While the precise biological function is unknown, amyloid precursor protein (APP) is a transmembrane protein considered to have an essential role in cell growth during development and aging (Hoglund et al., 2015). APP has three different isoforms: APP770, APP752, and APP695. The latter is the most dominant isoform in neuronal tissue.

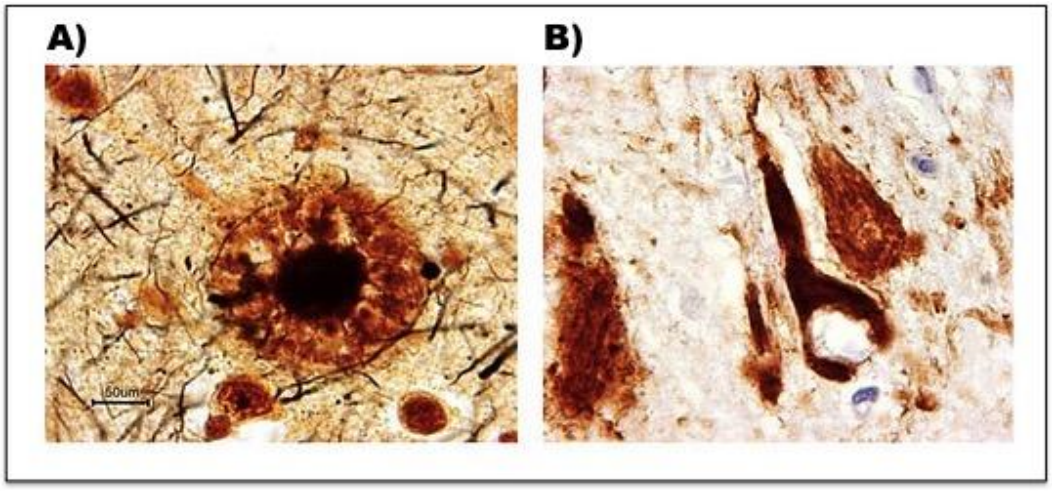


Figure 2. AD is characterized by the presence of extracellular Aβ plaques and intracellular NFTs. Light micrographs of post mortem human brains containing **A)** beta-amyloid plaques in the extracellular space between cells and **B)** neurofibrillary tangles within cells (Adapted from Big Think, 2016).

Aβ oligomers develop when APP is cleaved by β-secretase and ɣ-secretase at abnormal sites in the external surface of the membrane between amino acid residues (Lim, Ott, and Crowther, 2016) (Figure 3). Aβ polymerizes and creates antiparallel β-sheets in the extracellular spaces between cells, aggregating into fibrils that can enter cells and cause DNA damage (Wang et al., 2015). Aβ oligomers, the early intermediate aggregates of Aβ plaques, also induce synaptotoxicity, altered long-term potentiation, negative effects on neurotransmission, and defects in memory and cognition in rodent models. Although Aβ plaques are a characteristic in the late stages of AD, Aβ oligomers are thought to cause acute cell loss, which reflect the early cognitive deficits seen in the early stages of AD (Benilova, Karran, and De Strooper, 2012). Also, intracellular changes triggered by Aβ oligomers result in a neurodegenerative

triad of synaptic impairment, dendritic simplification, and cell death (Brandt and Bakota, 2017). Furthermore, studies with cultured cells and nonhuman models suggest that Aβ oligomers are the primary driving force of AD pathogenesis, but that these neurodegenerative changes require modifications of tau protein (Fath *et al*. 2002; Rapoport *et al*. 2002; Roberson *et al*. 2007; Shankar *et al*. 2007; Tackenberg and Brandt 2009). Thus, Aβ and tau work in conjunction in AD pathogenesis, and for the rest of this study, we will focus on the role of tau pathology in AD.

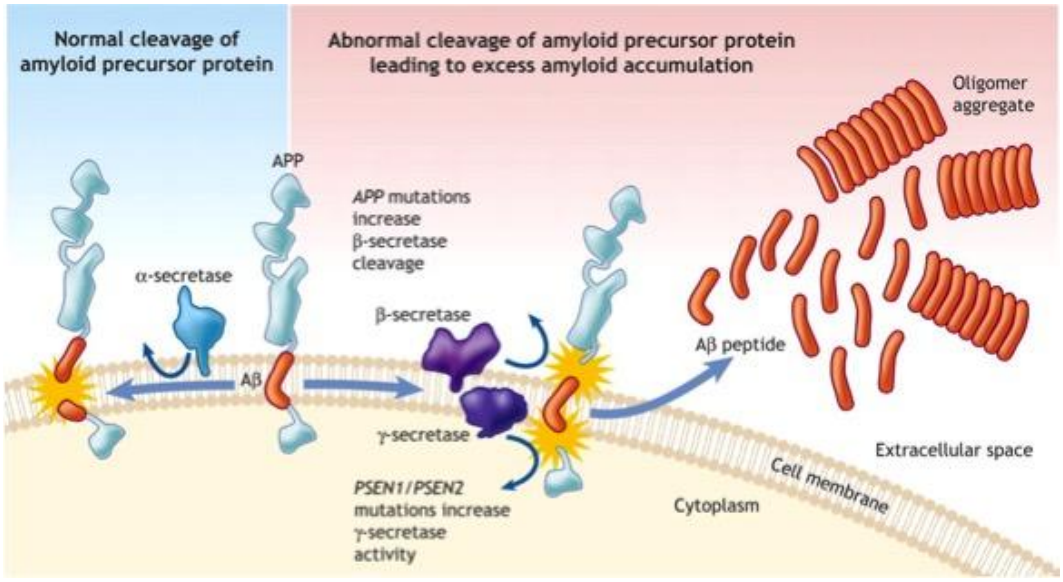


Figure 3. Schematic representation of processing pathways of APP. In the amyloidogenic pathway, APP is cleaved by β-secretase near the N-terminus of the corresponding Aβ domain at a distance of 16 amino acid residues from the non-amyloidogenic cleavage site of α-secretase, creating a build-up of Aβ oligomers (Adapted from Otwell et al., 2016).

1.2 Role of Tau

Tau is an intrinsically disordered protein that binds to and stabilizes microtubules (MTs). Tau is expressed in neurons of the Central Nervous System (CNS), where it is predominantly localized to neuronal axons. Microtubules are the main axonal transport system for cellular cargo, including mitochondria, synaptic vesicles, proteins and other important organelles within cells (Hoglund et al., 2015). Thus, tau is essential for the proper functioning of microtubules, which is important for cell survival.

Tau is encoded by the microtubule-associated protein tau (*MAPT*) *gene*, and contains 16 exons located on chromosome 17 (Iqbal et al., 2005). The protein structure can be divided into three main components: an acidic amino-terminal fragment (N inserts), a proline-rich fragment, and a neutral carboxyl-terminal fragment containing the microtubule-binding domains (R) (Figure 4; Buée, Bussière, Buee-Scherrer, Delacourte, and Hof, 2000).

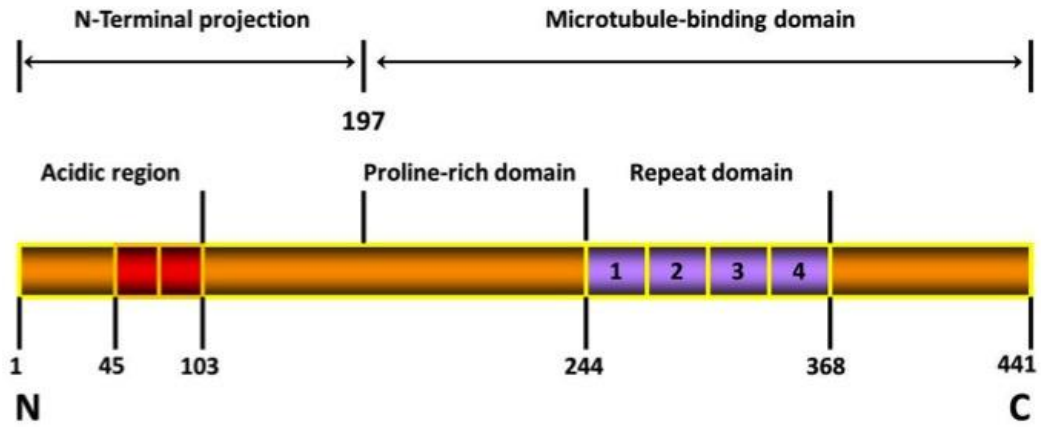


Figure 4. Schematic representation of the three major components of the longest tau isoform (2N4R). The N-terminal projection domain contains the N inserts (red), and the microtubule-binding domain contains the MT binding repeats (purple), which all contribute to the regulation of tau (Adapted from Mukrasch et al., 2005).

When alternative splicing of *MAPT* mRNA occurs, six different isoforms of tau can be produced (Figure 5). These isoforms can comprise between zero and two N-terminal inserts and three to four microtubule binding repeats, varying from 352-441 amino acids. Microtubule binding repeats (R) allow for binding interactions between tau and microtubules. Between a 4R and a 3R isoform, 4R has a stronger interaction with microtubules because of its extra microtubule-binding repeat (Hasegawa, 2016; Lee and Leugers, 2012). With alternative splicing, a tau isoform can affect how tau functions within a cell. It is important to note that all of these tau isoforms have been found in a class of tau-related diseases called tauopathies (Buee et al., 2000).

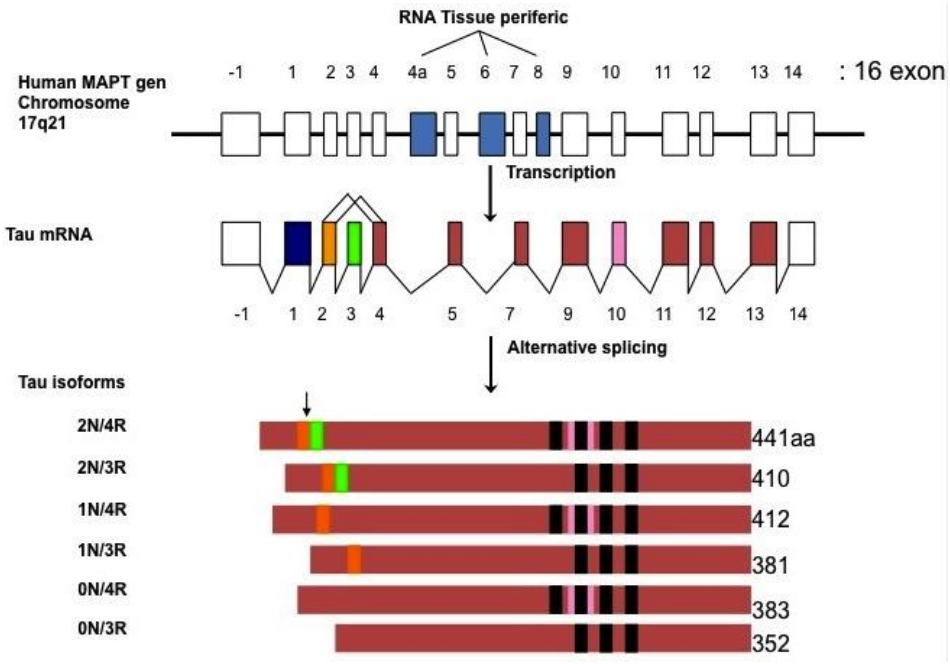


Figure 5. Schematic representation of human tau gene, the tau primary transcript and the six tau isoforms. It contains 16 exons with 13 exons present in the tau primary transcript. Exons 2, 3, and 10 are alternatively spliced, allowing for the production of 6 different CNS tau isoforms (Adapted from Zerr, 2015).

In addition, the phosphorylation state of tau can also influence how tau functions in CNS cells. Protein phosphorylation is a process that modifies tau on a post-translational level by adding phosphate groups at amino acid residues. Protein kinases and phosphatases are enzymes that regulate the phosphorylation of tau. Phosphatases dephosphorylate tau, which allow tau to appropriately bind to and stabilize microtubules. Kinases phosphorylate tau at serine, threonine, or tyrosine residues, which cause tau to dissociate from microtubules (Buee et al., 2000; Brandt et al., 2005). There are two major kinase phosphorylation sites: proline-directed or nonproline-directed sites. Proline-directed sites contain a threonine-proline (TP)-serine-proline (SP) sequence, which can be found in the N

or C-terminal away from MT-binding repeat domains. Nonproline-directed sites can be found within or adjoining MT-binding repeats (Zheng-Fischhoger et al., 1998). When tau is abnormally phosphorylated, it can result in severe functional deficits (Brandt et al., 2005).

The abnormal hyperphosphorylation of tau is the defining feature of a class of neurodegenerative diseases called tauopathies (Kovacs, 2015), which include AD. Hyperphosphorylation is defined as phosphorylation occurring on at least 8 residues, and this is more than the typical 2-3 residues undergoing phosphorylation (Buee et al., 2000). In the disease pathogenesis of all tauopathies, including AD, hyperphosphorylation of tau causes its unbinding from microtubules and subsequent aggregation into soluble oligomers. These oligomers eventually turn into paired helical filaments and proceed to form into large insoluble aggregates (Figure 6). The type of large insoluble aggregates is specific to each tauopathy (Higuchi, Trojanowski, and Lee, 2002).

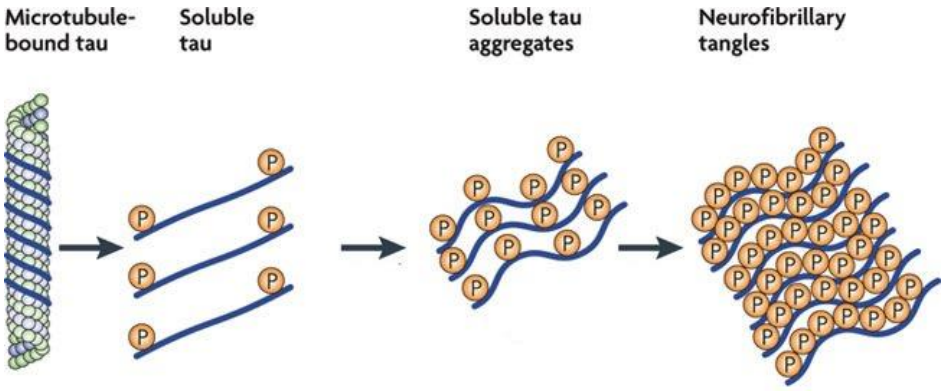


Figure 6. Hyperphosphorylated tau aggregates to form NFTs. Abnormal phosphorylation causes tau dissociation from microtubules, which develop into soluble aggregates that are toxic. These soluble aggregates in turn create tau inclusions that are specific to each tauopathy (Adapted from Citro, 2010).

In clinical studies, protein phosphatase 2A (PP2A), which regulates the dephosphorylation of tau, was discovered to be significantly lower in the brains of patients with dementia (Gong et al., 1995). In addition, glycogen synthase kinase-3β (GSK-3β), a protein kinase that regulates the phosphorylation of tau was found to be highly active in the brains of AD patients; this confirmed the relationship between the reduction of GSK-3β phosphorylation and the direct increase of tau phosphorylation by kinase activity (Baum et al., 1995; Leroy et al., 2007). All of these findings suggest that hyperphosphorylated tau plays a critical role as a toxic agent in neurodegeneration.

The accumulation of insoluble aggregates of hyperphosphorylated tau is a defining feature of AD and other related tauopathies, and suggests that tau may be the underlying toxic agent mediating disease progression. The pathology of tau dysfunction is evident in diseases involving mutations in the tau-encoding *MAPT*

gene, which lead to neurodegeneration and dementia (Hutton et al., 1998; Brandt et al., 2005; Ballatore et al., 2007). In addition, several studies overexpressing tau in animal models of tauopathy displayed shortened lifespan and age-dependent apoptotic cell death (Colodner and Feany, 2010), as well as cognitive deficits that resemble those seen in tauopathies (Morris et al., 2011). Studies have also found that the presence of tau is necessary for the toxicity associated with Aβ, the other agent of toxicity in AD, in cell culture (Rapoport et al., 2002), and in Aβ transgenic animal models (Roberson et al., 2007).

While specific mechanisms underlying how tau pathology influences neurodegeneration have not been established, the majority of research has identified abnormally hyperphosphorylated tau as the primary toxic species regulating neurodegeneration in AD and other tauopathies. In order to gain a clearer understanding of how tau toxicity may be promoting neurodegeneration, examining regions where neurodegeneration occurs, as well as pathways that influence these regions, may elucidate how AD originates and progresses, as well as shed light on how tau exerts its toxic effects to cause BPSD, like aggression.

3. Aggression & Alzheimer's Disease Patients

Aggression is a behavioral symptom of AD, commonly observed in male patients (Kitamura et al., 2012). Among institutionalized, late-stage AD patients, the rates of aggression correlate with cognitive decline, loss of independence, and other metrics of poor outcome (Bidzan, Bidzan, and Pachalska, 2012). Moreover,

the severity of cognitive decline and dementia correlate with physical agitation and verbal aggression in AD patients as measured by the Cohen-Mansfield Agitation Inventory (Li et al, 2014). Researchers have been unable to determine if the origins of these behavioral disturbances are attributed to the disease pathogenesis of AD or environmental provocations, such as frustration with memory loss, physical discomfort or poor communication between the caregiver and the patient (Borson and Raskind, 1997). To further understand how AD pathology affects aggressive behavior in AD patients, investigating the regions where AD pathology is found can provide insight on the underlying mechanisms of these behavioral disturbances.

Several studies have revealed the noradrenergic system is heavily damaged by subsequent Aβ plaques and NFTs (Weinshenker, 2008; Marien et al., 2004; Mathews et al., 2002; Hoogendijk et al., 1995). The noradrenergic system is the pathway for noradrenaline (NA), also known as norepinephrine, which is a neurotransmitter and hormone involved in the regulation of aggression (Marien et al., 2004; Matthews et al., 2002). One feature of AD pathogenesis is the degeneration of the locus coeruleus (LC), which is located in the pons and supplies NA to cortical and subcortical areas of the brain (Weinshenker, 2008), and as a result, appropriate levels of NA fail to reach cortical regions (Francis et al., 1985; Palmer et al, 1987). NA, destined to circulate in the CNS, is synthesized in the LC and released as a neurotransmitter from noradrenergic neurons, innervating regions such as the frontal cortex, thalamus, hypothalamus,

cerebellum, and limbic system (Figure 7; Marien et al., 2004). Thus, AD pathology may adversely affect NA neurons and result in a reduction of NA levels, which can lead to many BPSD, including aggression.

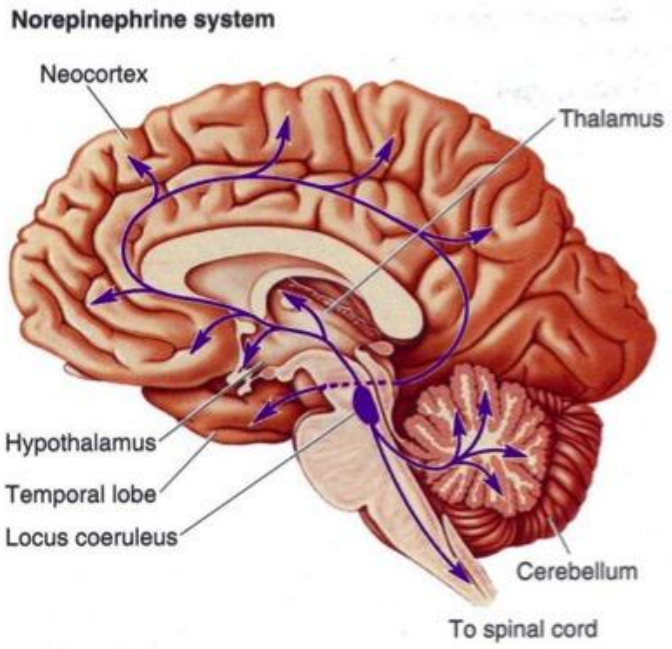


Figure 7. A Diagram of the Noradrenergic (Norepinephrine) Pathway. Noradrenergic neurons innervate multiple brain regions from the locus coeruleus, including regions that modulate aggression, such as the cerebral cortex, amygdala, hypothalamus, and limbic system, where neurodegeneration is seen (Adapted from Bear, Connors and Paradiso, 2007).

AD pathology can also be found in other regions of the brain. Clinical studies show hyperphosphorylated tau and NFT composition in cortical glutaminergic neurons are associated with cognitive degeneration and behavioral symptoms in AD patients (Arrigada et al., 1992), pinpointing a correlation between high accumulations of NFTs in the anterior cortical areas and high agitation scores, along with aberrant motor behavior in post mortem AD brains (Senanarong et al.,

2004; Tekin et al., 2001). In addition, high levels of aggression correlate with elevated phospho-tau to tau ratios at serine 396 of glutaminergic neurons in the prefrontal cortex and frontal lobe (Guadagna et al., 2012), and can be associated with greater atrophy in the frontal and cingulate cortices, insula, amygdala, and hippocampus (Trzepacz et al., 2013). While many studies have found correlations between AD pathology and aggressive behavior in AD patients, it is unclear if tau directly induces toxicity in the aforementioned neurons and brain regions. The majority of clinical studies with AD patients have been able to examine AD pathology in post mortem brains, which cannot provide a clear picture of tau pathology and how it interacts with neurons *in vivo*. In order to further study the effect of tau pathology in neurons and aggressive behavior, nonhuman models are utilized to investigate the underlying mechanisms of tau interaction and aggression *in vivo* of organisms with similar brain regions and signaling pathways to humans.

4. Rodent Models of Aggression

The rodent model is the most common nonhuman model utilized to study aggression. Aggression can have a phenomenological or functional definition. In phenomenological terms, aggression is a behavior that delivers intentional harm and injury to another organism (Buss, 1961), whereas in functional terms, aggression is perceived as a confrontational form of resource competition (Darwin, 1871; Archer, 2009; Lindenfors and Tullberg, 2011; Weiger and Bear,

1988). Aggression research using rodent models have explored the ethological significance of the behavior, gathering information like functionality in survival and reproduction, and its phylogenetic and ontogenetic development. Most research using nonhuman models to study aggression have investigated the functional definition in animals, observing the species-typical aggression essential for animals to obtain food, water, shelter and mating partners (Takahashi & Miczek, 2014).

Several brain regions and receptors have been discovered with the help of male rodent model experiments. Male rodents exhibit inter-male aggression when competing for resources, while escalated aggression is generated from social provocations and social isolation of the animal. Both forms of aggression are able to display the animal's species-typical aggression, and can be characterized by quantitative measures, such as decreased latency to attack, increased and persistent number of bites, and severe tissue-damaging attacks (Haller, 2017; Miczek et al. 2004; de Almeida et al., 2005).

Several techniques, like electrical stimulation and optogenetics with rodents, have found activation of the hypothalamus attack areas (HAA) to induce pathological aggressive behavior and that the arginine vasopression (AVP) regulates the HAA, which promotes aggressive behaviors (Siegel et al., 1999). Other studies have pinpointed the prefrontal cortex (PFC), which includes the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC), to have inhibitory effects on aggression and is regulated by 5-HT, depending on the

subtypes of receptors and brain region that they are expressed, as well as the type of aggression (Haller et al., 2006; Halasz et al., 2006; Wall et al., 2012). One study identified the dorsal raphe nucleus (DRN) as a crucial region for aggressive behavior, as it sends 5-HT neuron projections into the PFC (van der Vegt et al., 2003). In addition, there is an increase of dopamine in the nucleus accumbens (NAc) of animals during aggressive encounters (Miczek et al., 2007). The olfactory bulb has been found significant to process pheromonal information so that rodents show the appropriate social behaviors, like sexual or aggressive behavior, towards another animal (Clancy et al., 1984; Bean & Wysocki, 1989). Multiple studies have also revealed that blocking the vomeronasal organ (VNO) can eliminate aggression between males and activate sexual behavior (Stowers et al., 2002; Keverne, 2002; Norlin et al., 2003; Chamero et al., 2011). Lastly, it has been shown that balance is necessary between excitatory glutamate and inhibitory GABA to maintain aggressive behavior at the species-typical level (Herman et al., 2004; Miczek et al., 2007; Garcia-Garcia et al., 2009).

Genetic and pharmacological studies have identified subtypes of glutamate receptors involved in aggression, such as NMDA, AMPA, kainite receptors, and mGluRs. These studies have also identified the function of GABA receptors (GABAa and GABAb) in aggression (Takahashi & Miczek, 2014). Each receptor has a different role depending on the receptor subunits, localization and aggressive behavior being tested. In summary, the neurochemistry and brain

regions that regulate aggression have been successfully interrogated through rodent models.

4.1 Rodent Models of AD & Aggression

Studies have used transgenic mice to model AD pathology and have investigated its effects in several aggression assays. For example, one study used the dominant test tube to measure aggression between 12-month-old THY-Tau22 mice, expressing human 4-repeat tau to model NFTs in the hippocampus, and wild-type mice. A THY-Tau22 male mouse was placed on one end of the tube facing a wild-type male mouse with a diameter big enough to crawl through. The mouse that would dominate over their opponent would be labeled the winner and the most aggressive. The results of this study showed that THY-Tau22 male mice were consistently more aggressive than wild-type mice (Van der Jeugd et al., 2013). Thus, the study suggested that tau in the hippocampus induced aggressive behavior.

Similarly, a study utilizing APP23 transgenic mice to model the Aβ oligomers in familial AD, compared the aggression levels of APP23 mice and wild-type control in an isolation-induced and resident-intruder paradigm. In this paradigm, mice were isolated and undisturbed for 3 weeks to establish their territory in their cage and evoke aggressive behavior upon intrusion by another male mouse. Aggression levels were measured by number of attacks, duration of attack bouts, and latency to the first attack. The results of this study showed that APP23 mice

attacked intruder mice significantly more than wild-type littermates, displayed shorter latencies to first attack than wild-type mice, but there was no difference between their 6-month or 12-month groups (Vloeberghs et al., 2006). Both of these studies, as well as many others, report increased aggressive behavior in AD mouse models, but similar studies have yet to be undertaken in *Drosophila* models of AD.

5. *Drosophila* Models of Aggression

Drosophila have long been utilized to study human diseases, as 75% of human disease-related genes can be found in the fly genome (Cowan et al., 2011). As a result, many neuromodulators and key mediators of aggression have been discovered with *Drosophila* models. Like rodents, male-male pairings of flies display social dominance hierarchies when competing for food or a mate (Andrews et al., 2014; de Almeida et al., 2005). Dominance and territoriality are commonly studied in *Drosophila* male flies by analyzing males that win or lose fights and observing which male defends an area of food over a certain amount of time. Behavior modules of aggression in *Drosophila* can be divided into threat and attack behaviors. These modules include approaching, wing threats, lunging, boxing, tussling, fencing, kicking, chasing, and holding (Zwarts et al., 2012). Lunging is one of the behavior modules considered to be high intensity aggression, and it is one of the most common modules used to quantify aggression in these models (Zwarts et al., 2012; Certel et al., 2010; Chen et al., 2002;

Dierick, 2007). This study, like many *Drosophila* models of aggression, is focused on investigating the effect of neuronal tau expression on the species-typical aggression or attack behaviors between male flies.

Multiple studies have identified aggression neuromodulators in the fly. One study revealed that olfactory neurons (ORNs) detect food and pheromonal cues. If a combination of the two is detected, it will promote high levels of aggression in the fly (Chen et al., 2002; Hoffman, 1987; Lim et al., 2014). Another study suggests that two types of dopamine (DA) neurons can influence aggression via interactions of two different DA receptor subtypes in the central complex region of the brain (Alekseyenjo et al., 2013). Additionally, another study found that serotonergic (5HT)-PLP neurons can regulate aggression through signaling 5HT1A receptor-expressing neurons in two different regions of the brain, which causes reductions in aggression (Aleksevenko et al., 2014). Similar to the "fight or flight" decision-making response in the sympathetic nervous system of humans, octopamine (OA), the invertebrate homolog of norepinephrine, modulates the behavioral response in flies (Andrews et al., 2014; Certel et al., 2010; Certel et al., 2007). While many studies have allowed researchers to investigate the neuromodulators and brain regions of *Drosophila* aggression, the effect of tau expression on aggressive behavior in *Drosophila* is unknown.

5.1 Drosophila Models of AD & Aggression

There is currently no study that has developed a *Drosophila* model of neuronal tauopathy with the intention to study aggression. This study focuses to clear the gap between what we know about aggression in the fruit fly and tauopathies. As previously mentioned, there are specific OA and DA receptors expressed in the mushroom bodies of the fruit fly brain (Baier et al., 2002), and neuronal tau expression has a significant effect in the mushroom bodies, which suggests a link exists between tau expression and aggression in mushroom bodies (Cowan et al., 2011). Studies have also shown that interruptions in mushroom body output can completely diminish aggression, yet few findings have been gathered about the genes that influence aggressive behavior (Baier et al., 2002; Edwards et al., 2009). Another study revealed that an OA reduction caused a drop in frequency of lunges in male flies fighting one another, but it also showed that OA may not be necessary to trigger an aggressive response because there were still flies that periodically lunged without this amine (Hoyer et al., 2008). All of the studies aforementioned highlight how flies contain similar neuromodulators and brain regions to rodents and humans that regulate aggression. With this knowledge, the *Drosophila* model of neuronal tauopathy utilized in this study (see next section), adapted from Wittmann et al., 2001, will be able to shed light on the effect of neuronal tau expression on aggression.

5.2 Drosophila Model of Neuronal Tauopathy

In 2001, a *Drosophila* model of neuronal tauopathy was created to study the pathogenesis of Alzheimer's disease and other tauopathies. This model was designed to overexpress a non-mutant human tau in the neurons of fruit flies. It accurately replicates the common pathological characteristics of these tauopathies, including adult onset, progressive neurodegeneration, accumulation of abnormal tau, and early death (Wittmann et al., 2001). This model has been useful in understanding the role of tau phosphorylation states, tau toxicity, mitochondrial abnormalities, oxidative stress, and DNA damage in tau pathology (Frost et al., 2015; Sun & Chen, 2015). Using this model, all of these pathological features appear in adulthood but are not present during development, and the total tau and phosphorylated tau levels increase with age. Most of the neurodegeneration seen in this model include the loss of cholinergic neurons, as characterized in AD. Thus, this *Drosophila* model of neuronal tauopathy is capable of replicating multiple critical features of human disease, like AD.

AIM OF STUDY

The previously mentioned studies show that there is still a lot to learn about the relationship between tau pathology and aggression, and the *Drosophila* model of neuronal tauopathy may provide critical insight into this relationship. By compiling methods from several behavioral studies with *Drosophila* (Certel & Kravitz, 2012; Hoyer et al., 2007), we can quantify aggression in male flies expressing tau in neurons. With studies that have tracked the neuromodulation and brain regions involved in aggression, this study aims to apply this information to its findings. Most of the aggression paradigms of AD have shown that an increase in any of the two pathological hallmarks of AD can enhance aggression in male flies (Vloeberghs et al., 2006; Van der Jeugd et al., 2013). This proposed experiment sets out to explore how the underlying pathways of aggression in a *Drosophila* model of AD are affected by the presence of tau.

We hypothesized that if wild-type human tau is expressed in the neurons of *Drosophila*, a difference in the levels of aggression between control flies and those expressing tau will be seen. For our second hypothesis, we thought flies expressing tau in neurons will exhibit higher levels of aggression than control flies because symptomatic aggressive behavior in AD is associated with neurodegeneration, a process induced by the formation of NFTs. Our findings showed that there was a difference in the aggressive behavior between tau-expressing flies and flies not expressing tau, but contrary to our second hypothesis, a reduction in aggression was observed in 5-day old tau flies. No

effect was seen in 2-day old tau flies. These findings suggest that age-dependent increases of tau pathology in AD do have an effect on aggressive behavior, but additional experiments will be necessary to further define this effect.

METHODS

1. The GAL4-UAS System

The GAL4-UAS system is an effective and widely used tool to study targeted gene expression in *Drosophila*. The method was discovered by Brand and Perrimon in 1993, and it consists of two components: the yeast transcription factor galactosidase-4 (GAL4) and an upstream activating sequence (UAS). GAL4, a transcription activator protein, possesses a domain that detects and binds to enhancer UAS sites, allowing gene transcription (Figure 8). The components are carried in two separate lines: the driver line and the responder line. The driver line promotes tissue-specific GAL4 expression, and the responder line carries the gene of interest under the control of UAS sites. Neither the GAL4 or UAS component independently activate the target gene. When the GAL4 gene is under the control of a native gene promoter, GAL4 will solely be expressed in cells where the native gene promoter is active. After GAL4 is expressed, it will bind to the UAS sites and activate the expression of the gene of interest only in these cells (Duffy, 2002; Sun & Chen, 2015).

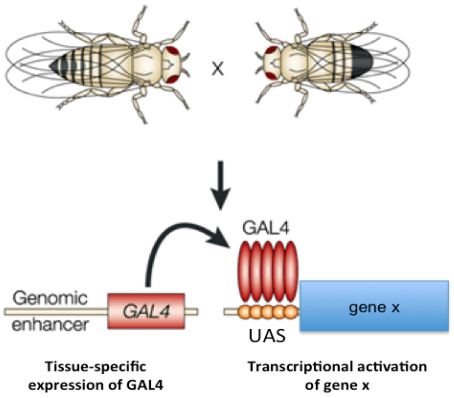


Figure 8. Schematic diagram of the GAL4/UAS expression system in *Drosophila*. In crossing a female and male fly, the yeast transcriptional activator, *GAL4*, binds to *UAS* and drives tissue-specific expression of a gene of interest (Adapted from Muqit & Feany, 2002).

2. *Drosophila* Stocks & Genetics

To create our *Drosophila* model of neuronal tauopathy, the GAL4-UAS system was utilized to exclusively express human-wild type tau, containing zero N-terminal inserts and four microtubule binding domains (0N,4R), in neurons, consistent with previous *Drosophila* models of tauopathy (Wittmann et al., 2001; Colodner and Feany, 2010).

All stocks were kept in an incubator with a temperature set at 25°C. The following are the genotypes of the three fly stocks used in this experiment:

1. *elav-GAL4/elav-GAL4*
2. *UAS-Tau^{WTII} / TM3, Sb*
3. *W*

The experimental tau transgenic fly line contains the neuronal-specific driver *elav-GAL4*, coupled with the *UAS-Tau*^{WTII} responder. The *GAL4* gene was inserted downstream of a neuronal-specific *elav* promoter, allowing GAL4 protein to be expressed in neurons. GAL4 protein will then bind to the UAS region, activating the expression of the *tau* gene downstream of the UAS sequence in neurons (Figure 9).

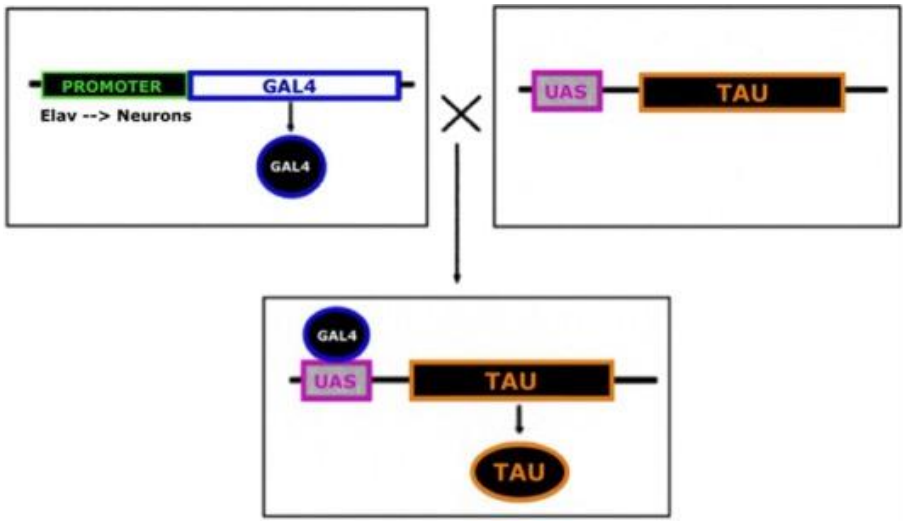


Figure 9. Neuronal-specific tau expression in *Drosophila* achieved via the GAL4/UAS Driver system. The *elav-GAL4* construct is present in the genome of the female fly and once crossed to a male fly with the *UAS-Tau*^{WTII} construct, GAL4 binds to UAS regions to activate the expression of tau protein in neurons. (Adapted from Herberlein et al., 2004).

The *elav-GAL4* construct is homozygous and inserted on the X-chromosome of the first stock aforementioned. In the second stock, the UAS region upstream of the *tau* gene is inserted on the third chromosome and balanced by the *TM3, Sb* chromosome. The purpose of the *TM3, Sb* balancer chromosome is to prevent recombination from occurring. This chromosome contains a marker gene, *Sb*,

which is the dominant allele for bristles with a stubble. By selecting against this bristle phenotype, we ensure that our experimental progeny will be expressing tau in their neurons.

The third stock, the *W*$^{-}$ strain lacks a functional copy of the *white* gene on the X-chromosome, and as a result loses the red eye color typically seen in wild-type fruit flies. Both *elav-GAL4* and *UAS-Tau*^{WTII} stocks were created in a *W*$^{-}$ genetic background, so the *w*$^{-}$ serves as a genetic background control.

3. *Drosophila* Crosses

For this experiment, we wanted to measure the aggression levels of three different progenies of interest (Table 1). The *elav-GAL4/+* flies control for *GAL4* transgene and the presence of the GAL4 protein. In the same way, the *UAS-Tau*^{WTII}*/+* flies control for the *UAS* transgene and for the presence of *UAS-Tau*^{WTII}.

Table 1. Genotypes of offspring utilized in aggression assay.

Genotype	**Fly Type**
elav-GAL4/+; UAS-Tau^{WTII}*/+*	Tau
elav-GAL4/+	Control I
UAS-TauW^{TII}*/+*	Control II

In order to assemble our three *Drosophila* crosses, we collected the appropriate virgin females to guarantee that our progenies would eclose with the proper genotypes. We virgined from the *elav-GAL4* and *W*$^{-}$ stocks. Vials and bottles of these two stocks were cleared, getting rid of all the males and females that may

have already mated with one another and their progeny. Female *Drosophila* are virgins between 4-6 hours after eclosion in 25°C and 12-14 hours after eclosion in 17°C. Vials were checked following these conditions, making sure to identify the sex of all the flies. The virgin females that were collected were kept in 17°C until there were enough virgins to set up crosses. For each cross, we ensure that approximately 6-8 female virgins of one strain and 10-12 male flies of the other strain in one vial. All crosses were maintained in an incubator at 25°C with a 12-hour light and 12-hour dark cycle. All of the flies utilized in our aggression assay were males and aged to either 2-days old or 5-days old.

3.1 Tau Flies

To generate the progeny of our tau flies (experimental), *elav-GAL4/elav-GAL4* female virgins were crossed with *UAS-Tau*^{WTII} */ TM3, Sb* males to obtain our *elav-GAL4/+; UAS-Tau*^{WTII}*/+* males (Figure 10).

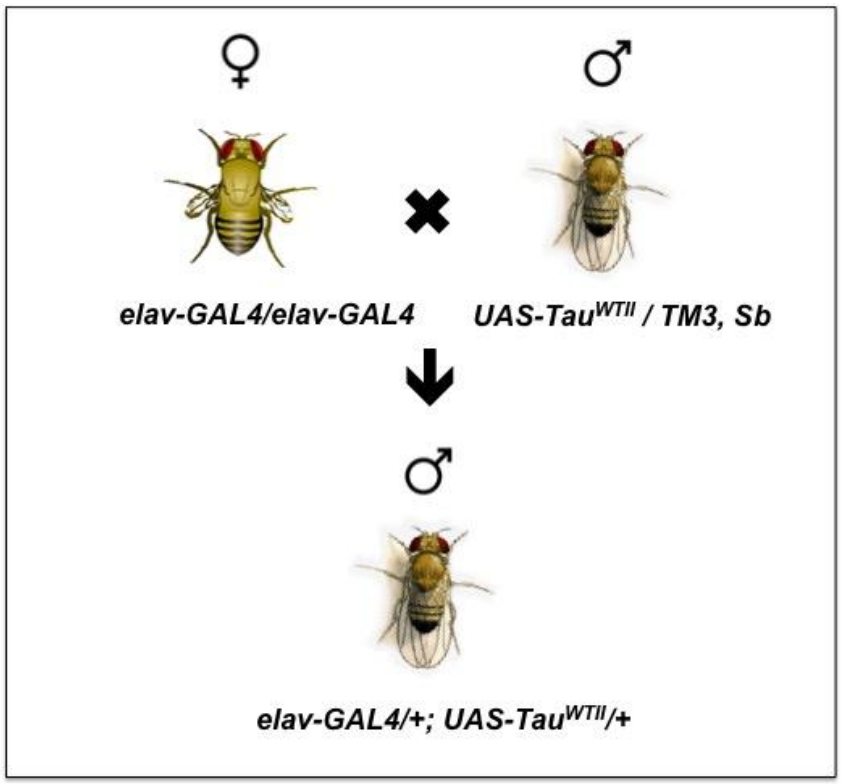


Figure 10. *elav-GAL4/UAS-Tau^{WTII}* allowing neuronal tau expression in *Drosophila*. Females carrying a *elav-GAL4* neuronal-specific driver were mated to males carrying the wild-type human tau responder, *UAS-Tau^{WTII}*. Progeny carrying both parts of the system will express GAL4 in neurons, which will bind to UAS sites and drive neuronal expression of wild-type human tau.

After isolation and eclosion (see below), flies are sorted according to their phenotypic identifiers. For this experimental cross, we selected against any flies with stubble bristles (marker gene) in the progeny. Our progeny of interest were males with no stubble, red eyes, and expressing tau.

3.2 Control I Flies

To generate the progeny of our control I flies, *elav-GAL4/elav-GAL4* female virgins were crossed with *W* males to obtain our *elav-GAL4/+* males (Figure 11).

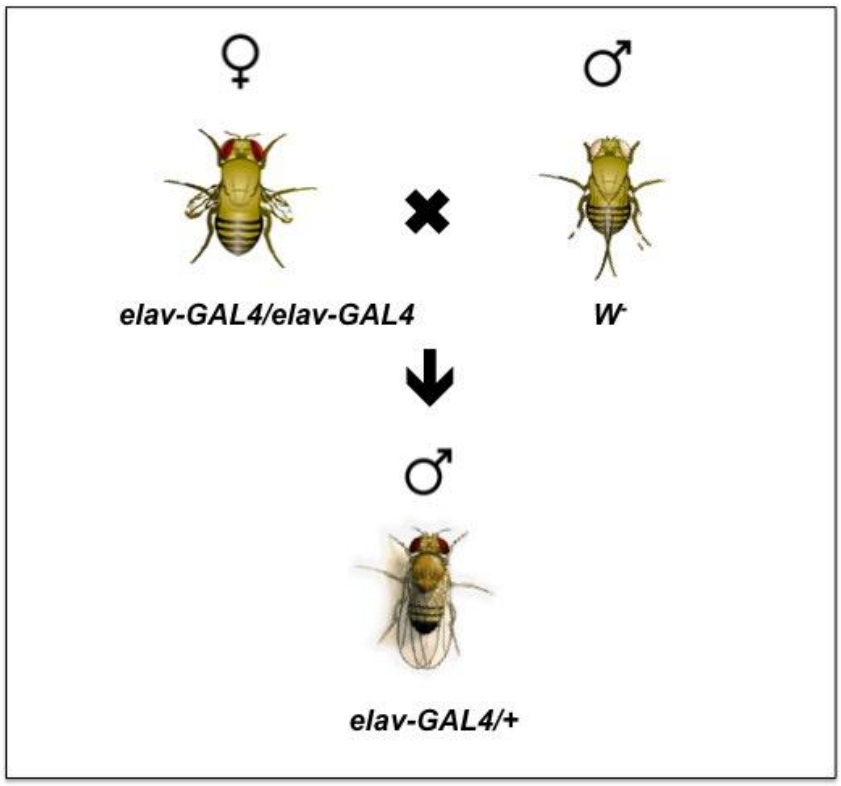


Figure 11. *elav-GAL4* construct without *UAS-Tau^{WTII}* in *Drosophila* results in GAL4 expression in neurons. Females carrying a *elav-GAL4* neuronal-specific driver were mated to males from the *W-* strain, which lack functional copies of the *white* gene in the X-chromosome causing this *Drosophila* parent to have white eyes. All progeny contain the *elav-GAL4* construct, which results in red-eyed progeny expressing GAL4 in neurons, without tau.

The progeny were sorted according to their phenotypic identifiers. For this control I cross, all male flies with red eyes would be the progeny of interest. These flies are not expressing tau, but contain the *elav-GAL4* construct allowing GAL4 expression in neurons.

3.3 Control II Flies

To generate the progeny of our control II flies, W^{-} female virgins were crossed with *UAS-Tau*^{WTII} */ TM3, Sb* males to obtain our *UAS-Tau*^{WTII}/+ males (Figure 12).

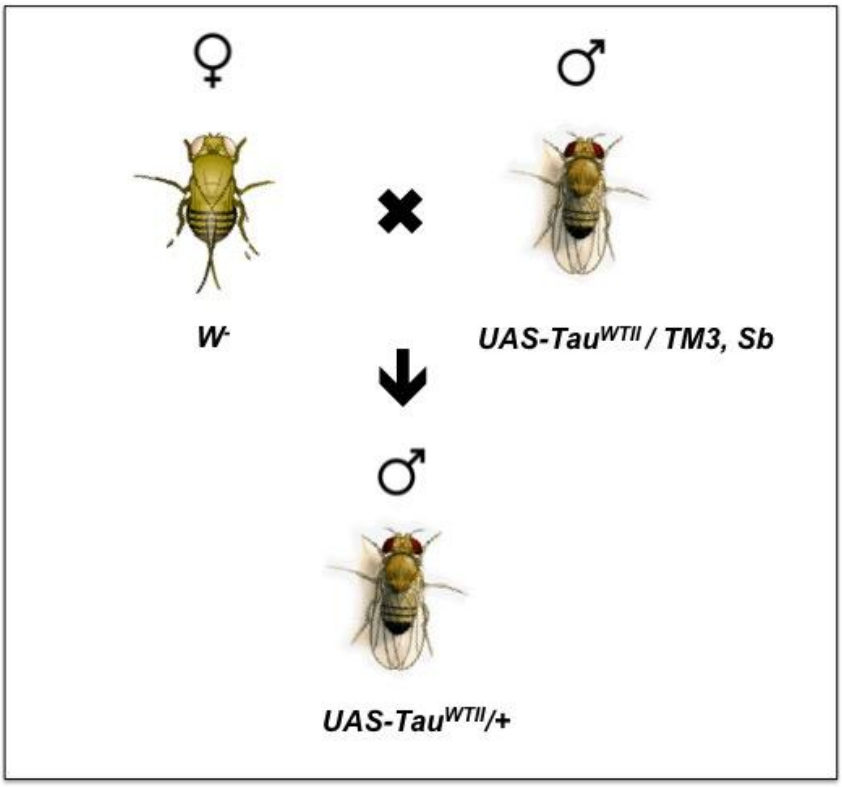


Figure 12. *UAS-Tau*^{WTII} without the *elav-GAL4* construct in *Drosophila* does not result in GAL4 or tau expression in neurons. Wild-type females were mated to males carrying the wild-type human tau responder, *UAS-Tau*^{WTII}. Our progeny of interest will have the *UAS-Tau*^{WTII} responder without the *elav-GAL4* construct. In the absence of the *elav-GAL4* construct, there is no GAL4 expression in neurons that will be able to bind to UAS sites and drive tau expression.

As with every cross, the progeny were sorted according to their phenotypic identifiers. For this control II cross, our progeny of interest were male flies

without stubble and pale orange eyes. These flies are not expressing GAL4 or tau in their neurons.

4. Aging & Isolation

After mating the flies, it takes approximately nine days for *Drosophila* to become late stage pupae. At this stage in their life cycle, we isolated male flies from each cross, identified by the presence of male sex combs (Figure 13A). Isolation involves using a paintbrush to gently move a single male pupa into a separate vial without any other flies (Figure 13B). This prevents the male fly from experiencing any social interaction after eclosion.

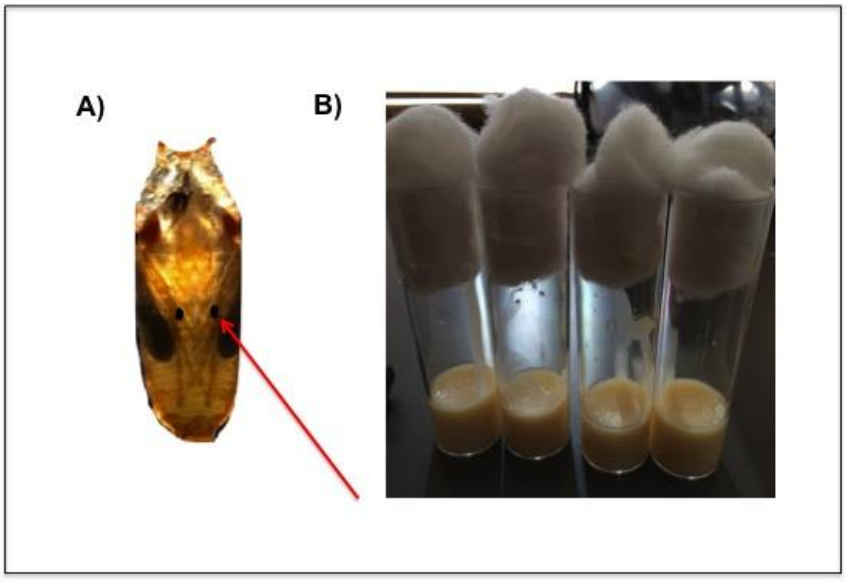


Figure 13. Isolation allows *Drosophila* males to remain socially naive. A) A male pupa can be identified by its sex combs, which appear as two black dots located on the two front legs of the male fly (arrow). The two legs are crossed over the abdomen of the fly when encapsulated in its pupal case. **B)** Isolation vials are filled 1 cm high with Nutri-Fly food, 2-3 yeast pellets and covered with a cotton ball to allow for easy aspiration access.

Once all male pupae from each cross were collected and placed into isolation, the flies were checked to see if they had eclosed each day. On the day that a fly ecloses, that marks its first day in age. For this experiment, we aged all of our flies for 2 days or 5 days. Aging allows for the flies to express more tau protein and accumulate in nerve cells over time.

After flies have aged to their appropriate ages, the flies were aspirated into an arena to fight. The fight pairings were the same for 2-day old and 5-day old flies (Figure 14). All fights were held in the morning after the lights turned on in their incubator. All fights started 30 minutes to an hour after their wake up time, which is when the lights in the incubator would turn on.

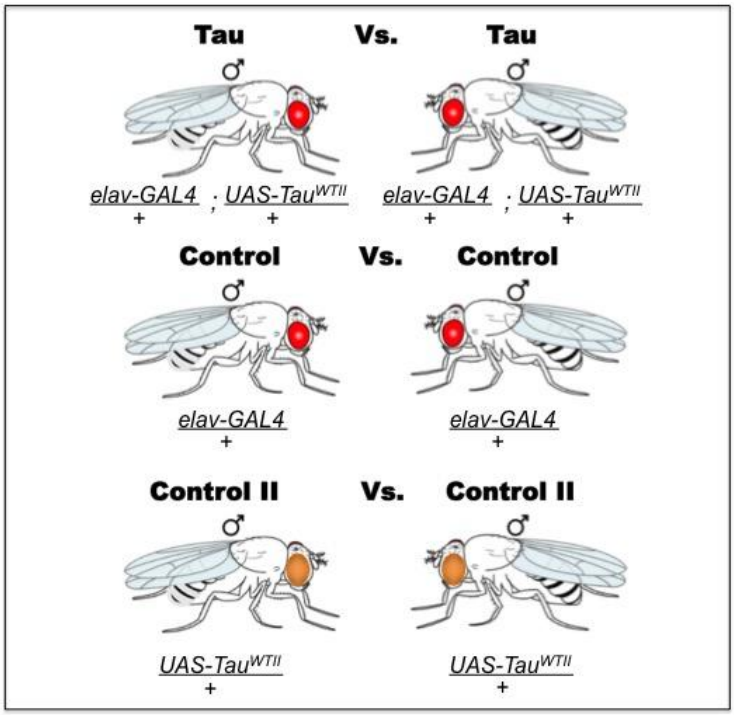


Figure 14. Fight pairings and their corresponding genotypes of *Drosophila*. Fights for each pairing were carried out for 2-day old flies and 5-day old flies.

5. Behavioral Assay

All fights were held in a room with a relative humidity of 60% and a room temperature of 78°C. To ensure that the room reached our desired relative humidity, a humidifier with a timer was set the night before to turn on 2 hours prior to a fight. This technique was adapted from Certel & Kravitz, 2010. In order to create our behavioral assay, we assembled an arena adapted from previous studies (Nilsen et al., 2004; Certel & Kravitz, 2012). The foundation of the arena was made from a single well of a 12-well plate (Figure 15A). The walls of the arena, the rim of the food cap, and one side of the microscope slide were covered in Fluon Slip Insect Barrier. This liquid solution kept the flies off of the surfaces of the well and microscope slide, centralizing them on the food cap. To make the food cap, the cap of an eppendorf tube was tightly packed with Nutri-Fly food. To make our yeast paste, yeast pellets were mixed with 0.2 M sucrose in water. A small drop of the yeast paste was placed in the center of the food cap to give the male flies something to compete over. After assembling the arena, two male flies were taken out of their isolation vials together and introduced to the arena at the same time using gentle aspiration (Figure 15C; adapted from Nilsen et al., 2004). Two hour footage was recorded by an EverFocus camera positioned over the well (Figure 15B). After the fight, all flies were transferred to an eppendorf tube and kept in a freezer that maintains a temperature of -80**°C** to perform western blot analysis.

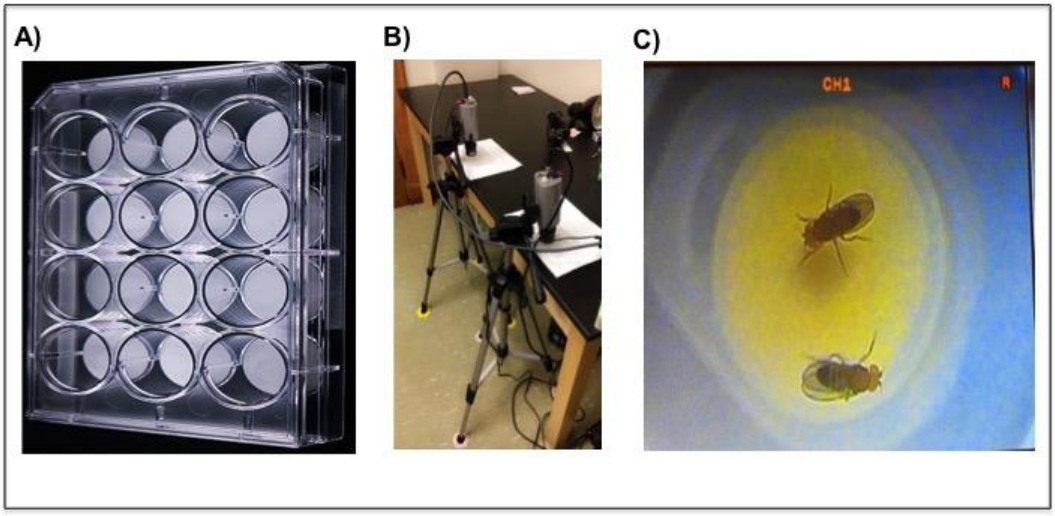


Figure 15. *Drosophila* males loaded into aggression assay. A) The food cap was centralized in a single well of a 12-well plate. **B)** EverFocus cameras capture 2 hour fights and run on 30 frames per second. **C)** Male flies fight over the yeast paste on the food cap.

6. Scoring Criteria

Each fight was blinded and hand-scored using QuickTime software, in order to slow down camera footage frame-by-frame. A small subset of fights were blinded and scored by a second scorer to assess inter-rater reliability. Aggressive behavior was measured by the number of lunges, latency to first lunge, total time both flies were on the food, and the number of lunges per minute. A lunge is defined as when a fly leans back on its hind legs, snaps forward at a fast velocity towards another fly, and pulls the opposing fly towards itself (Figure 16). All lunges observed on the Nutri-Fly food and the rim of the cap were recorded, and lunges observed off of these parameters were not counted. This was our operational definition of the lunge behavior measured in our assay.

Figure 16. The *Drosophila* lunge is a stereotypical high-intensity aggressive act exerted primarily by males when dominance hierarchies form (Adapted from Neith, 2014).

Latency to first lunge is defined as the time it takes before the first lunge is observed from either fly on the food. Fights that had zero lunges were not included the statistical analysis of latency to first lunge between groups. Total time on food data is computed after collecting the timestamps for when both flies are on and off the food. The sum of these intervals equals the total time that the flies were on the food for the entire fight. Flies are considered on the food when both are on the Nutri-Fly food or on the rim of the cap. When one fly is on the food and the second fly is entering the food, the timestamp at which one front leg enters the Nutri-Fly food is recorded. When the first male flies off the cap or crawls off the rim, that timestamp is recorded. Lunges per minute can be calculated by taking the total number of lunges of a fight and dividing it by the

total time on food of the fight. All time is recorded in minutes converted to decimal form.

7. Sucrose Experiment

7.1 Drosophila Stock

Initial experiments to confirm the effect of sucrose on aggression levels (Lim et al., 2014) were performed with a control fly strain (*Canton S*) that is commonly used in *Drosophila* aggression studies. There were 12 fights in total, 6 fights with sucrose (0.2 M in water) and 6 fights without sucrose.

8. Quantification of Tau

8.1 Western Blotting

Western blots were used to determine tau expression levels, specifically the total amount of human total tau and phosphorylated tau in *Drosophila* brains. The analysis was performed on 5-day old *Drosophila* males that fought in our aggression assay. The heads of frozen males flies expressing tau were isolated and homogenized in 2x Laemli's buffer (Figure 17A). After ten minutes of boiling, each sample was loaded into a separate well (Figure 17B) and subjected to SDS-PAGE in 10% separating gels (Figure 17C). The proteins were then transferred from the gel to a nitrocellulose membrane (Figure 17D). Proteins were blocked in 2% milk in PBS with 0.05% Tween 20 in order to prevent non-specific binding

between the membrane and the antibody utilized to detect human tau. The proteins were then immunoblotted overnight at 4°C using primary (1°) antibodies: phosphorylation-independent rabbit polyclonal anti-tau C-terminal antibody (1:200,000) and a mouse-anti-actin antibody (1:250). Actin was used as a loading control, which is expected to be present at similar levels across conditions. A loading control shows that the differences in tau protein levels were not a result of differential loading technique, but actual differences in brain tissue. The following day the membrane was washed and treated for 2 hours at room temperature with secondary antibodies (2°): goat anti-mouse antibody (1:20,000) and goat anti-rabbit antibody (1:20,000). These antibodies are conjugated to the horseradish peroxidase enzyme, which allows them to be detected via chemiluminescence. The relative intensity of each protein band corresponds to total protein levels in each sample.

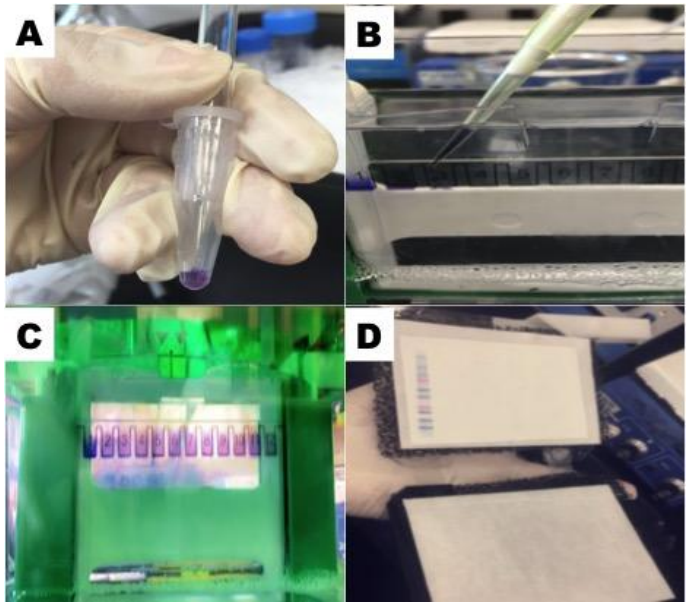


Figure 17. Western blot analysis of *Drosophila* brain tissue. A) Isolation and homogenizing process of fly brain in 2x Laemli's buffer. **B)** Each sample is loaded into a separate well of the gel. C) Proteins become separated by electrophoresis via SDS-PAGE. D) Proteins are transferred to nitrocellulose membrane and then treated with blocking solution, followed by antibody treatment.

8.2 Stripping and Reblotting

In order to determine the human phosphorylated tau levels in the male *Drosophila*, western blots that had been probed with the total tau antibody were stripped and reprobed. Membranes were incubated in stripping buffer at 50°C for 30 minutes. The stripping buffer is comprised of 62.5 mM Tris-HCL pH 6.8, 2% SDS, and 1% β-mercaptoethanol. After incubation, the membranes were washed three times with 1X TBS with Tween. The membranes were immunoblotted with mouse anti-AT8 primary (1°) antibody (1:1,000), which detects specific phosphorylation at pSer199/pSer202/Thr 205 sites. The membranes were then treated with goat anti-mouse secondary (2°) antibody (1:20,000) conjugated to the

horseradish peroxidase enzyme, and imaged via chemiluminescence. The actin protein loading control was obtained prior to reprobe. The relative intensity of each protein band corresponds with tau phosphorylation levels in *Drosophila* brains.

8.3 Densitometry

In order to quantify tau levels, the density of each tau band in each lane was calculated using densitometric analysis in Image J. Once each band density was obtained, the data was normalized to either actin or total tau for statistical analysis. Every two sample lanes were males of one fight, so the average of the two adjusted ratios for the fight were calculated prior to statistical analysis.

9. Statistical Analysis

To determine if there was a statistical difference in the aggression measures of *Canton S* fights with or without sucrose, independent samples t-tests via SPSS were performed. To determine if there was a statistical difference in the aggression measures of 2-day old flies and 5-day old flies, one-way ANOVAs were performed via SPSS. If significance was obtained, a Games-Howell post hoc was performed to assess the differences among the means. In order to determine if age, genotype or both had an effect on the aggression measures of our 2-day and 5-day old flies, we performed a factorial ANOVA via SPSS. If significance was obtained, a Least Significant Difference (LSD) post hoc was performed to assess the differences among the means. To discern if there was a relationship between

the number of lunges in 5-day old flies and quantified tau expression levels, the mean of the adjusted ratios of every 2 tau bands were compared to the corresponding lunges observed between the two flies using a Pearson correlation. In this study, there were two hand-scorers for a small subset of fights, and a Bland-Altman test via SPSS was completed to establish the inter-rater reliability between the sets of scores. For all statistics, a p value of $< .05$ indicates a statistically significant difference.

RESULTS

1. Sucrose Experiment

To investigate the relationship between aggression and neuronal tau expression in a *Drosophila* model of AD, we first had to establish the aggression paradigm in our laboratory. To examine if our aggression set-up was sufficient to monitor and record aggression in *Drosophila*, experimental trials were performed to determine if yeast paste with or without sucrose would generate typical aggressive acts, like the lunge. This robust aggressive activity had not been observed by previous students in the lab and we hypothesized that more lunges would be seen in fights using yeast paste with sucrose diluted in water, consistent with previous findings (Lim et al., 2014; Lewis, 1960).

To determine if using a yeast paste with sucrose (0.20 M of sucrose in water) or without sucrose created a significant difference in the levels of aggressive behavior, a total of 12 fights were conducted with male *Canton S*, and six fights were carried out for each condition. There was no significant difference in the total number of lunges between the yeast paste with no sucrose (M = 21.83) flies and the yeast paste with sucrose (M = 18.83) flies (Figure 18).

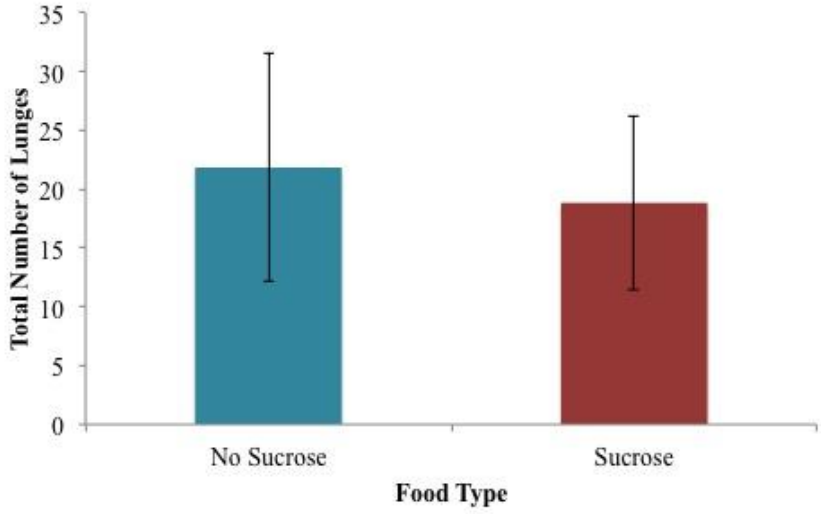


Figure 18. Levels of aggressive behavior remain unchanged independent of sucrose in *Canton S*. Total number of lunges of *Canton S* with Nutri-Fly food containing yeast paste without sucrose (n = 6) and yeast paste with sucrose (n = 6) showed no statistical significance between groups. Independent samples t-test, $p > .05$. Error bars represent ± SEM. All flies were males.

Similarly, there was no difference in the time it took for the flies to initiate aggressive behavior between the yeast with no sucrose (M = 3.60 min) and the yeast paste with sucrose (M = 6.85 min) groups (Figure 19).

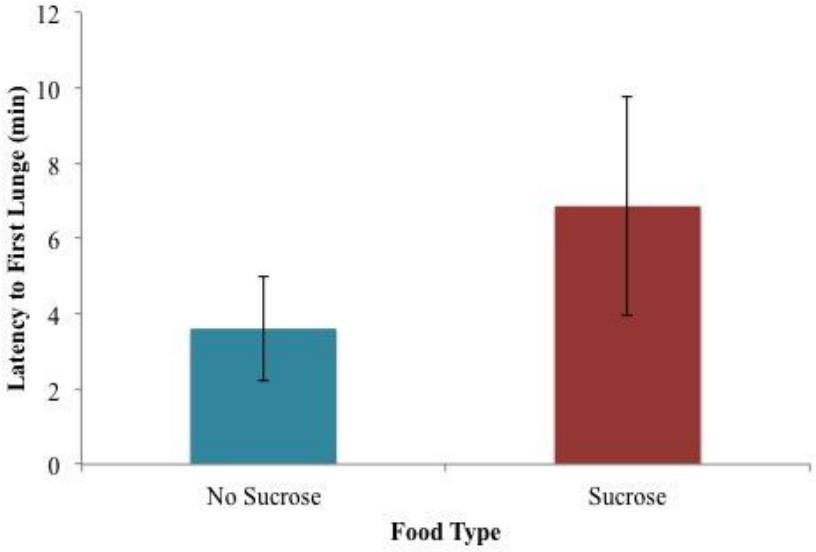


Figure 19. Food type showed no change in the time it took to initiate aggressive behavior in *Canton S*. Latency to first lunge of *Canton S* flies between the yeast paste with no sucrose (n = 6) and yeast paste with sucrose (n = 6) groups showed no statistical significance. Independent samples t-test, $p > .05$. Error bars represent ± SEM. All flies were males.

There was no difference in the total time on food between the yeast paste without sucrose (M = 48.70 min) flies and the yeast paste with sucrose (M = 20.12 min) flies, showing that food type had no significant effect in the amount of time the flies spent on the food cap (Figure 20).

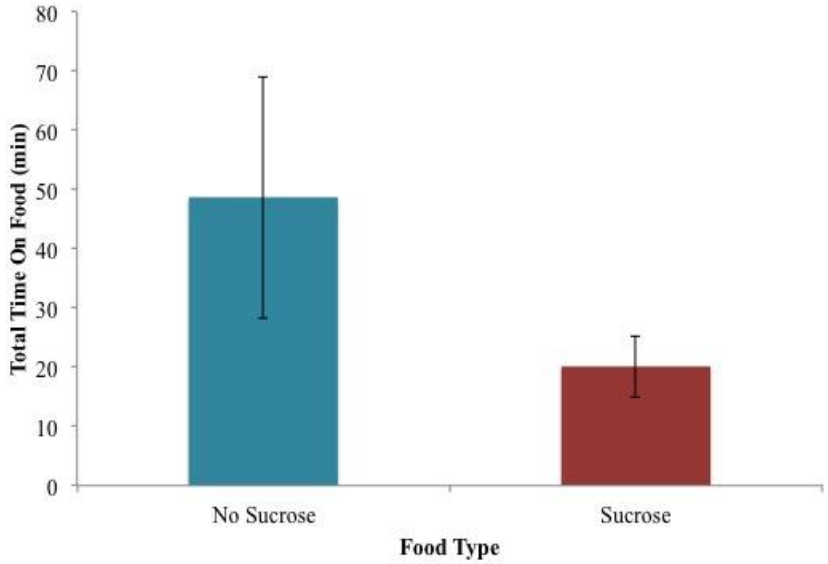


Figure 20. Food type had no effect on the total time that *Canton S* spent on the food cap. The total time *Canton S* flies spent on the food with no sucrose (n = 6) and with sucrose (n = 6) showed no statistical difference. Independent samples t-test, $p > .05$. Error bars represent ± SEM. All flies were males.

There was no significant difference between the number of lunges per minute on the food of the two food types, as the sucrose group (M = 1.31) and the no sucrose group (M = 1.88) lunged approximately the same amount per minute (Figure 21).

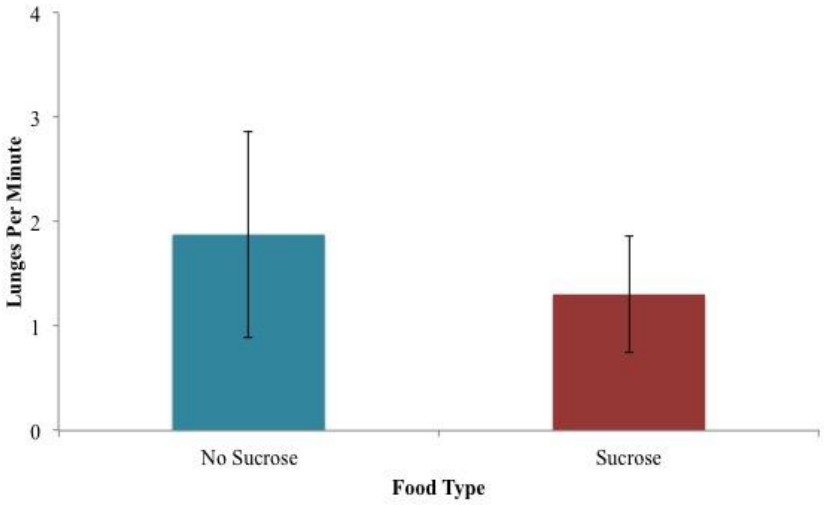


Figure 21. Food type had no effect on the amount of lunges seen per minute on the food in *Canton S*. The number of lunges that *Canton S* flies performed per minute showed no statistical significance between yeast paste with no sucrose (n = 6) and with sucrose (n = 6). Independent samples t-test, $p > .05$. Error bars represent ± SEM. All flies were males.

2. 2-Day Old Flies

To determine if 2-day old tau-expressing flies showed a significant difference in the levels of aggressive behavior, a total of 18 fights were conducted using our aggression assay. Our independent variable was fly type, which had three levels to be compared. The fly types in our aggression assay were tau, control I and control II. The four dependent variables (total number of lungs, latency to first lunge, total time on food, and lunges per minute) were compared between the 3 *Drosophila* genotypes to determine if there was any significant difference between aggression levels.

The total amount of lunges seen between the fly types were not significantly different for tau flies (M = 4.50), control II flies (M = 18.83) or control I flies (M = 33.83) (Figure 22).

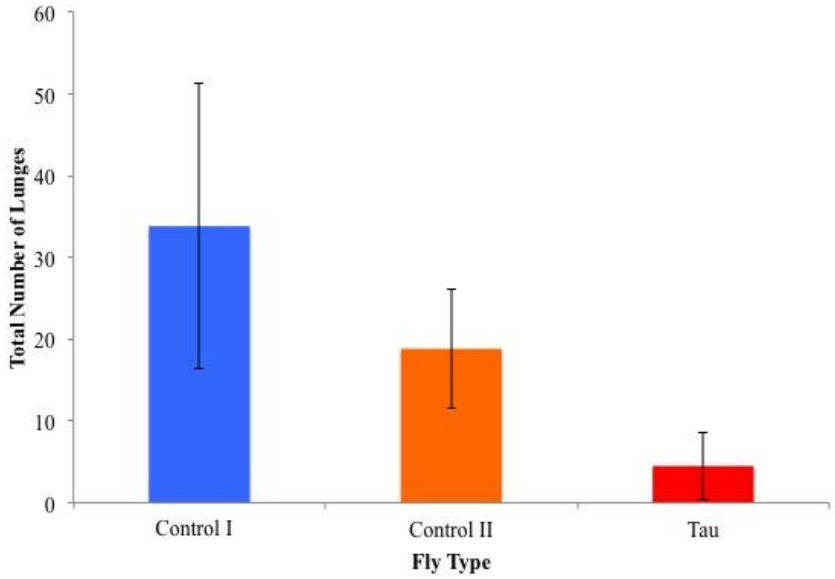


Figure 22. Tau expression showed no difference in the total number of lunges in 2-day old *Drosophila*. There was no significant effect between tau (n = 6), control (n = 6) or control II (n = 6) flies. One-way ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

In order to determine if 2-day old tau-expressing flies showed a significant difference in the time it took to initiate the first aggressive act, we compared the time the first lunge was observed in each fight between fly types. Fights that had no lunges could not be measured for latency to first lunge, and as a result, brought our sample sizes down for each fly type. There were a total of 10 fights computed for this statistic.

Latency to first lunge between fly types was not significantly different between groups, as tau flies (M = 32.13 min), control flies (M = 31.08 min), and control II flies (M = 30.88 min) all initiated their first lunge around the same time (Figure 23).

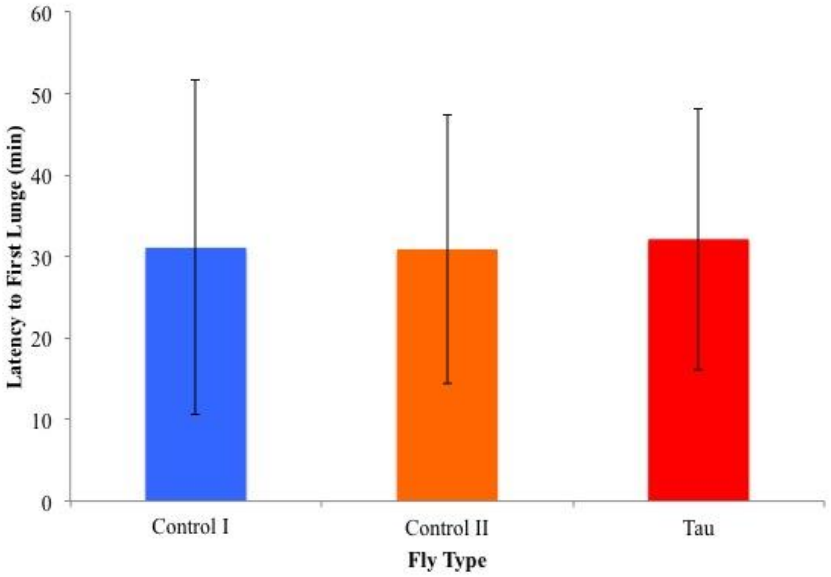


Figure 23. Tau expression showed no difference in latency to first lunge in 2-day old *Drosophila*. There was no significant effect between tau (n = 2), control (n = 4) or control II (n = 4) flies. One-way ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

The total time that the flies spent on the food remained unchanged between each fly type, showing that tau flies (M = 33.10 min), control flies (M = 39.55 min), and control II (M = 37.38 min) flies spent the same amount of time on the food cap (Figure 24).

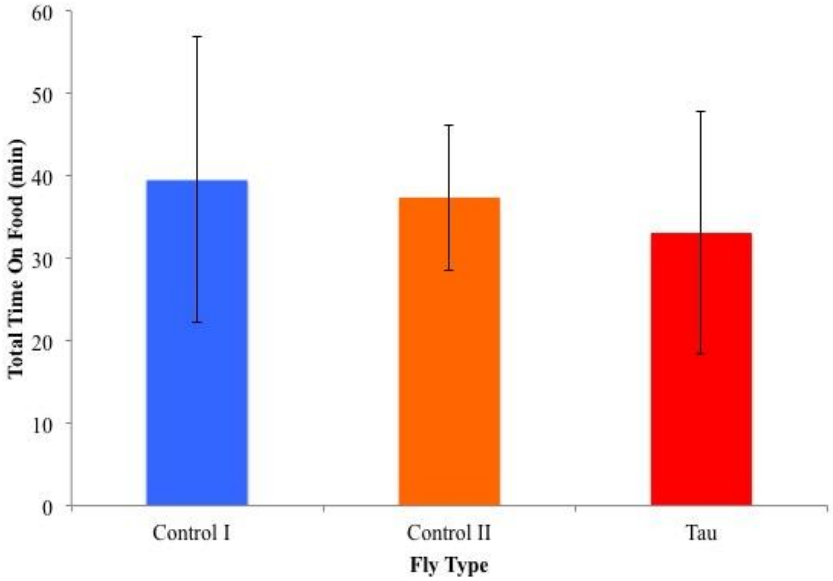


Figure 24. Tau expression showed no difference in the time that 2-day old *Drosophila* spent on the food. There was no significant effect between tau (n = 6), control (n = 6) or control II (n = 6) flies. One-way ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

There was no significant difference in the average number of lunges per minute on the food for tau flies (M = 0.60), control flies (M = 1.40), and control II flies (M = 0.80) (Figure 25).

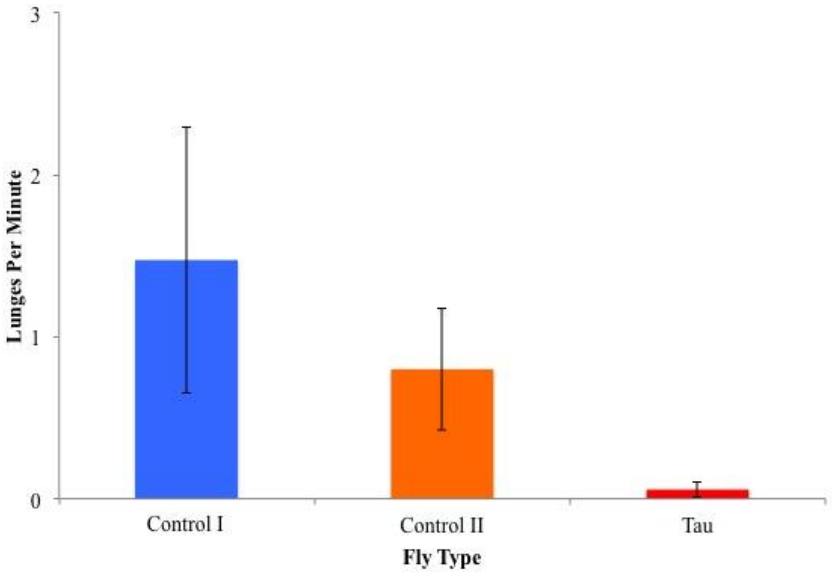


Figure 25. Tau expression showed no difference in the lunges per minute of 2-day old *Drosophila*. There was no significant effect between tau (n = 6), control (n = 6) or control II (n = 6) flies. One-way ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

3. 5-Day Old Flies

In order to determine if there was an effect of tau expression on aggression in older flies, we assayed aggressive behavior in 5-day old flies. A total of 54 fights were conducted with similar independent and dependent variables.

In 5-day old flies, tau flies lunged significantly less than control I and control II flies, as there was a significant difference in lunges between our tau flies (M = 24.00) and control I (M = 107.33) and control II flies (M = 74.67) (Figure 26). There was no significant difference between control I and control II groups in regards to the total of lunges observed.

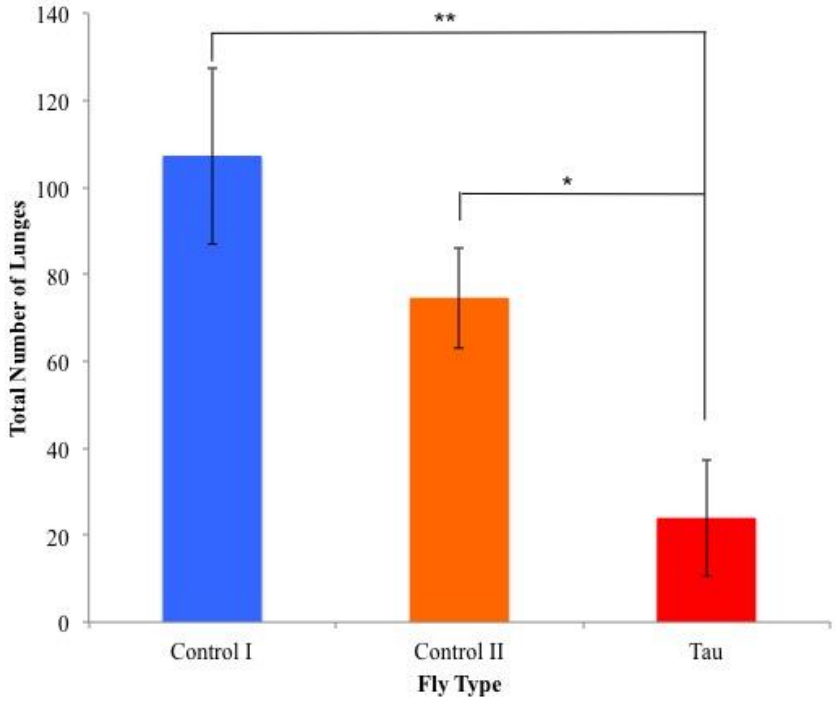


Figure 26. Tau expression showed a significant decrease in the total number of lunges in 5-day old *Drosophila*. Less lunges were observed in tau (n = 18) fights in comparison to the number of lunges seen in control I (n = 18) or control II (n = 18) fights. One-way ANOVA, * $p < .05$, ** $p < .01$; Games-Howell Post Hoc Test. Error bars represent ± SEM. All flies were males.

In order to determine if 5-day old tau-expressing flies showed a significant difference in the time it took to initiate the first aggressive act, we compared the time the first lunge was observed in each fight between fly types. Fights that had no lunges could not be measured for latency to first lunge, and as a result, there were 46 fights included in this analysis.

Latencies to first lunge between fly types were significantly different between tau and control groups, as tau flies (M = 63.15 min) took longer to initiate aggression than the control I flies (M = 10.38 min) and control II (M = 19.33 min) (Figure 27).

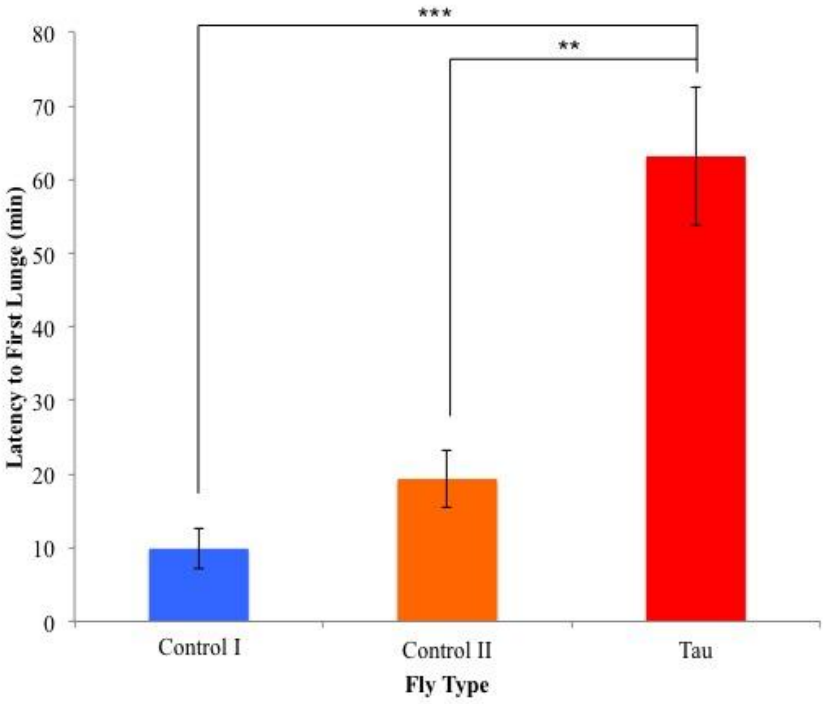


Figure 27. Tau expression showed a significant increase in the latency to first lunge in 5-day old *Drosophila*. An increase in latency to first lunge was observed in tau (n = 14) fights in comparison to the latency seen in control I (n = 15) or control II (n = 17) fights. One-way ANOVA, ** $p < .01$, *** $p < .001$; Games-Howell Post Hoc Test. Error bars represent ± SEM. All flies were males.

In 5-day old *Drosophila*, a statistical difference between fly type was observed in regard to total time on food. Tau flies (M = 33.10 min) spent more time on food than control II flies (M = 16.78 min), but did not spend significantly more time on food compared to control I flies (M = 48.02 min), whereas control II flies spent less time on the food than control I flies (M = 48.02 min) (Figure 28).

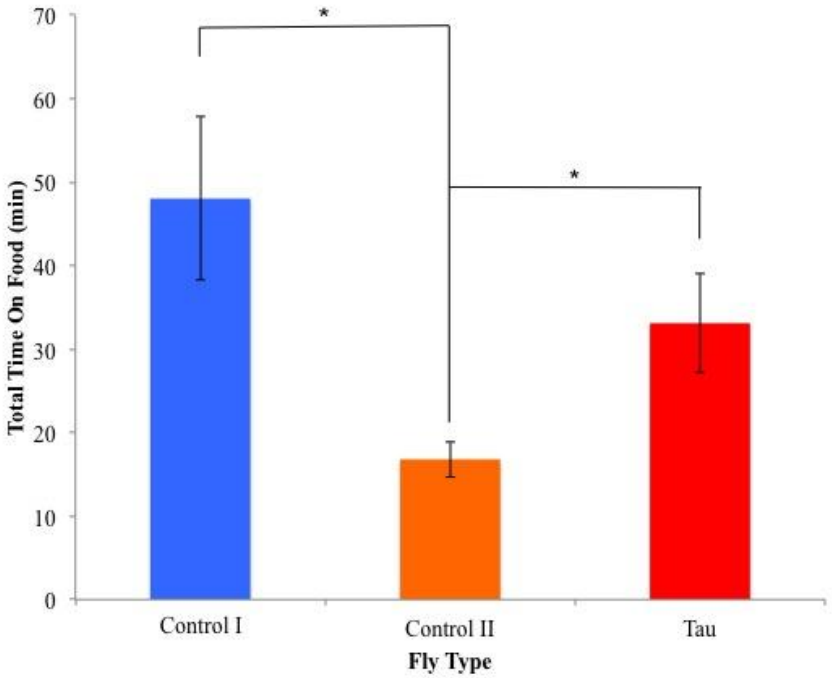


Figure 28. 5-day old tau *Drosophila* showed a significant increase in the total time spent on the food when compared to control II flies, not expressing GAL4 or tau. An increase in total time on food was observed for tau (n = 18) fights was observed when compared to control II (n = 18) fights, and control II (n = 18) fights compared to control I (n = 18). One-way ANOVA, * $p < .05$; Games-Howell Post Hoc Test. Error bars represent ± SEM. All flies were males.

In 5-day old *Drosophila*, there was a statistical difference in the average number of lunges seen per minute on food between tau flies and both control types, as tau flies (M = 1.12) lunged significantly less per minute than control I (M = 5.37) and control II flies (M = 5.03) (Figure 29).

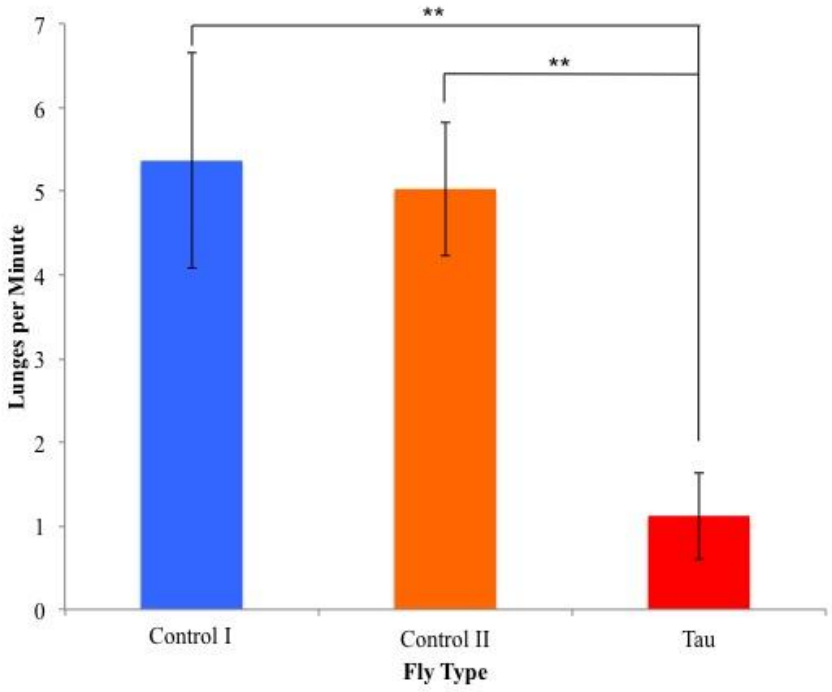


Figure 29. Tau expression showed a significant decrease in lunges per minute in 5-day old *Drosophila*. Less lunges per minute were observed in tau (n = 18) fights in comparison to the number of lunges seen in control I (n = 18) or control II (n = 18) fights. One-way ANOVA, ** $p < .01$; Games-Howell Post Hoc Test. Error bars represent ± SEM. All flies were males.

4. Interaction of Age & Fly Type

4.1 Age

In order to determine if there was an effect of age on aggression in all flies, we compared the means of each dependent variable between 2-day old and 5-day old flies. There were a total of 72 fights compared in this statistical analysis.

Five-day old flies lunged significantly more than 2-day old flies, as there was a significant difference in the lunges between our 2-day old flies (M = 19.06) and our 5-day old flies (M = 68.67) (Figure 30).

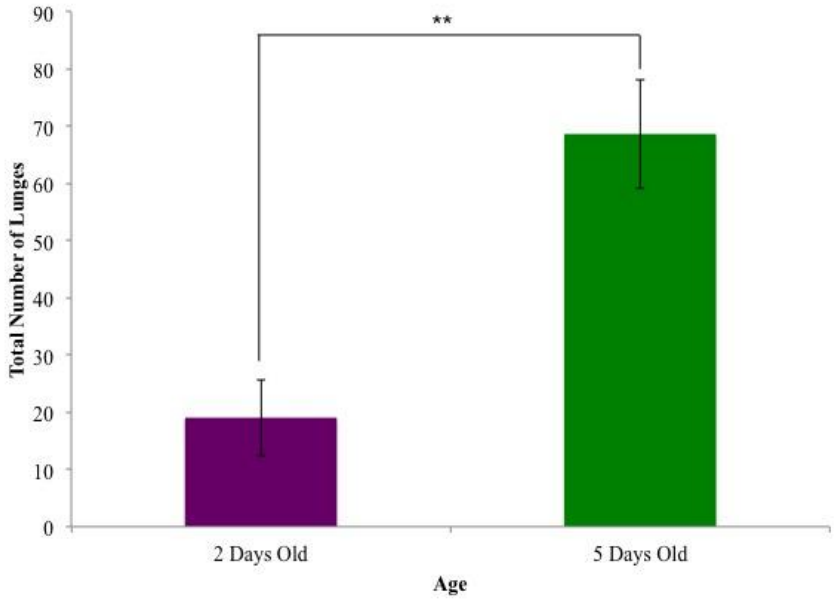


Figure 30. Age showed a significant increase of lunges in 5-day old *Drosophila*. Less lunges were observed in 2-day old flies (n = 18) in comparison to 5-day old flies (n = 54). Factorial ANOVA, ** $p < .01$; LSD Post Hoc Test. Error bars represent ± SEM. All flies were males.

In order to determine if age showed a significant difference in the time it took to initiate the first aggressive act, we compared the time the first lunge was observed in each fight between 2-day old and 5-day old flies. Fights that had no lunges could not be measured for latency to first lunge, and as a result, there were 56 fights included in this analysis.

Latencies to first lunge between fly types were not significantly different between 2-day old and 5-day old flies, as 2-day old flies (M = 31.22 min) took approximately the same amount of time to initiate aggression when compared to 5-day old flies (M = 29.76 min) (Figure 31).

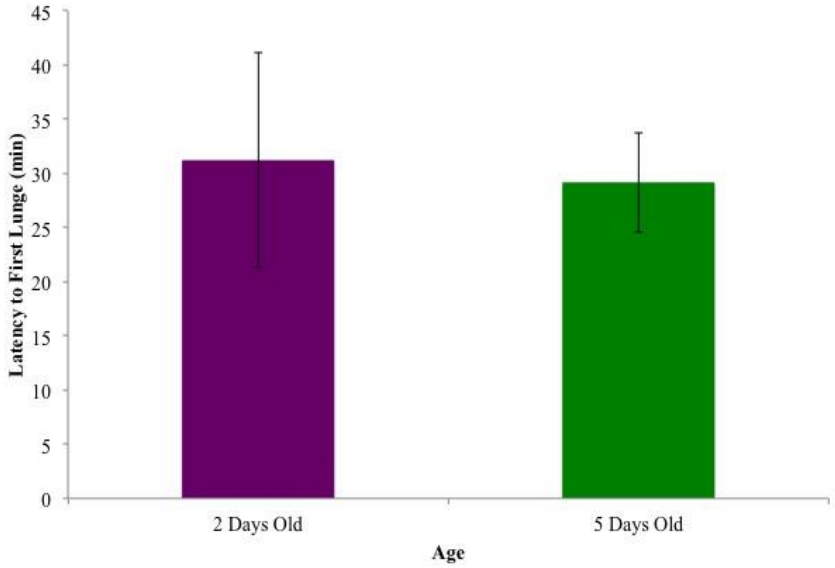


Figure 31. Age showed no significant effect in latency to first lunge of *Drosophila*. The 2-day old flies (n = 10) took approximately the same amount of time to initiate aggression when compared to 5-day old flies (n = 46). Factorial ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

There was no statistical difference observed between 2-day old flies and 5-day old flies in regard to total time on food. The 2-day old flies (M = 36.69 min) spent approximately the same amount of time on the food when compared to 5-day old flies (M = 32.64 min) (Figure 32).

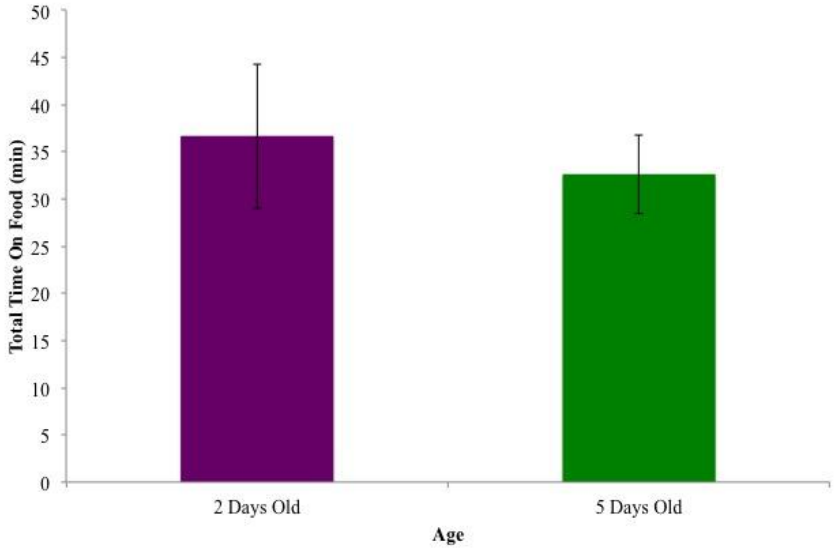


Figure 32. Age showed no significant effect in the total time of food of *Drosophila*. The 2-day old flies (n = 18) spent approximately the same amount of time on the food compared to 5-day old flies (n = 54). Factorial ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

Age showed a statistical difference in the average number of lunges seen per minute on food between 2-day old flies and 5-day old flies, as 5-day old flies (M = 3.84) lunged significantly more per minute than 2-day old flies (M = .78) (Figure 33).

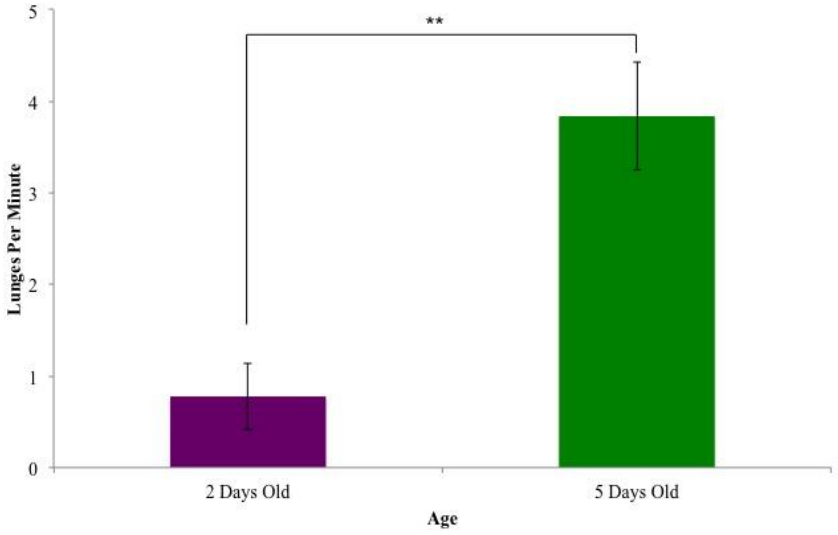


Figure 33. Age showed a significant increase of lunges per minute on food in 5-day old *Drosophila*. More lunges per minute on food were observed in 5-day old flies (n = 54) in comparison to 2-day old flies (n = 18). Factorial ANOVA, ** $p < .01$; LSD Post Hoc Test. Error bars represent ± SEM. All flies were males.

4.2 Main Effect of Age & Fly Type

In order to determine if there was an effect of age and fly type on aggression in all flies, we compared the means of each dependent variable between corresponding fly types of 2-day old and 5-day old flies. There were a total of 72 fights compared in this statistical analysis.

Age and fly type, together, did not show a significant difference in the lunges of 2-day old flies (M = 19.06) and 5-day old flies (M = 68.67) (Figure 34).

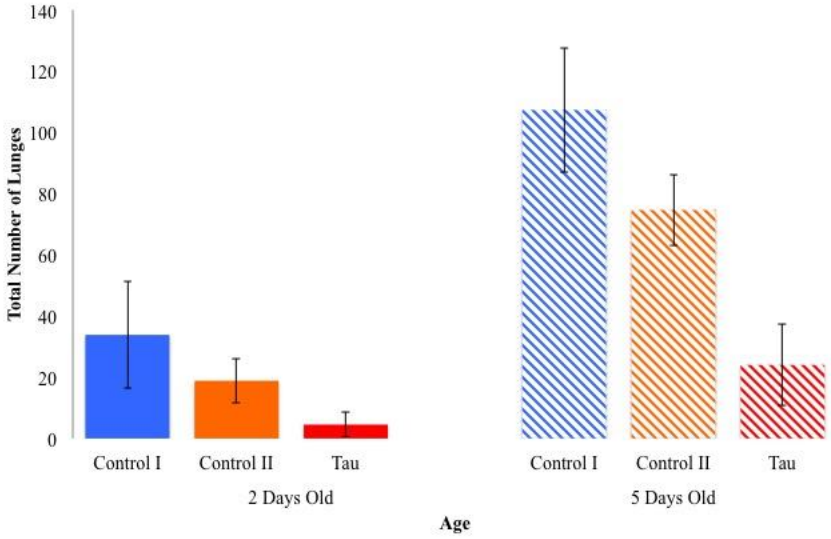


Figure 34. Age and fly type showed no significant effect in the number of lunges of *Drosophila*. Age and condition do not interact as a main effect in the number of lunges between all groups (n = 72). Factorial ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

In order to determine if there was an effect of age and fly type on the time it took to initiate the first aggressive act, we compared the time the first lunge was observed in each fight between all groups. Fights that had no lunges could not be measured for latency to first lunge, and as a result, there were 56 fights included in this analysis.

Age and fly type, together, did not show a significant difference in the latencies to first lunge between fly types or 2-day old (M = 31.22 min) and 5-day old flies (M = 29.76 min) (Figure 35).

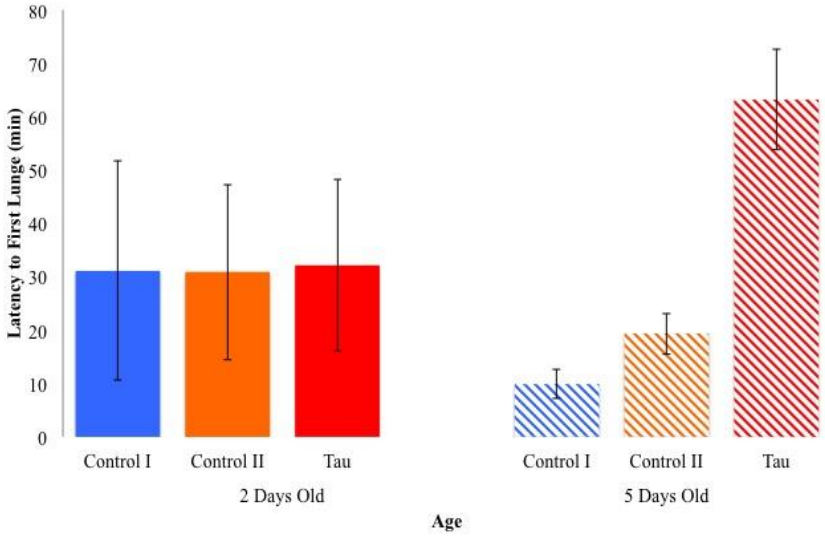


Figure 35. Age and fly type showed no significant effect on the latency to first lunge of *Drosophila*. Age and condition do not interact as a main effect in the time it takes to initiate aggression between all groups (n = 72). Factorial ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

Age and fly type, together, show no statistical difference observed between 2-day old flies (M = 36.69) and 5-day old flies (M = 32.64) in regard to total time on food (Figure 36).

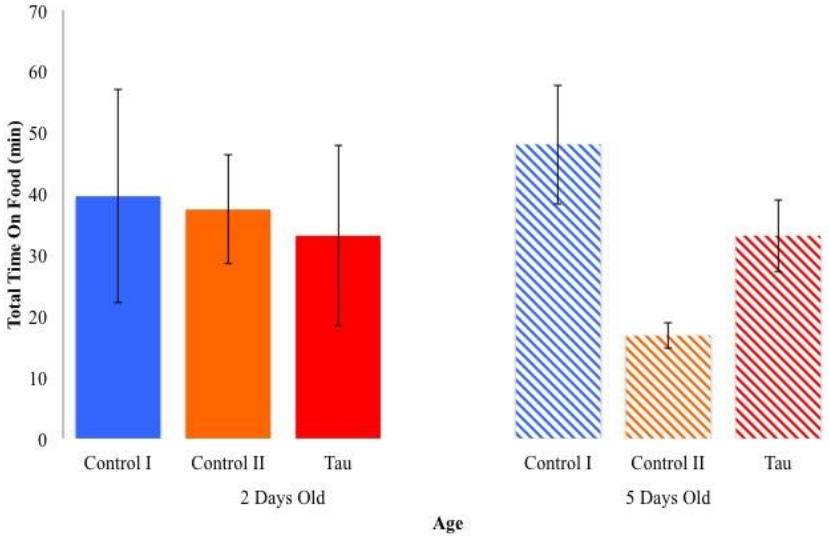


Figure 36. Age and fly type showed no significant effect on the total time on food of *Drosophila*. Age and condition do not interact as a main effect in the total time on food spent between all groups (n = 72). Factorial ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

Age and fly type, together, showed no statistical difference in the average number of lunges seen per minute on food between 2-day old flies (M = .78) and 5-day old flies (M = 3.84) (Figure 37).

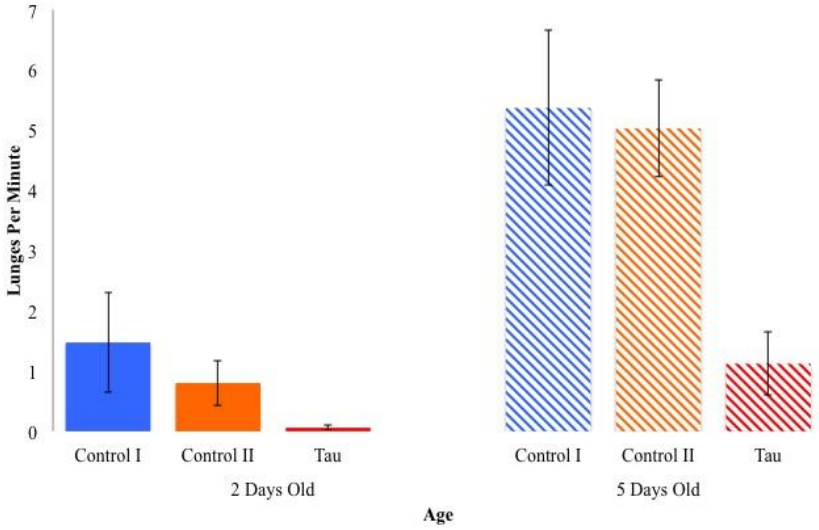
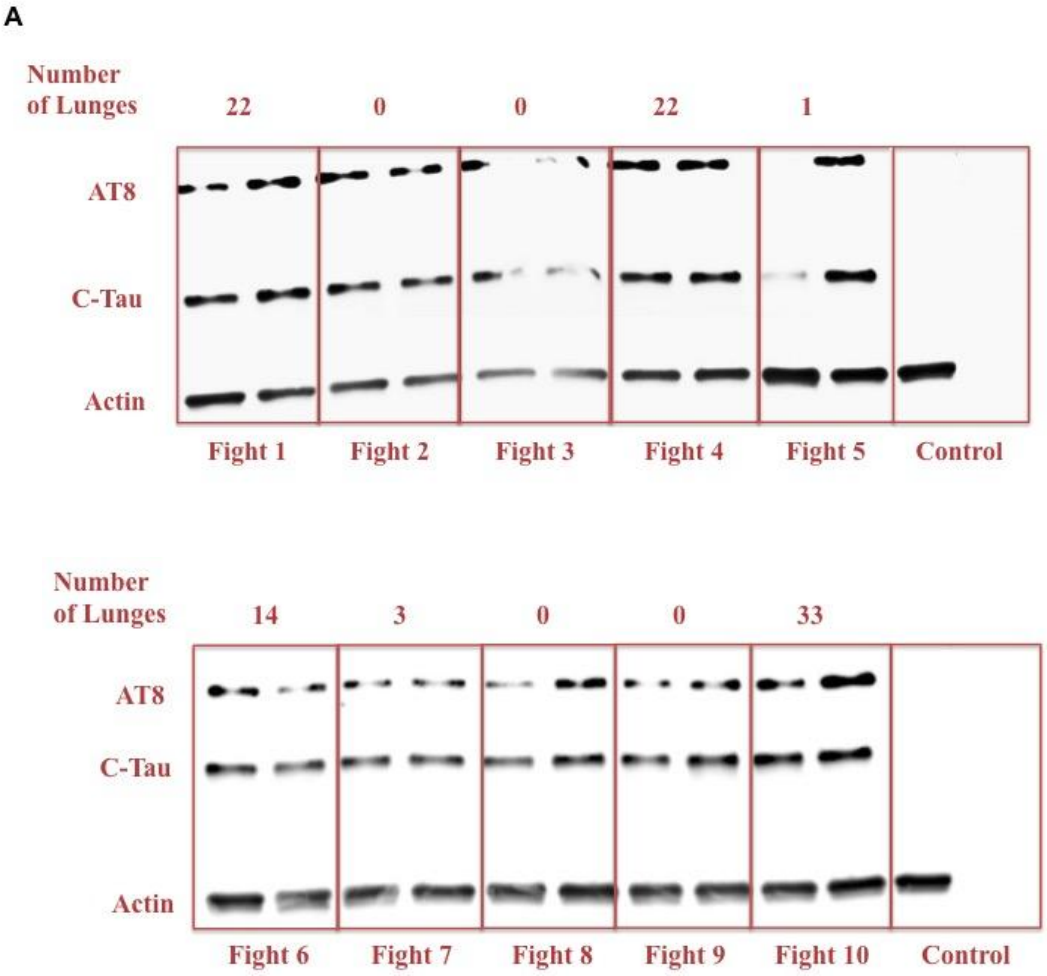


Figure 37. Age and fly type showed no significant effect on the lunges per minute on food in *Drosophila*. Age and condition do not interact as a main effect in the lunges per minute on food observed between all groups (n = 72). Factorial ANOVA, $p > .05$. Error bars represent ± SEM. All flies were males.

5. Comparing Tau Levels & Aggressive Behavior

To quantify the levels of total tau and phosphorylated tau in our 5-day old flies, we performed Western blot analysis on 10 sets of tau fighting pairs. Densitometric analysis using C-Tau, and AT8 antibodies was used to quantify total tau expression, phosphorylated tau expression, respectively. Actin was used as our loading control (Figure 38A). To determine if there was a correlation between tau expression levels and the number of lunges in 5-day old flies, we ran a Pearson correlation between total number of lunges per fight and the relative levels of total and phosphorylated tau. There was no correlation found between the total number of lunges and the total tau (C-Tau) levels in flies (Figure 38B). In

addition, there was no correlation in the total number of lunges and phosphorylated tau (AT8).



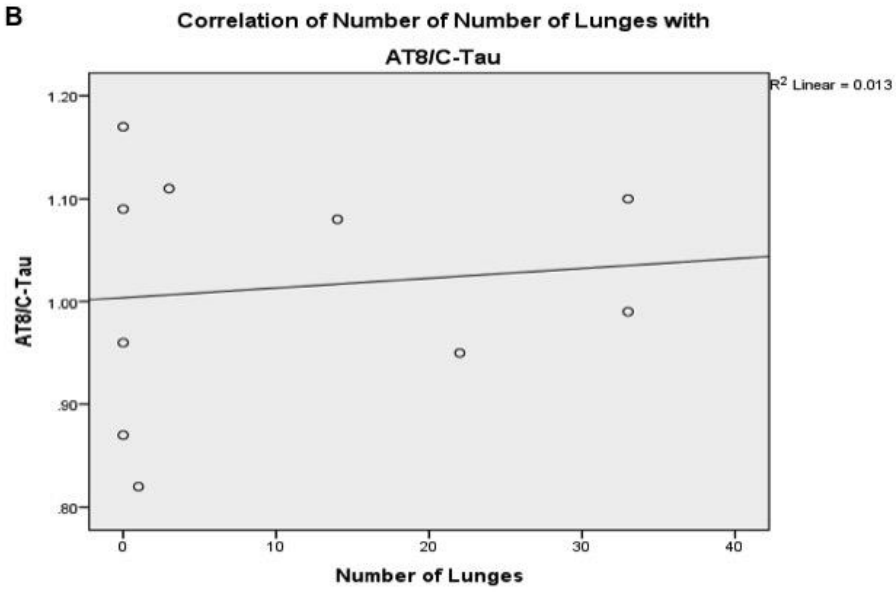


Figure 38. No correlation found between tau levels and number of lunges in 5-day old *Drosophila.* A) Means of adjusted phosphorylated tau (AT8 antibody) and total tau (C-Tau antibody) ratios with their corresponding number of lunges per fight showed no significant relationship. **B)** Pearson Correlation, $r(8) = 0.116$, $p > 0.05$. There were 20 tau male flies and 2 control male flies.

6. Inter-Rater Reliability

In order to determine inter-rater reliability between the two raters in this study, a Bland-Altman was performed in order to assess if there was a level of agreement between a subset of scores. The two dependent variables compared in this analysis were number of lunges and latency to first lunge. The mean differences of each dependent variable of 5-day old flies were compared between raters. Both analyses showed a proportional bias between the sets of scores, however, a level of agreement between the two raters was met, as all scores fell between ±2 standard deviations (SD) from the mean (Figure 39).

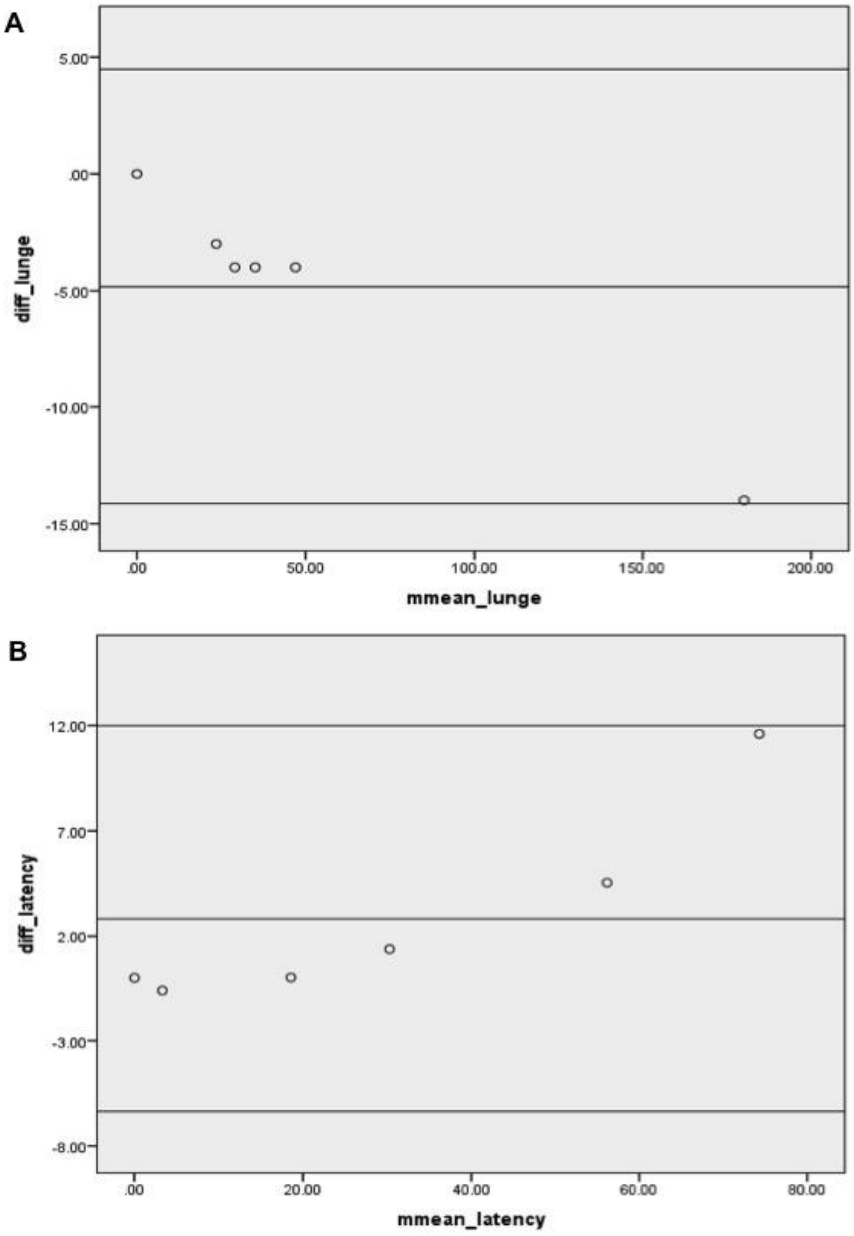


Figure 39. Bland-Altman limits of agreement plots showed a high consensus among raters 1 and 2. Pairwise analysis of inter-rater reliability between raters 1 and 2 reporting **A)** lunges (n = 6) and **B)** latency (n = 6) to first lunge showed that 100% of all data points fell within ±2SD of the mean difference.

DISCUSSION

1. Review of Results

In order to determine whether neuronal tau expression alters aggression in a *Drosophila* model of AD, we quantified and compared aggressive behavior in 2-day old or 5-day old tau transgenic flies and control transgenic flies. We hypothesized that there would be a difference in the aggression levels of tau transgenic flies and control tau transgenic flies. Secondly, we hypothesized that tau transgenic flies would exhibit higher aggression levels than control transgenic flies, and that a correlation would be seen between tau and aggression levels in 5-day old tau transgenic flies.

In support of our first hypothesis, our results revealed that neuronal tau expression had an effect on the aggressive behavior of tau transgenic flies, but contrary to our second hypothesis, a reduction of aggressive behavior was observed in 5-day old tau transgenic flies compared to control tau transgenic flies. This is not consistent with the effect reported in clinical studies and rodent models, as the presence of hyperphosphorylated tau and NFTs correlate with higher aggression levels of AD patients (Guadagna et al., 2012; Arrigada et al., 1992), and mice with AD pathology exhibit increased levels of aggression (Van der Jeugd et al., 2013; Vloeberghs et al., 2006). Furthermore, this study revealed using Western blot analysis that there was no correlation between the hyperphosphorylated or total tau levels and aggression levels in the tau transgenic

flies. Although the effects were different between our *Drosophila* model of AD and what has been reported in clinical and rodent studies of AD, applying what is known from aggression studies and AD pathological studies with *Drosophila* will elucidate how tau may exert its effects on fly aggression.

In *Drosophila* models of AD, neuronal death following the expression of human wild-type tau (0N, 4R) has been reported in cholinergic neurons, sensory neurons and mushroom bodies (Yeh et al., 2010; Williams et al., 2000; Wittmann et al., 2001; Kosmidis et al., 2010). This toxicity appears to be a general feature of tau overexpression, as neurodegeneration has been reported following the expression of bovine, rodent or the overexpression of *Drosophila* tau (Chen et al., 2007; Williams et al., 2000; Kosmidis et al., 2010). Thus, tau overexpression or misexpression is toxic regardless of the host species from which it is harvested. Based on these findings, tau overexpression is causing neuronal death in our tau transgenic flies. While a cellular mechanism by which high tau expression is toxic has not been established, studies suggest an imbalance in the normal tau isoform ratios following the expression of exogenous tau or tau phosphorylation can, independently or in conjunction, cause neurodegeneration (Cowan et al., 2011). In our *Drosophila* model of AD, increases of tau phosphorylation at AT8 and AT100 sites occur, mediating tau toxicity in neurons (Wittmann et al., 2001). Our Western blot analyses confirmed the presence of hyperphosphorylated tau at the AT8 sites of our tau transgenic flies, implying phosphorylation of tau was occurring and may be a source of neurodegeneration in our model. While studies

have shown that tau phosphorylation tightly correlates with degenerating neurons (Nishimura et al., 2004), our study found no correlation between the tau phosphorylation levels (AT8) or total tau levels (C-Tau) and aggression levels of our 5-day old tau transgenic flies, as measured by the total number of lunges. Interestingly, the reduction in aggression levels was present in 5-day old tau transgenic flies, whereas 2-day old tau transgenic flies were unaffected. This is consistent with a study utilizing a glial model, in which human wild-type tau (0N, 4R) is expressed, that determined there was an age-dependent increase in tau phosphorylation at AT8 and AT100 sites, increasing insoluble tau and glial tangles and apoptotic cell death (Colodner and Feany, 2010). It is possible that the lack of an effect on aggression observed in 2-day old tau transgenic flies and control transgenic flies is due to reduced levels of toxic phosphorylated tau. In contrast, 5-day old tau transgenic flies, should have increased levels of tau phosphorylation, and that subsequence neuronal death can cause the observed effect on aggression.

To understand this decrease effect on aggression, one must get a sense of where neurodegeneration occurs in the brains of humans and *Drosophila*. In AD patients, the LC is found degenerated (Weinshenker, 2008), and as a result, NA pathway reduction has been implicated to be a part of the disease progression (Francis et al., 1985; Palmer et al., 1987). Furthermore, NA, a neuromodulator of aggression, is synthesized in the LC and released as a neurotransmitter from noradrenergic neurons at several CNS regions, such as the frontal cortex,

thalamus, hypothalamus, cerebellum, and the limbic system (Marien et al., 2004). Thus, AD pathology impairs NA neurons and causes a reduction of NA content, which can lead to many BSPD, including aggression. In male and female *Drosophila*, a reduction of OA, the fly homolog of NA, has been shown to decrease in aggression, as *Drosophila* mutants lacking OA do not initiate aggression (Zhou et al., 2008; Hoyer et al., 2008). While there is no study that has developed a *Drosophila* model of neuronal tauopathy with the intention to study aggression, this study aimed to clear the gap between what we know about aggression in *Drosophila* and tauopathies, like AD. In our 5-day old data, tau transgenic flies lunged less, were slower to initiate the first aggressive act, spent more time on the food, and lunged less per minute on the food, in comparison to both control transgenic flies. It is important to note that there are 120 octopaminergic neurons in the fly brain with clusters located in regions of the protocerebrum, fan-shaped body, central complex, optic lobes, subesophageal ganglion (SOG) and antennal lobe (Potter and Luo, 2008). In addition, specific OA and DA receptors are expressed in the mushroom bodies of *Drosophila* (Baier et al., 2002). Overexpression of human wild-type tau (0N, 4R) has been shown to ablate the mushroom bodies in adult fly brains (Kosmidis et al., 2010), which suggests a link between tau-mediated toxicity and the control of aggression by mushroom body (Cowan et al., 2011). Studies have also shown that interruptions in mushroom body output can altogether eliminate aggression, however there are no findings about the genes that influence aggressive behavior (Braier et al., 2002;

Edwards et al., 2009). Thus, a reduction in OA mediated by tau-toxicity may be implicated in our 5-day old tau transgenic flies. Moreover, while the effect of tau expression on aggression is divergent between humans and *Drosophila*, it is possible that tau expression alters aggression through the degeneration of NA/OA neurons, and reduction of the NA/OA neurotransmitter in the CNS/mushroom bodies.

2. Limitations and Future Directions

While this study displayed compelling evidence on the effect of tau toxicity on neurons and aggression in *Drosophila*, a series of sophisticated experiments need to be carried out in order to properly confirm these findings. First, while there was a reduction in aggression levels of 5-day old tau transgenic flies, there are other factors that should be considered. For example, the tau transgenic flies could be moving less and showing less aggression because they are lethargic or less hungry than control transgenic flies. In order to determine if this effect can be attributed to the flies being lethargic, a series of locomotion experiments can be carried out with all of our genotypes to confirm its effect. For example, a climbing assay experiment can be implemented, where a few flies from each genotype are placed in an individual vial with a line drawn across the top of it, and the flies will be timed and observed to see how many will reach the top of the line, which will be used as an indicator of activity. In order to discern if this study's effect can be attributed to the flies being hungry, a series of respirometry experiments can be

carried out with all of our genotypes that will measure the CO_2 output and O_2 input to calculate the metabolic rate of each genotype.

To establish if specific subpopulations of neurons are mediating the reduced aggression observed in tau transgenic flies, additional aggression assays should be performed with flies expressing tau in a subset of octopaminergic neurons. Recently, a study using the TDC2-GAL4 driver, which expresses tyrosine decarboxylase (TDC) in OA neurons of the antennal lobe and subesophageal ganglion, fully restored the wild-type aggression levels of tyramine-β-hydroxylase (TβH) mutants (Potter and Luo, 2008). This study was able to identify two populations of OA neurons that are the primary sources for the modulation of aggression. The next steps would be to replicate this study using this driver that will express tau in these specific OA neurons and observe if it yields similar results to our preliminary findings.

CONCLUSION

While there are multiple experiments that need to be performed to verify and strengthen the findings of this investigation, this study found that the expression of human wild-type tau in *Drosophila* neurons significantly reduced aggression, though the role of tau phosphorylation in mediating this effect remains unclear. Future experiments investigating whether tau-induced degeneration of OA brain regions promotes the reduced aggression in the fly should be informative.

This is the first study to use a *Drosophila* model of AD to investigate the effect of neuronal tau on aggression, and the first to link *Drosophila* aggression studies to tauopathies, like AD. Though the reason why AD patients are more aggressive is still unclear, these findings shed light on the significance of tau toxicity and aggression, and can potentially aid in the development of therapies and treatments for AD and related tauopathies.

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