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Birdy Newman 5/14/25

Effect of Tau Expression on Subperineurial Glia in *Drosophila*

by

Birdy Newman

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ABSTRACT

Tauopathies, neurodegenerative disorders exhibiting symptoms such as dementia and motor deficits, are characterized by pathological aggregates of a microtubule-associated protein called tau. While prior research has focused largely on the ramifications of neuronal tau pathology, tau pathology also affects glial cells, a diverse group of non-neuronal cells that play key roles in the nervous system. *Drosophila melanogaster* (fruit flies) have been used to effectively model glial tauopathy. However, the specific effect of tau expression on subperineurial glia (SPG), a glial cell subtype involved in the fly's blood-brain barrier and sleep patterns, remained unknown. This study transgenically expressed human tau in SPG to investigate how the presence of human tau affects SPG nuclear quantity and morphology in the fly brain. Tau was found to decrease the number of SPG nuclei in young male flies and decrease SPG nuclear sphericity in general. The newfound age- and sex-dependent toxicity of human tau to *Drosophila* SPG contributes to the growing base of knowledge about glial tauopathy that will lay the foundation for innovative tauopathy treatments.

INTRODUCTION

1. Human Tau

In the mid-1970s, a team of researchers at Princeton University discovered a protein that would change the future of neuroscience (Weingarten et al., 1975). These researchers were interested in how microtubules—the hollow, filamentous protein structures involved in crucial cellular functions such as vesicular transport and mitosis—undergo rapid assembly during dynamic cellular processes. Accordingly, they isolated a protein from porcine brains that has a role in microtubule assembly, which they called tau. A decade later, tau made it into the limelight when a team of researchers based at the New York State Institute for Basic Research in Developmental Disabilities uncovered its role in Alzheimer's disease (AD) pathology (Iqbal & Grundke-Iqbal, 2006). Firstly, these researchers identified tau as a major component of a type of AD-associated neuronal abnormality termed paired helical filaments (Grundke-Iqbal et al., 1986a). Additionally, they found that tau is abnormally phosphorylated in the brains of AD patients, meaning that it has been covered with additional phosphate groups not found on tau in healthy brains (Grundke-Iqbal et al., 1986b). Finally, they suggested that pathological tau causes defects in microtubule assembly in AD (Iqbal et al., 1986). Collectively, these studies provided early evidence for the role of tau pathology in neurodegeneration. In the years since, researchers have identified an entire class of neurodegenerative disorders characterized by the aggregation of hyperphosphorylated tau, termed tauopathies (Cummings et al., 2023; Kahlson & Colodner, 2015). Tauopathy research is now a thriving subfield of neuroscience.

In order to fully understand the role of tau in pathological states, it is helpful to begin by understanding how the protein functions in the healthy brain. Tau is expressed predominantly in neurons, where it plays a valuable role in microtubule assembly and stabilization as well as axonal transport, kinase regulation, DNA integrity, and regulation of NMDA receptor signaling (Tapia-Rojas et al., 2019). The protein is encoded by a gene on chromosome 17 known as *MAPT*, which functions as a template for the synthesis of tau mRNA (Neve et al., 1986; Tacik et al., 2015). This mRNA recipe contains exons that can be spliced together in different combinations, which are then translated into six different isoforms of the tau protein (Tapia-Rojas et al., 2019).

These isoforms are divided into two major classes: 3R tau, which has three microtubule-binding repeats, and 4R tau, which has four. After being translated into a protein, tau can undergo posttranslational modifications such as phosphorylation (addition of a phosphate group), prolyl-isomerization (alteration of bond structure), and ubiquitination (addition of a ubiquitin protein). These modifications impact tau's ability to bind to microtubules as well as its tendencies to degrade, aggregate, and undergo additional posttranslational modifications. Ubiquitination, for example, is involved in the degradation of tau because it tags the protein to be cleaved by specialized cylindrical complexes called proteasomes. Phosphorylation, on the other hand, may reduce the degradation of tau by enzyme-filled lysosomes (Caballero et al., 2018; Tapia-Rojas et al., 2019).

While tau plays a valuable role in the healthy human brain, its abnormal function in tauopathies presents a serious risk to nervous system function. These neurodegenerative disorders are caused by diverse factors such as genetic mutations, infections, and head trauma, but they share one key pathological hallmark: the buildup of hyperphosphorylated tau (Creekmore et al., 2024). Researchers have only begun to understand the mechanisms of tau pathology. According to experiments in transgenic mice and cultured neurons, hyperphosphorylation directs tau to synapse-associated protrusions called dendritic spines, where it impairs synaptic function by disrupting the proper recruitment of glutamate receptors (Hoover et al., 2010). Hyperphosphorylation additionally reduces tau's affinity for microtubules, leading to the destabilization of the neuronal cytoskeleton and encouraging tau aggregation (Guo et al., 2017). The resulting tau aggregates can propagate throughout the central nervous system, where they sequester molecular structures involved in normal protein turnover and thus disrupt each cell's ability to maintain a balanced collection of proteins (Creekmore et al., 2024). Synaptic dysfunction, cytoskeletal destabilization, and impaired proteostasis represent three components of the complex pathways involved in tauopathy pathogenesis.

While the mechanisms of tau pathology have been best characterized in neurons, glial cells are also implicated in tauopathy pathogenesis (Kahlson & Colodner, 2015). Glial cells, initially understood to serve a purely structural and supportive role in neuronal survival, turn out to perform a range of critical functions in the human nervous system (Magni et al., 2024). For

example, they are involved in cell number regulation, neuronal differentiation and migration, synaptic communication and plasticity, and ion homeostasis (Allen & Lyons, 2018). There are different subtypes of glia to perform these diverse functions. Thus, the glial cells of the vertebrate central nervous system (CNS) are divided into five major categories based on their distinct morphologies and functions: astrocytes, oligodendrocytes, microglia, ependymal cells, and radial glia (Rasband, 2016). Tau pathology is seen in astrocytes, oligodendrocytes, and microglia (Kahlson & Colodner, 2015). Thus, it is worth taking a closer look at these three categories of cells.

2. Human Glial Subtypes in Health and Disease

Astrocytes are the most numerous glial cell subtype in the CNS (Rasband, 2016). These cells, whose fine processes interact with both neurons and blood vessels, perform a variety of important functions. For example, they help maintain homeostasis within the nervous system by regulating properties such as local blood flow, brain volume, and extracellular potassium and glutamate concentrations (Magni et al., 2024; Rasband, 2016). They also contribute to metabolism by providing surrounding neurons with lactate to maintain their activity and survival; this process is known as the astrocyte-neuron lactate shuttle (Roumes et al., 2023). Astrocytes not only support individual neurons but also play a role in the development and modulation of interneuronal connections called synapses, which suggests that they are involved in cognitive activities such as learning and memory (Rasband, 2016). Additionally, astrocytes contribute to waste clearance in the brain and inflammatory responses to damage (Magni et al., 2024). In sum, they are incredibly versatile cells that support diverse aspects of proper nervous system function.

Functionally, oligodendrocytes are more easily defined than astrocytes. Their primary role is to wrap neuronal axons in a fatty substance called myelin, which provides insulation to speed up the transmission of electrical signals. Only some axons are myelinated, resulting in a selective increase in conduction velocity that contributes to the precise timing of interneuronal communication (Allen & Lyons, 2018; Simons et al., 2024). This system is highly dynamic: Myelin undergoes structural changes in response to life experience and neuronal activity that in turn functionally alter neuronal circuits (Simons et al., 2024). The dynamism of oligodendrocytes

is particularly important when myelin is damaged. Research in mice demonstrates that in response to myelin degeneration, oligodendrocyte precursor cells differentiate into functional oligodendrocytes to rapidly remyelinate axons (Chapman et al., 2023). However, this reparative response does not occur in older mice, suggesting that myelin plasticity decreases with advanced age.

Microglia too exhibit a diminished response to damage with aging (Hefendehl et al., 2014). This is significant given their generally dramatic response to injury. By default, microglia are highly branched cells, extending long processes to monitor the environment within the brain (Colonna & Butovsky, 2017). However, upon detection of severe damage, microglia undergo cell body enlargement and process shrinkage to take on a more blob-like shape. This latter morphology is associated with phagocytic activity in which microglia ingest bacteria, dying cells, and protein aggregates (Colonna & Butovsky, 2017; Levtova et al., 2017). As phagocytes, microglia also contribute to synaptic pruning by engulfing key components of unnecessary synapses (Colonna & Butovsky, 2017). Outside of their role as the brain's resident garbage collectors, they secrete chemical signals such as ROS and BDNF that alter synaptic activity and thus further contribute to synaptic plasticity.

Astrocytes, oligodendrocytes, and microglia are crucial to nervous system function, from efficient interneuronal communication to recovery from injury. Accordingly, disruption to the proper functioning of these glial cells is associated with a range of pathological conditions. For instance, demyelination of axons is a major pathogenic event in multiple sclerosis, which leaves patients managing symptoms such as limb weakness, incontinence, and electric-shock-like pain (Brownlee et al., 2017; Reich et al., 2018). Additionally, imbalanced astrocytic glycogen metabolism may be an important mechanistic factor linking depression, sleep disturbances, and migraine (Petit et al., 2021). Finally, these three cell types are implicated in tauopathy pathogenesis: Tau pathology leads to functional deficits in astrocytes and oligodendrocytes, while microglia contribute to the propagation of tau pathology throughout the brain (Kahlson & Colodner, 2015).

3. Human Tauopathies

Given the complex ways in which tau can affect different brain cells, it is unsurprising that tauopathies make up an incredibly diverse collection of disorders. In order to organize their similarities and differences, tauopathies are divided into categories based on their characteristics. The most commonly discussed distinction is between primary tauopathies, in which tau is the main protein abnormality, and secondary tauopathies, in which tau aggregates exist alongside other protein abnormalities (Cummings et al., 2023). Primary tauopathies include Pick's disease, frontotemporal dementia with parkinsonism linked to chromosome 17, and primary age-related tauopathy, among many others. Secondary tauopathies include AD and Down syndrome. Interestingly, recent research has suggested that long Covid, which is associated with impaired cognition known as brain fog, may function as a (presumably secondary) tauopathy (Sfera et al., 2023). In addition to the primary/secondary distinction, tauopathies are distinguished based on whether they are associated with a clear genetic cause, which isoforms of tau are preferentially expressed, and the ratio of neuronal tau aggregates to glial tau aggregates (Creekmore et al., 2024; Cummings et al., 2023; Kahlson & Colodner, 2015).

This study focuses on primary sporadic 4R tauopathies with prominent glial tau pathology. Notable disorders in this category include progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease, and aging-related tau astrogliopathy (Creekmore et al., 2024; Cummings et al., 2023; Ikeda et al., 2018; Kahlson & Colodner, 2015; McCann et al., 2021). A brief review of these four pathologies will highlight the significance of the present investigation.

Progressive supranuclear palsy (PSP) is typically characterized by deficits in motor function and is associated with neuronal, astrocytic, and oligodendrocytic tau pathology (Creekmore et al., 2024; Giagkou et al., 2019). The impact of the disease is difficult to quantify given that estimates of its population prevalence range from 1-18 per 100,000 people (Lyons et al., 2023). The age-specificity of its incidence is better characterized: In a study of 100 patients with autopsy-confirmed PSP, the average age at onset was 65.2, and the average patient lived to be 73.3 (Respondek et al., 2014). Patients with PSP can experience a variety of clinical manifestations. The classic presentation is Richardson's syndrome (PSP-RS), characterized by

impaired eye movements accompanied by symptoms such as balance issues, behavioral changes, executive dysfunction, impaired speech, and slowed movement (Boxer et al., 2017; Giagkou et al., 2019). However, PSP can take on other phenotypes such as PSP-parkinsonism and PSP with progressive gait freezing (Boxer et al., 2017). Which clinical phenotype arises depends in part on the anatomical location of tau pathology (Giagkou et al., 2019). One symptom widely present across various manifestations of PSP is pseudobulbar palsy, or difficulty speaking and swallowing.

Like PSP, corticobasal degeneration (CBD) is associated with neuronal, astrocytic, and oligodendrocytic tau pathology (Saranza et al., 2019; Figure 1). Research indicates that astrocytic tau pathology precedes neuronal tau pathology in CBD, suggesting an important role for astrocytes during early disease progression (Ling et al., 2016; Saranza et al., 2019). Clinically, CBD can result in cognitive impairment, changes in behavior and speech, difficulty with purposeful limb movements, abnormal eye movements, and motor symptoms such as limb rigidity, slowed movement, and balance issues (Armstrong et al., 2013; Saranza et al., 2019). On average, patients live just 6.6 years with the condition before death, and the mean age at onset is 63.5 (Saranza et al., 2019). Fortunately, CBD is a rare disorder. However, it may be underrecognized in the elderly population (Kovacs et al., 2013; Saranza et al., 2019).

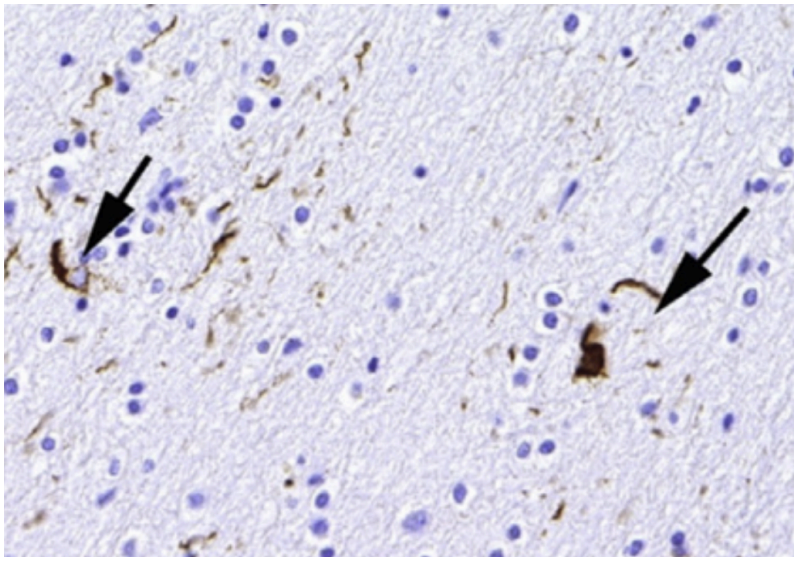

Figure 1. Oligodendrocytic tau inclusions in CBD. Coiled bodies (black arrows) are a form of oligodendrocytic tau inclusion observed in CBD patients. Adapted from Saranza et al., 2019.

Unlike CBD and PSP, argyrophilic grain disease (AGD) is not associated with prominent motor symptoms (Das & Ishaque, 2018). Common symptoms instead include slowly progressive cognitive decline and irritability. In fact, among neurodegenerative diseases, AGD is considered the second most common cause of dementia in elderly patients (after Alzheimer's disease). The disease can also be associated with psychiatric manifestations such as depression and bipolar disorder (Das & Ishaque, 2018; Shioya et al., 2015). Alternatively, AGD can present without any obvious symptoms (Das & Ishaque, 2018). Due to its nebulous symptomatology, AGD can be definitely diagnosed only after death. Its eponymous neuropathological features are argyrophilic grains, which are small, spindle-shaped lesions with an affinity for silver stains located in neuronal processes (Braak & Braak, 1998; Das & Ishaque, 2018). However, the disease is also associated with oligodendrocytic tau pathology (Das & Ishaque, 2018).

The final primary sporadic 4R tauopathy to be reviewed here is aging-related tau astrogliopathy (ARTAG), a neuropathology found primarily in people over 60 years old (Kovacs et al., 2016; McCann et al., 2021). As the name suggests, ARTAG is associated with prominent astrocytic tau pathology (McCann et al., 2021). There are two conflicting hypotheses describing how tau propagates in ARTAG. According to one hypothesis, astrocytes take up tau released by neurons, while the other hypothesis suggests that astrocytes express their own tau, which is then transmitted to other cells. Given that ARTAG was only identified in 2016, this is not the only area of uncertainty regarding the disorder (Kovacs et al., 2016; McCann et al., 2021). It is also unclear whether ARTAG is associated with clinical manifestations (McCann et al., 2021). The results of a study of 185 individuals aged 90 or older indicate that ARTAG may be associated with dementia only when it manifests in particular brain regions (Robinson et al., 2018). Additional research suggests an association between ARTAG and dysfunction of the blood-brain barrier (McCann et al., 2021). Evidence for the clinical relevance of ARTAG itself remains slim; however, neuroscientists have suggested that the disorder may be a precursor to other tauopathies.

The primary sporadic 4R tauopathies with prominent glial tau pathology described above, with the possible exception of ARTAG, can have a drastic negative impact on the lives of affected individuals. This is of particular concern given the absence of approved treatments

(Feldman et al., 2024). Efforts to develop drugs or other interventions to improve patient outcomes will benefit from a strengthened understanding of the mechanisms underlying tauopathy pathogenesis, including the role of glial cells.

4. Modeling Tau Pathology

Mammalian Models of Glial Tau Pathology

Much of the evidence for the adverse effects of glial tau pathology comes from mouse models. In a transgenic mouse model engineered to express human tau in oligodendrocytes, researchers found fine structural alterations in myelin, impaired axonal transport, and axonal degeneration (Higuchi et al., 2005). Other researchers used a neuronal tau knockdown mouse model to show that oligodendrocytic tau can propagate throughout the brain over time even in the near absence of neuronal tau pathology (Narasimhan et al., 2020). Microglia also play a role in the spread of tau between brain regions, as indicated by a study using a mouse model of rapid tau propagation (Asai et al., 2015). As for astrocytes, a transgenic mouse model of astrocytic tau pathology demonstrated neuronal degeneration and mild blood-brain barrier disruption in the same regions as astrocytic tau pathology (Forman et al., 2005). Collectively, these studies have leveraged mouse models to build a strong case for a glial contribution to tauopathy pathogenesis.

Work in mammalian cell culture models has provided further insight into the complex relationship between tau toxicity and glial cells. One study analyzed the ratio of 4R:3R tau in astrocytes and neurons derived from induced pluripotent stem cells from carriers of the 10+16 intronic mutation in *MAPT*, which causes frontotemporal lobar degeneration via increased expression of 4R tau (Setó-Salvia et al., 2022). Results revealed that the 4R:3R ratio was elevated—skewed toward 4R tau—in both astrocytes and neurons bearing the mutation compared to healthy control cells. However, the elevated 4R:3R tau ratio decreased over time in mutant neurons, while it was maintained in mutant astrocytes, suggesting a potential astrocytic contribution to the pathogenesis of frontotemporal lobar degeneration. Another study examined the effect of tau expression on astrocytic cells derived from a rat brain tumor (Benda et al., 1968; Galland et al., 2019; Torres-Cruz et al., 2016). Morphological analysis revealed that tau expression led to bulging of the plasma membrane as well as changes in the distribution of

filamentous actin, a component of the cytoskeleton (Torres-Cruz et al., 2016). While these changes were not associated with any significant cell death, the authors argue that they may contribute to neurodegenerative processes. Thus, the above studies have leveraged mammalian cell culture models to highlight potential roles of glia in pathways of tau toxicity.

Modeling Tauopathy in Flies

While mammalian systems have contributed a great deal to the mechanistic dissection of tauopathies, the humble fruit fly (*Drosophila melanogaster*) has been comparably impactful in the field. *Drosophila* are incredibly useful in biomedical research. They are relatively easy to genetically manipulate, they reproduce quickly, and they present minimal ethical concerns compared to rodents (Cotterill & Yamaguchi, 2023). Most importantly, findings in fruit flies can be relevant to human health: 85% of genes related to human diseases have homologs in the *Drosophila* genome (Baldridge et al., 2021; Cotterill & Yamaguchi, 2023). Thus, *Drosophila* have been used to study a range of biomedical topics, including immunomodulation, diet, addiction, and tau pathology (Colodner & Feany, 2010; Cotterill & Yamaguchi, 2023).

Drosophila have a gene homologous to human tau (Giong et al., 2021). Accordingly, some genetic constructs used to model tau pathology in flies are designed to alter the expression of fly tau (Gistelinck et al., 2012). Meanwhile, others are built to induce the expression of human or even bovine tau. In total, over 35 genetic constructs have been used to create transgenic fly models of tau pathology. These models have in turn been used to elucidate important aspects of tau toxicity. One study used flies expressing human tau to evaluate the role of the enzyme *tyramine β hydroxylase* in tau toxicity (Nangia et al., 2021). This enzyme is involved in the production of octopamine, the *Drosophila* homolog to human noradrenaline. The researchers found that genetic reduction of *tyramine β hydroxylase* suppresses the pathogenic effects of tau independent of tau phosphorylation. This finding is particularly relevant given the complex relationship between noradrenergic signaling and tau toxicity in humans. In another study, researchers overexpressed different types of mutant human tau to investigate the protein's interaction with the kinase PAR-1, which helped them to better understand the phosphorylation

events involved in tau pathology (Nishimura et al., 2004). In sum, transgenic *Drosophila* have uncovered various components contributing to tau toxicity.

One area in which flies have been particularly useful is the effort to understand aspects of tau toxicity affecting the nucleus. A group of researchers investigated the effect of neuronal 4R tau expression on mitotic processes in *Drosophila* (Malmanche et al., 2017). They found that tau impairs proper allocation of chromosomes to daughter cells, resulting in aneuploidy (extra or missing chromosomes) in postmitotic neurons. Another research team demonstrated that panneuronal expression of mutant human tau leads to increased DNA damage as well as reduced levels of the nuclear membrane protein Lamin (Frost et al., 2016). Flies with a partial loss-of-function *Lamin* mutation (but no human tau expression) replicate this neuronal DNA damage, suggesting that Lamin disruption occurs upstream of DNA damage in tau-expressing flies. These experiments pinpoint the nucleus as a key site of neuronal tau toxicity.

Of great importance to the present study, *Drosophila* have also been used to model glial tau pathology. Fruit flies, like humans, have glial cells. In fact, there is significant overlap in the functions served by glia in flies and in mammals, including neurotransmitter metabolism and ensheathment of neuronal axons (Freeman & Doherty, 2006). Despite this functional similarity, the categorization of glial subtypes in *Drosophila* is quite different from in humans. The glia of the fruit fly CNS are divided into the following five subtypes: perineurial glia, which form the outer layer of the blood-brain barrier; subperineurial glia, which form the inner layer of the blood-brain barrier; cortex glia, which engulf neuronal cell bodies and contribute to neurogenesis; ensheathing glia, which include cells compartmentalizing the brain into distinct regions (neuropil ensheathing glia) and cells ensheathing the neuronal projections running through the brain (tract ensheathing glia); and astrocyte-like glia, which are involved in neurotransmitter homeostasis and phagocytosis (Kremer et al., 2017; Yildirim et al., 2019). Researchers are interested in how tau expression and aggregation might disrupt the ability of these glial cells to carry out their proper functions. By using complex genetic tools to target manipulations to particular *Drosophila* cell types, they have been able to investigate the specific effects of glial tau pathology in flies.

In 2010, a *Drosophila* model of glial tauopathy was developed to express human wild-type tau in the glial cells of adult flies (Colodner & Feany, 2010). This panglial human tau expression led to several pathological effects in flies: development of fibrillary inclusions of phosphorylated tau, similar to human glial tangles; age-dependent apoptosis of both neurons and glia; and reduced lifespan. However, these effects could not be traced to the impact of tau in different subtypes of glial cells. To examine glial subtype-specific effects of tau in *Drosophila*, a previous undergraduate researcher in the Colodner Lab overexpressed human tau in astrocyte-like glia and cortical glia (in separate populations of flies) and measured the effects of tau expression on each subtype's cell count (Kang, 2023). They found that tau expression in cortical glia leads to extensive cell death across sex, while tau expression in astrocyte-like glia leads to an increase in cell count in young female flies. These results demonstrate that glial subtypes respond differently to human tau expression.

5. Subperineurial Glia and the Blood-Brain Barrier

One of the *Drosophila* glial subtypes on which the effects of human tau expression had not been examined prior to this study is subperineurial glia (SPG). These large, flat cells surround the brain just underneath the perineurial glia, forming a tight epithelium of overlapping cells (Kremer et al., 2017; Yildirim et al., 2019). They are small in number, making up roughly 300 cells between the brain and ventral nerve cord (Kremer et al., 2017). Because they can replicate their genetic material without undergoing full cell division, they become polyploid (Yildirim et al., 2019).

This polyploidization is necessary for SPG to perform their most well-known function as part of the *Drosophila* blood-brain barrier (Unhavaithaya & Orr-Weaver, 2012). In humans, the blood-brain barrier (BBB) controls the passage of cells and molecules between the blood and the CNS (Michalicova et al., 2020). Since *Drosophila* do not have blood or blood vessels, the nervous system floats in a fluid called hemolymph (Yildirim et al., 2019). Thus, their BBB divides the nervous system from the hemolymph. SPG establish a paracellular barrier by expressing pleated septate junctions that prevent molecules from traveling through intercellular space (Contreras et al., 2024; Yildirim et al., 2019). Aside from their role in preventing

paracellular diffusion to maintain the BBB, SPG express channels that enable neural stem cell reactivation in response to nutritional signals (Spéder & Brand, 2014). They are also involved in regulating sleep: A recent study found that knockdown of the *kismet* gene in SPG decreases nighttime sleep time in flies (Coll-Tané et al., 2021). In sum, SPG perform a range of key functions in the fly brain.

SPG are relevant in the context of tauopathy research because some of their roles in *Drosophila* correspond to aspects of human brain function that are disrupted in tauopathies: sleep regulation and the BBB. Tauopathy patients frequently struggle with sleep disturbances. In a study of ten patients with lateralized CBD, researchers noted alterations to the sleep/vigilance pattern (Moretti et al., 2005). Another study surveyed the caregivers of 90 patients with suspected PSP with speech and language problems and found that sleep disturbance was present in at least a third of these patients (Hokelekli et al., 2021). Disruptions to the BBB are also common in tauopathies, from capillary surface irregularities in Alzheimer's disease to twisted vessels in Pick's disease (Michalicova et al., 2020). Chronic traumatic encephalopathy (CTE), the neurodegenerative pathology associated with repetitive mild traumatic brain injury, offers a particularly clear connection between tau abnormalities and BBB perturbations: In two separate CTE patients, loss of the tight junction protein claudin-5, which is involved in BBB function, was observed in areas with dense perivascular accumulation of phosphorylated tau (Doherty et al., 2016; Farrell et al., 2019). Leakage of blood components into the brain was also noted. The present study can provide insight into how glial tau pathology might contribute to the development of BBB dysfunction and sleep disturbances.

6. Aim of My Study

The purpose of this study was to examine the effects of human tau on SPG in the *Drosophila* CNS and answer the following research question: How does the presence of human tau in *Drosophila* SPG affect the quantity of these cells in the brain? To answer this question, I used the GAL4/UAS system to transgenically express 0N4R human tau in SPG. I then compared the quantity of SPG nuclei within the brain between experimental tau-expressing flies and control flies not expressing human tau, incorporating fly age and sex into the experimental design and data analysis as additional explanatory variables. Because an age- and sex-dependent

effect of human tau expression has previously been observed in *Drosophila* astrocyte-like glia, it is worthwhile to include these variables when using flies to investigate glial subtype-specific effects of tau expression (Kang, 2023). This may also enhance the specificity of fly models of human tauopathies given the complex impact of age and sex on tauopathy pathogenesis (Gao et al., 1998; Mahale et al., 2022). During the process of experimentation and observation of preliminary data, two additional questions emerged: How does human tau affect SPG nuclear morphology, and how does the dissection process affect SPG integrity? In order to effectively address these questions, I completed two supplementary analyses beyond my original research plan. Ultimately, my study contributes to the growing base of knowledge about glial tauopathy that will in turn lay the foundation for innovative tauopathy treatments.

MATERIALS AND METHODS

1. Genetic Crosses

GAL4/UAS Expression System

Nearly every cell in an organism contains the same genetic code within its nucleus. However, different cell types are morphologically and functionally distinct because they express different genes by transcribing them into mRNA and translating them into functional proteins. A cell's gene expression profile is controlled in part by which transcription factors it contains. Transcription factors are specialized proteins that bind to different promoter sequences, in turn triggering the transcription of different genes. The GAL4/UAS system is a way for researchers to manipulate gene expression in organisms such as *Drosophila* in order to drive cell type-specific expression of exogenous genes.

In yeast (*Saccharomyces cerevisiae*), GAL4 is a transcription factor that binds to a promoter called the upstream activation sequence (UAS) to induce the transcription of genes involved in sugar metabolism (Johnston & Hopper, 1982; Phelps & Brand, 1998). This binary expression system can be translated into other organisms that do not naturally express GAL4 or encode the UAS promoter in order to trigger the expression of genes of interest in specific populations of cells (Jones, 2009). To leverage the GAL4/UAS system in flies, researchers must begin by identifying a genetic promoter naturally activated only within the *Drosophila* cell population of interest as well as a gene that they would like to express in these cells. Next, they generate two lines of flies: one containing the identified promoter followed by the gene encoding GAL4, and one containing the UAS region followed by the gene they would like to express (Brand & Perrimon, 1993; Phelps & Brand, 1998). By crossing these two lines, they can collect fly progeny with both of the above genetic constructs. In these progeny, GAL4 will be expressed only within the cell population of interest, where GAL4 will bind to the UAS region to trigger the expression of the exogenous gene of interest.

Balancer Chromosomes

When researchers create a transgenic fly line, they frequently rely on an inverted, rearranged chromosome known as a balancer (Miller et al., 2019). A balancer prevents crossing over of genetic material between chromosomes so that the entirety of the inserted genetic construct is retained on a single (normal) chromosome. Balancers contain recessive lethal or sterile mutations to avoid the production of flies homozygous for the balancer. They typically also contain dominant visible markers to allow for the identification of flies containing the balancer (Figure 2).

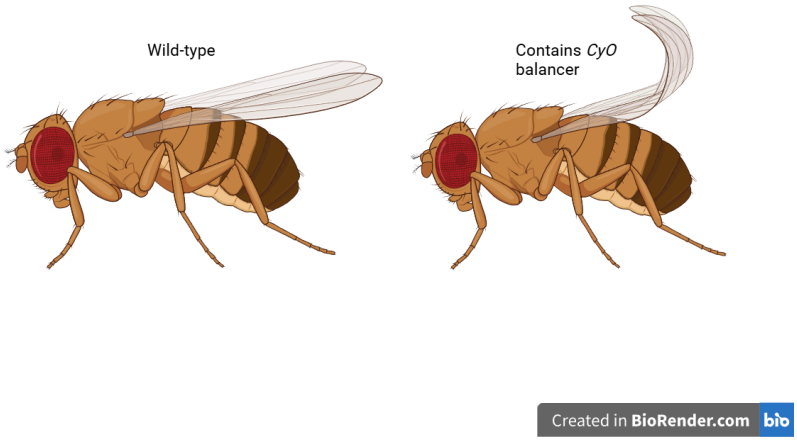

Figure 2. Example of a balancer-associated dominant marker. Wild-type *Drosophila* have flat wings, while those containing the *CyO* balancer have curly wings. Created in BioRender.com

Crosses

The fly genotypes included in this study were generated using the promoter *R54C07* to drive gene expression in subperineurial glia (Kremer et al., 2017). This promoter is associated with the *Mdr65* gene, which encodes a BBB transporter protein that protects the brain from cytotoxic chemicals (Mayer et al., 2009; Pfeiffer et al., 2011). By using *R54C07* to drive the expression of various exogenous genes, three groups of flies were created: a control group, an experimental tau-expressing group, and a GFP-expressing group used for exploratory analysis of SPG sheath integrity.

The control group was created by crossing virgin female white-eyed (*w*-) flies with male flies expressing two genetic constructs: GAL4 under the control of *R54C07*, and histone-bound red fluorescent protein (hisRFP) under the control of UAS. The progeny, described henceforth as

the control genotype, expressed hisRFP in their subperineurial glia (Figure 3). The experimental tau-expressing group was created by crossing virgin female flies expressing human wild-type 0N4R tau under the control of UAS with male flies expressing two genetic constructs: GAL4 under the control of *R54C07*, and hisRFP under the control of UAS (Colodner & Feany, 2010). Some offspring contained the *TM3*, Sb^1 balancer used in the *UAS-tau* line but not the tau construct itself; these were identified and discarded. The rest of the progeny, described henceforth as the tau genotype, expressed both hisRFP and human tau in their subperineurial glia (Figure 4).

Finally, the exploratory group expressing green fluorescent protein (GFP) was created by crossing male flies expressing membrane-tethered GFP under the control of UAS with virgin female flies expressing two constructs: GAL4 under the control of *R54C07*, and hisRFP under the control of UAS (Banerjee et al., 2017). Some offspring contained the *CyO* balancer used in the *UAS-mCD8::GFP* line but not the GFP construct itself; these were identified and discarded. The rest of the progeny, described henceforth as the GFP genotype, expressed both hisRFP and membrane-tethered GFP in their subperineurial glia (Figure 5).

2. Fly Maintenance

To perform a genetic cross, roughly 3-5 flies per sex were combined in a vial containing cornmeal-based food (Nutri-Fly® Bloomington Formulation) prepared with propionic acid and Tegosept, and supplemented with dry yeast. The vial was stored at 25℃, and small amounts of distilled water were added as needed to maintain adequate food consistency over time. No later than seven days after setting up the cross, the parents were removed from the vial, at which point they were either discarded or used to propagate a new cross. Around 10 days after setting up the cross, adult progeny began to eclose. They were collected as needed through 17 calendar days after the cross was established, ensuring the exclusive collection of the first generation of progeny. To enable accurate tracking of progeny age, the vial was always cleared of adult flies no sooner than one calendar day before collection. Upon collection, progeny were placed at 25℃ in a vial containing cornmeal-based food (Nutri-Fly® Bloomington Formulation) prepared with propionic acid and Tegosept. They were flipped into a new vial containing fresh food roughly

every 2-3 days. Progeny were used for dissection on Day 3 (two days following collection) or Day 10 (nine days following collection).

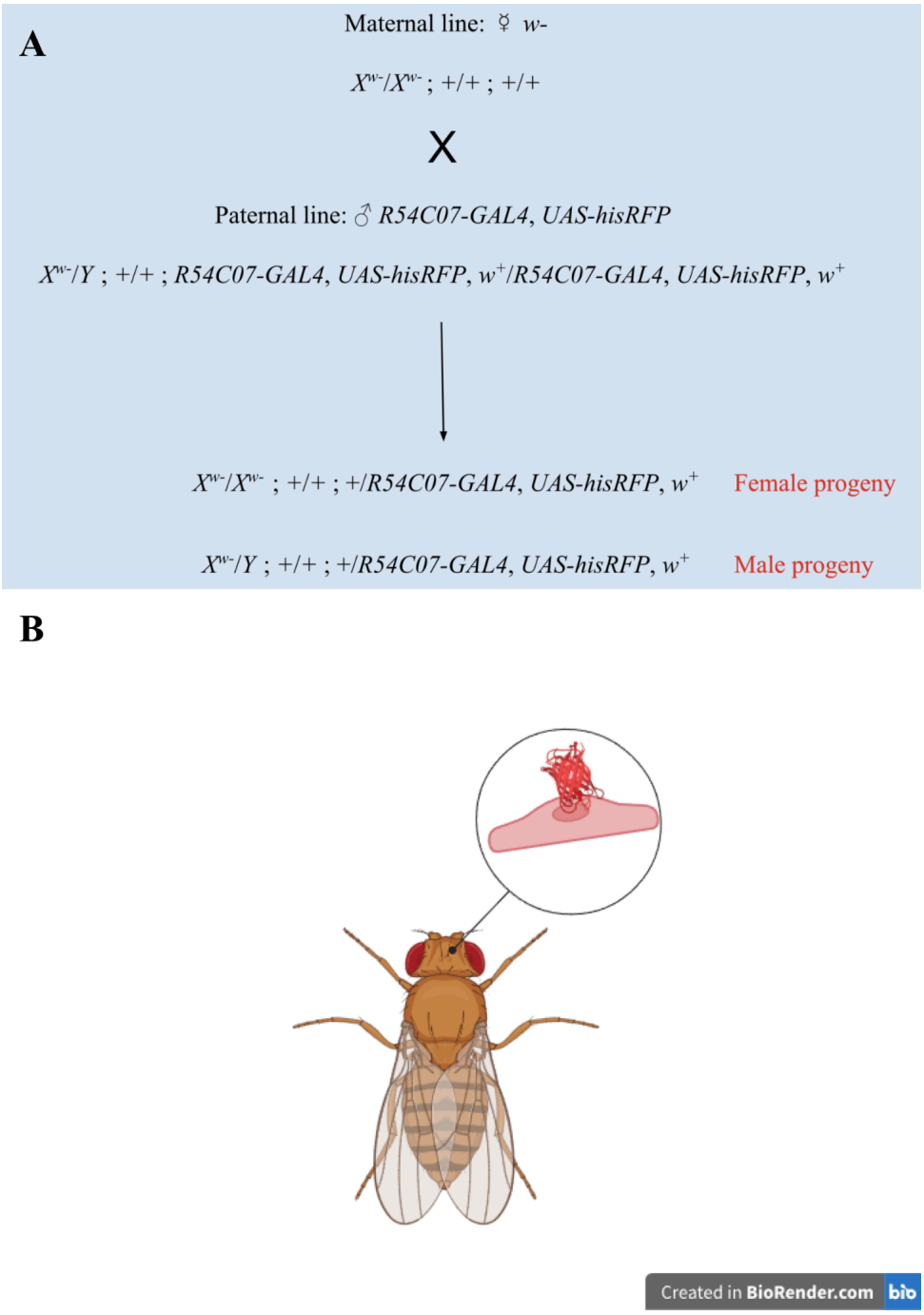

Figure 3. Construction of control genotype. (A) Crossing scheme used to create genotype. **(B)** Progeny expressed hisRFP in subperineurial glia. Created in BioRender.com.

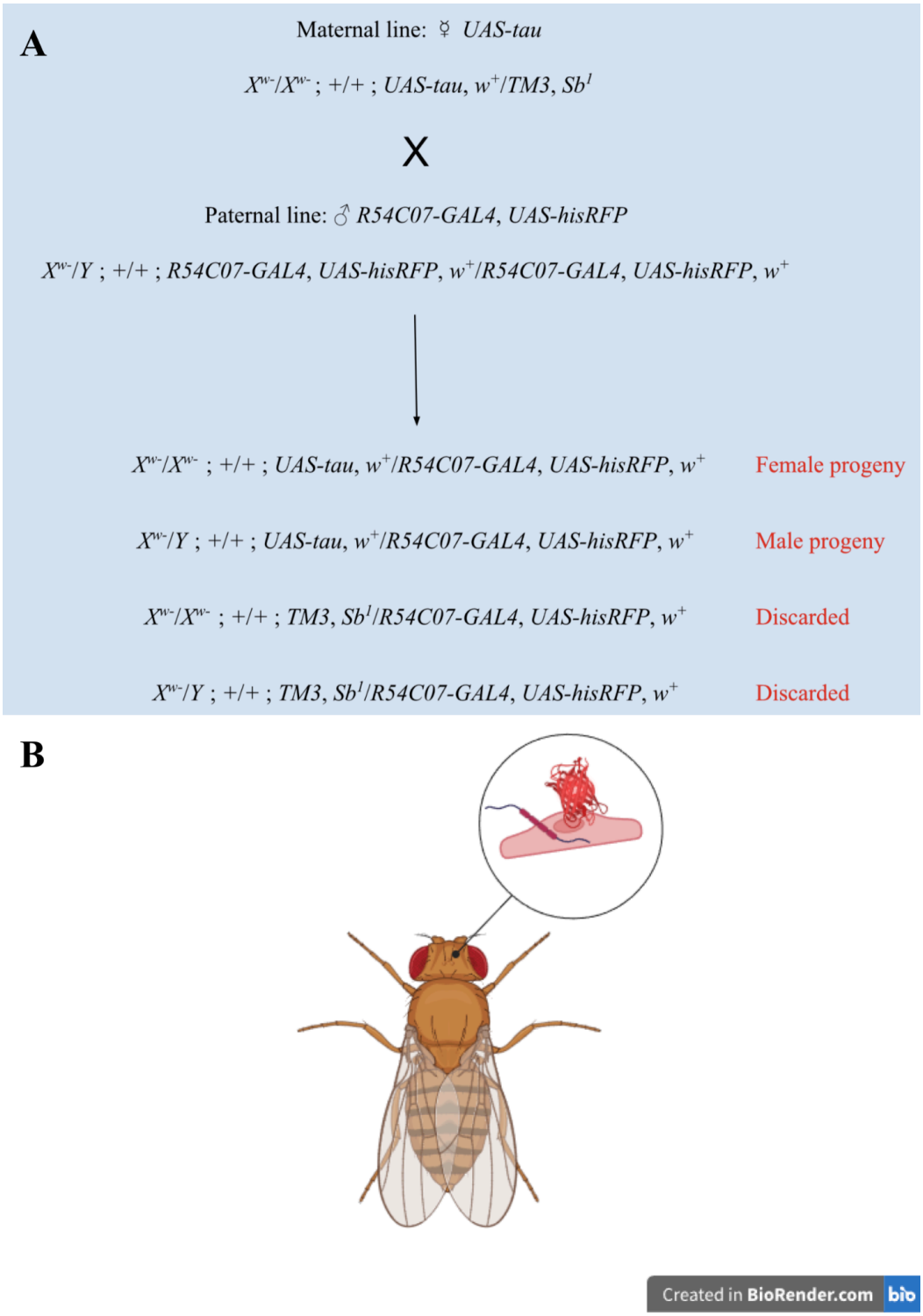

Figure 4. Construction of tau genotype. (A) Crossing scheme used to create genotype. In practice, virgin females homozygous for the *UAS-tau* construct were typically selected from the maternal line to avoid the need to sort progeny. **(B)** Progeny expressed hisRFP and human tau in subperineurial glia. Created in BioRender.com.

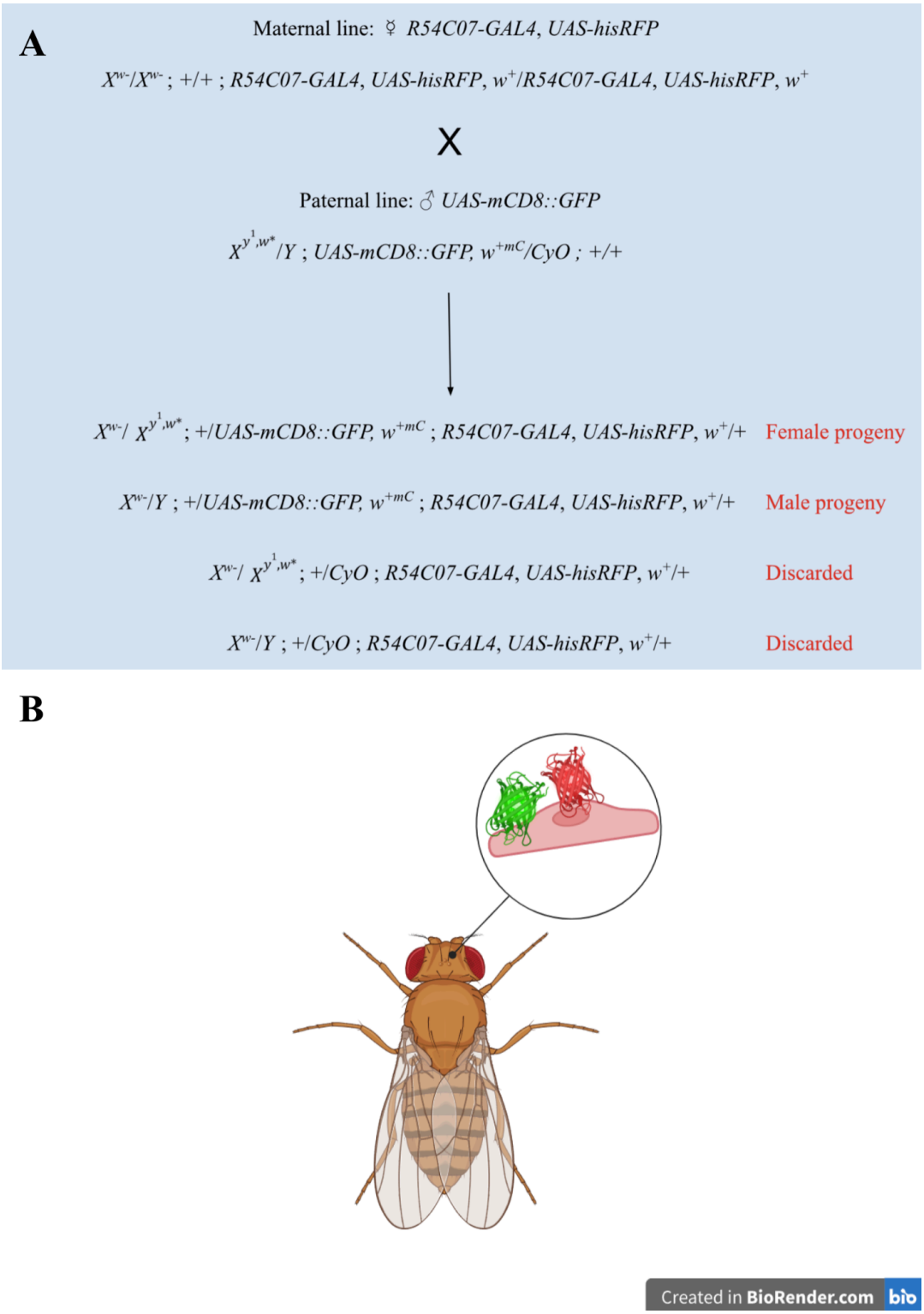

Figure 5. Construction of GFP genotype. (A) Crossing scheme used to create genotype. **(B)** Progeny expressed hisRFP and membrane-tethered GFP in subperineurial glia. Created in BioRender.com.

3. Brain Dissection and Fixation

Flies of a designated age, sex, and genotype were anaesthetized over ice. In order to prevent air bubbles from later adhering to their heads, flies were dipped in 70% ethanol for about three seconds before being rinsed twice in 1x phosphate-buffered saline (PBS) (Kang, 2023; Tito et al., 2016). They were then placed in a 1.5mL microcentrifuge tube containing 500µL 4% paraformaldehyde (PFA) in PBS to fix at room temperature for 15 minutes. The 4% PFA solution was removed, and the flies were rinsed twice with 500µL PBS. The second rinse was replaced with 500µL fresh PBS, at which point the tube of flies was placed over ice.

Fly brains were dissected out in a large drop of PBS on a Sylgard plate. Directly after dissection, brains were placed in 10µL 4% PFA solution within one well of a Terasaki plate (Ahmed et al., 2023). After placement of the first brain in the fixative, the remaining brains were collected within 20 minutes to prevent overfixation. The 4% PFA solution was then removed and replaced with 10µL fresh 4% PFA solution. The brains were nutated in the fixative for 20 minutes. This and all subsequent nutations were performed at room temperature.

After fixation, the 4% PFA solution was removed, and the brains were rinsed twice with 10µL PBS. The second rinse was removed, and the brains were washed with 10µL PBS for 2 x 20 minutes while nutating. The PBS was removed, and the brains were washed with 10µL 0.5% Triton X-100 in PBS for 20 minutes while nutating. The Triton X-100 solution was removed, and the brains were washed with 10µL 40% glycerol in PBS for 10 minutes, then 10µL 60% glycerol in PBS for 10 minutes, then finally 10µL 80% glycerol in PBS for 10 minutes, all while nutating. This cleared the tissue to control its light scattering properties and thus improve the quality of microscopic imaging later on (Richardson & Lichtman, 2015).

The brains were transferred through two wells of the Terasaki plate each containing 10µL VECTASHIELD® PLUS Antifade Mounting Medium with DAPI. They were then placed into a large drop of the mounting medium nestled between two halves of a no. 2 coverslip affixed to a microscope slide using clear nail polish (Kang, 2023). When possible, the brains were placed anterior side up near the bottom of the mounting medium. Finally, they were covered with a no. 1.5 coverslip whose edges were sealed with nail polish. The prepared slide was stored in the dark at 4℃ until imaging.

4. Microscopy and Imaging

Several of the compounds used in this study—namely DAPI, RFP, and GFP—exhibit fluorescent properties. These compounds, termed fluorochromes, absorb particular wavelengths of light that excite some of their electrons (Ishikawa-Ankerhold et al., 2012). When the electrons relax, energy is released in the form of a different, longer wavelength of light. Thus, DAPI absorbs ultraviolet light and emits blue light; RFP absorbs green light and emits red light; and GFP absorbs blue light and emits green light (Brejc et al., 1997; Kang, 2023; Karg & Golic, 2018). By exposing a fluorochrome to the appropriate wavelength of light to excite the electrons within, it can be detected based on the light it emits. This is the principle behind fluorescence microscopy.

Confocal laser scanning microscopy (CLSM) is a specific type of fluorescence microscopy designed to reject out-of-focus light and maintain high resolution even when taking images from deep within thick tissue (Elliott, 2020). In CLSM, a laser beam is swept rapidly across a horizontal plane of the sample, illuminating one tiny point at a time for imaging. Multiple planes can be combined to create a z-stack covering the full depth of the sample.

The present study imaged fluorescent samples using CLSM on a Nikon ECLIPSE Ti2 microscope paired with NIS-Elements software. The settings used to image each fluorochrome were held consistent across brains (Table 1). Z-stacks were obtained using the 20x objective with 2µm between focal planes, and the top and bottom coordinates were set to capture the entire brain as viewed using DAPI (Kang, 2023). For relatively large brains, two fields with 15% overlap were scanned, and the images were stitched together using the software's automatic blending settings. Each z-stack was saved as an nd2 file for analysis. Additionally, z-stacks were converted to maximum intensity projection (MIP) TIFF files for ease of visualization and quality control. A MIP collapses a three-dimensional volume into a two-dimensional image by representing each pixel as the most intense point at that x-y coordinate throughout the depth of the sample (Kwon et al., 2015). MIPs were set to combine the two (Figure 6) or three (Figure 7) laser channels used to image each brain.

Table 1. Laser power and gain settings used to image each fluorochrome.

Fluorochrome	Laser power	Gain
DAPI	20	20
RFP	20	16
GFP	20	12

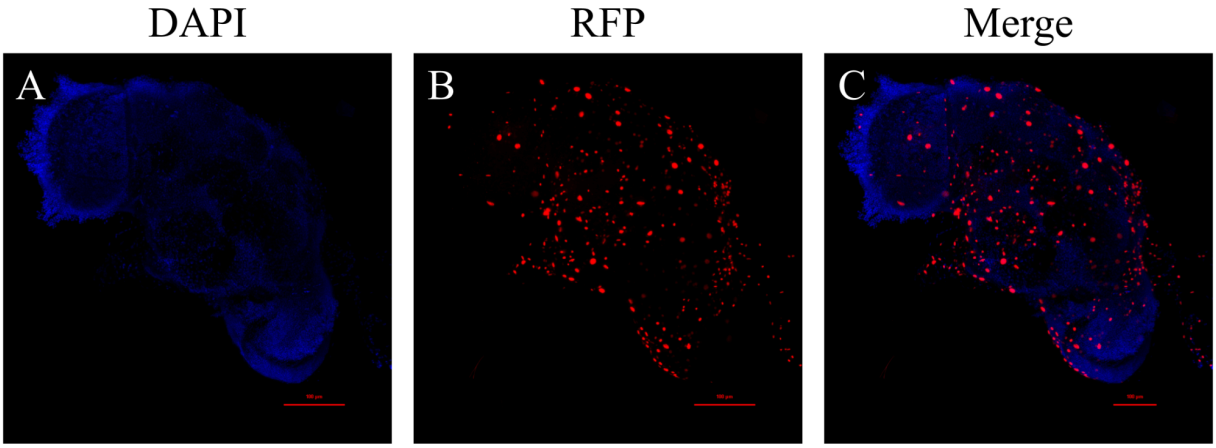

Figure 6. Channels used to create images of control and tau brains. Maximum intensity projections of **(A)** DAPI, showing all cell nuclei in blue, and **(B)** RFP, showing SPG nuclei in red, were combined to create **(C)** a merged image mapping SPG nuclei throughout the brain. The brain pictured belongs to a 3-day-old female fly (*D. melanogaster*) of the control genotype. Scale bar = 100µm. Control genotype = *+/R54C07-GAL4,UAS-hisRFP*.

5. Quality Control

Careful quality control of the 103 dissected brains was conducted to ensure data validity. Because establishing the proper settings at which to image brains took several weeks, 16 brains were at least 30 days old by the time they could be imaged at the correct settings. All of these brains were excluded from analysis due to a tendency to under fluoresce. Five additional brains were excluded due to poor mounting resulting in image obscurement either by air bubbles or by the no. 2 coverslip spacers. Finally, eight brains were excluded for miscellaneous reasons outlined in detail below (Table 2). This left 74 brains for analysis.

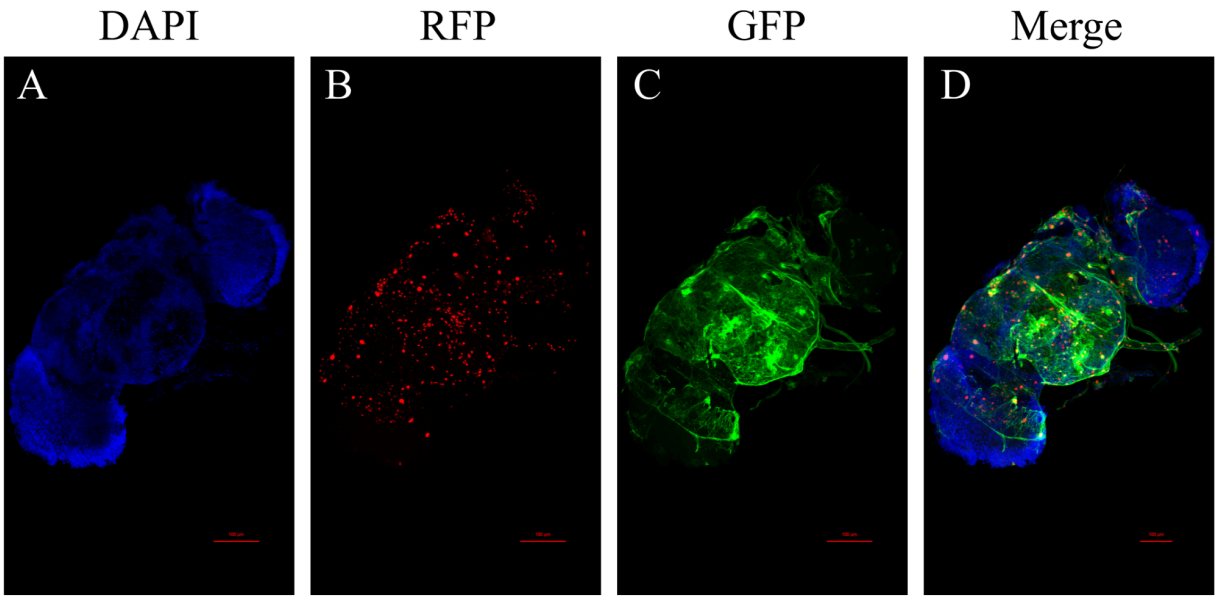

Figure 7. Channels used to create images of GFP brains. Maximum intensity projections of **(A)** DAPI, showing all cell nuclei in blue, **(B)** RFP, showing SPG nuclei in red, and **(C)** GFP, showing SPG cell membranes in green, were combined to create **(D)** a merged image mapping SPG nuclei and processes throughout the brain. The brain pictured belongs to a 3-day-old female fly (*D. melanogaster*) of the GFP genotype. Scale bar = 100µm. GFP genotype = +/*UAS-mCD8::GFP*; *R54C07-GAL4,UAS-hisRFP*/+.

Table 2. Brains excluded from analysis listed by rationale.

Reason for concern	**Number of brains excluded**
≥30 days old when ready to image with established settings	16
Mounting issues	5
Known brain processing issues paired with suspected under fluorescence of ≥1 brain on slide	3
Substantial damage to brain	2
Suspected under fluorescence due to multiple imaging while troubleshooting settings	1
Presence of large fluorescent filament	1
Suspected cell duplication during imaging	1

6. Image Analysis

Image analysis was conducted in Imaris 9.3.1 (Bitplane). The spot detection tool was used for initial quantification of SPG nuclei. Background subtraction was applied, and the nuclear diameter was estimated at 5µm. A minimum intensity center threshold of 1800 was chosen to avoid overcounting (detection of spots in the absence of probable SPG nuclei) or undercounting (failure to detect spots in the presence of probable SPG nuclei). The threshold selection process is illustrated below (Figure 8).

The surface detection tool was used for secondary analysis of SPG nuclei (Figure 9). Surface smoothing was applied with a grain size of 1µm. Background elimination was applied with the "Diameter of Largest Sphere" set to 3.6µm (Ryabova et al., 2021). A background subtraction threshold minimum of 500 was chosen, as well as a volume threshold minimum of 24μm^3. In addition to the quantity of surfaces, the average surface volume and sphericity were calculated for each brain.

7. Statistical Analysis

To determine adequate sample size, an a priori power analysis was conducted in G*Power. A two-tailed *t* test of the difference between two independent means was selected, with allocation ratio set to 1, alpha to 0.05, and power to 0.9. Effect sizes were calculated from relevant data extracted from prior research (Table 3). The resulting sample size recommendations ranged from 3-16 per group. A sample size of 8-10 brains per age-sex-genotype combination was chosen for flies of the control and tau genotypes. For the exploratory analysis using the GFP genotype, two brains per sex were used.

After data collection, statistical analysis was conducted in GraphPad Prism 10.4.1. Three-way ANOVAs with Šidák's multiple comparisons tests were used to compare SPG spot count, SPG surface count, average sphericity, and average volume between groups. This method accounted for the three differentiating variables of genotype, age, and sex. When evaluating ANOVA results, $P \leq 0.05$ was considered significant. For Šidák's tests, means were compared only between groups differing by a single factor. A multiplicity adjusted P value was reported using a family-wise alpha threshold of 0.05.

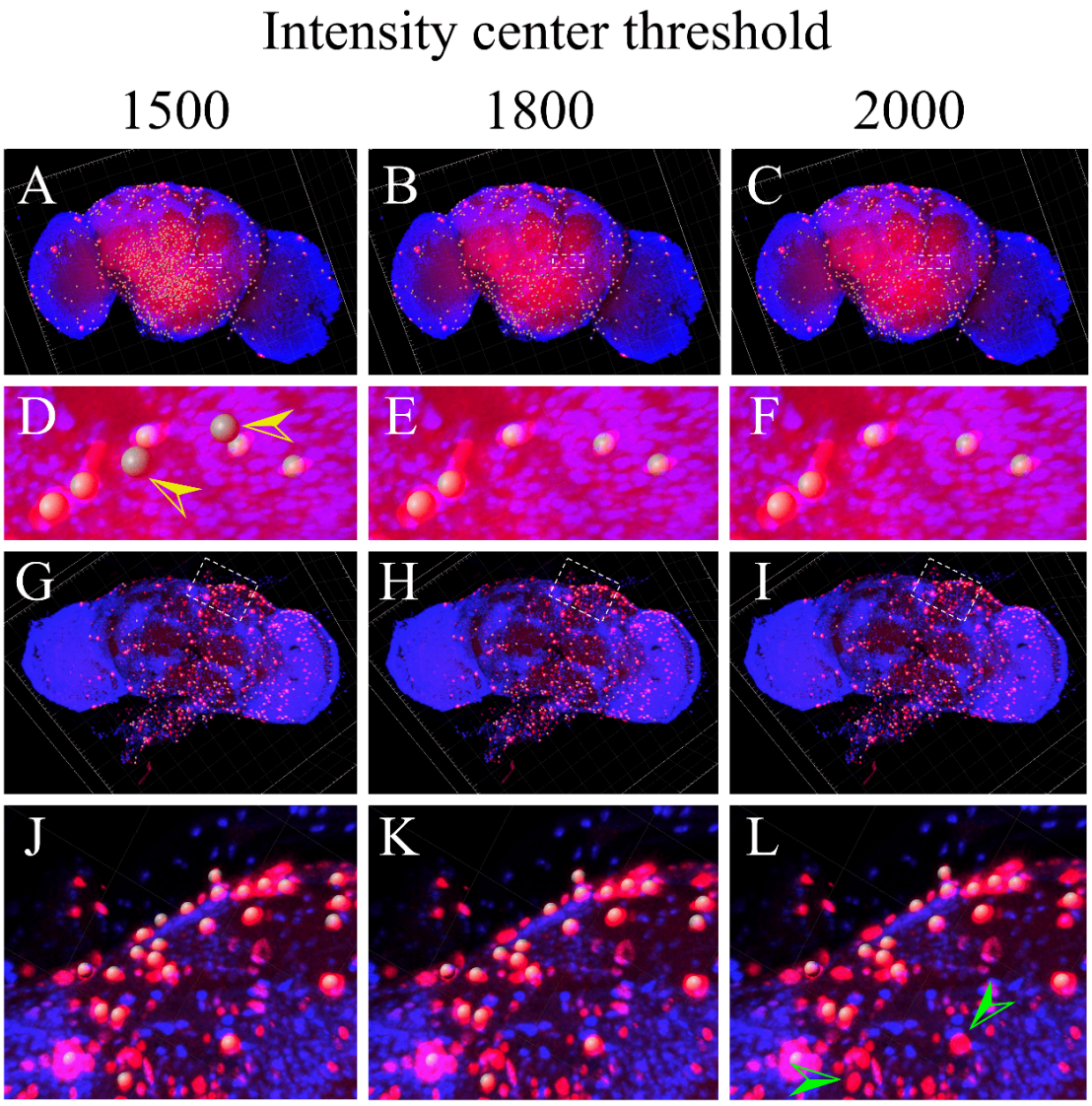

Figure 8. Selection of minimum intensity center threshold for SPG spot count. (A-C) 3D reconstruction of a relatively bright brain belonging to a 10-day-old male fly (*D. melanogaster*) of the tau genotype. **(D-F)** Zoomed in sections of annotated brain reconstructions from A-C. **(G-I)** 3D reconstruction of a fainter brain belonging to a 10-day-old female fly of the control genotype. **(J-L)** Zoomed in sections of annotated brain reconstructions from G-I. Dotted rectangles highlight approximate regions shown in zoomed in images. White dots indicate SPG nuclei detected by Imaris spot detection tool. Minimum intensity center threshold differs by column: 1500 in the leftmost column, 1800 in the central column, and 2000 in the rightmost column. Yellow arrows indicate spots detected in the absence of probable SPG nuclei (overcounting). Green arrows indicate probable SPG nuclei unmarked by spot detection tool (undercounting). Tau genotype = *UAS-tau/R54C07-GAL4,UAS-hisRFP*. Control genotype = *+/R54C07-GAL4,UAS-hisRFP*.

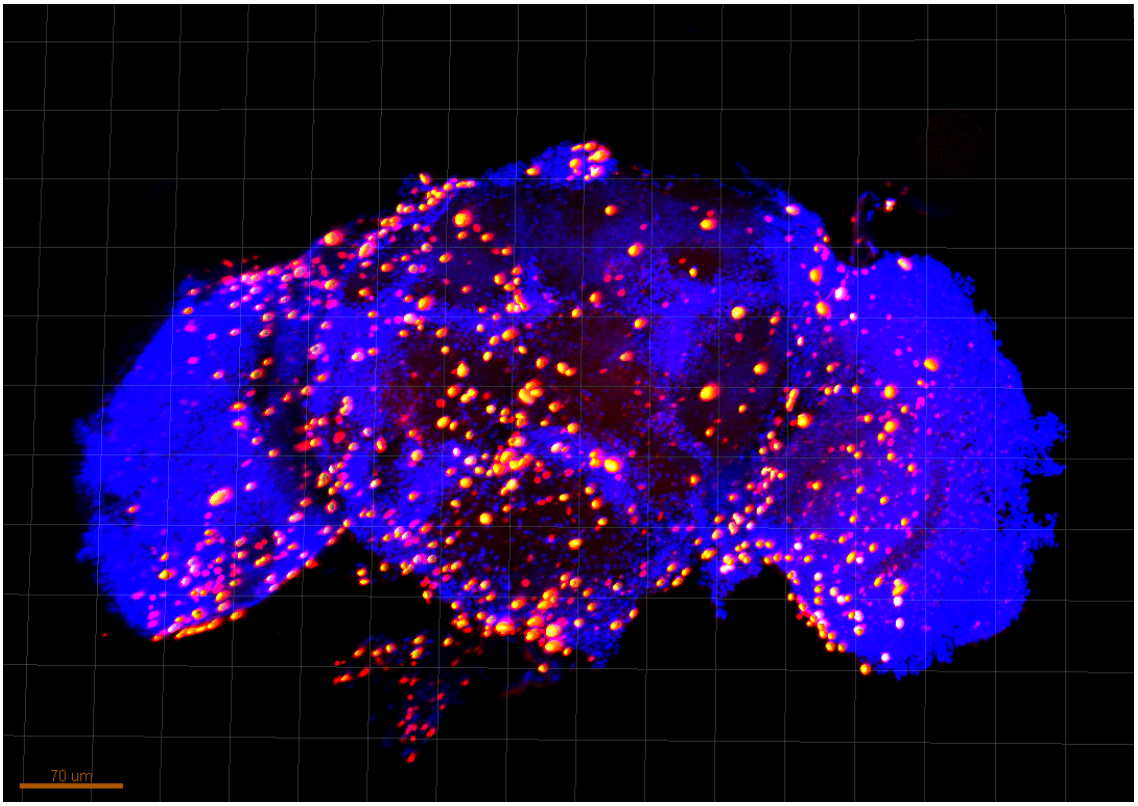

Figure 9. Surface detection in Imaris. 3D reconstruction of a brain belonging to a 10-day-old male fly (*D. melanogaster*) of the control genotype. Yellow blobs indicate SPG nuclei detected by Imaris surface detection tool. Scale bar = 70µm. Control genotype = +/*R54C07-GAL4,UAS-hisRFP*.

Table 3. Results of a priori power analysis. Two-tailed *t* test of the difference between two independent means. Allocation ratio = 1. α = 0.05. Power = 0.9. Conducted in G*Power.

Study	**Extracted comparison**	**Recommended sample size**
Bukhari et al., 2024	Number of vacuoles: 10-day-old control flies vs 10-day-old heterozygous tau knock-in flies	3 per group
Kang, 2023	Astrocyte count: 3-day-old male control flies vs 3-day-old female control flies	8 per group
Coll-Tané et al., 2021	Total dark period sleep time: SPG *kismet* knockdown flies vs *SPG-GAL4*/+ control flies	16 per group

In order to evaluate the variability of SPG spot count, the coefficients of variation were calculated for 3-day-old male and female flies of the control genotype. As a point of comparison, the coefficients of variation were also calculated for data extracted from prior research quantifying astrocytes (also known as astrocyte-like glia) in 3-day-old male and female control flies (Kang, 2023).

RESULTS

1. Human Tau Expression Reduces Quantity of SPG Nuclei in Young Male Flies

Primary Quantification Method: Spot Count

In order to examine the effect of human tau expression on the SPG cell population, SPG nuclei were quantified in control and tau flies using the spot detection tool in Imaris. These data were analyzed using a 3-way ANOVA, which identified genotype ($P = 0.0055$), genotype-age interaction ($P = 0.0163$), and genotype-sex interaction ($P = 0.0024$) as statistically significant sources of variation. This indicates an age- and sex-dependent effect of tau on SPG nuclear quantity.

To identify notable pairwise differences between groups, Šidák's multiple comparisons test was used. The test was set to exclusively compare groups differing by only one factor, allowing for the examination of one explanatory variable at a time. After adjusting for multiple comparisons, the only significant pairwise difference was between 3-day-old male flies of the control genotype and those of the tau genotype (adjusted P value = 0.0012). This suggests a toxic effect of tau on SPG exclusive to young male flies (Figure 10).

Secondary Quantification Method: Surface Count

SPG are known for their relatively large size (Kremer et al., 2017). However, due to the imperfect specificity of the *R54C07* driver, some very small nuclei likely belonging to other cell types were marked with histone-bound RFP (Figure 11). In an effort to limit the analysis to SPG-specific nuclei, a secondary quantification was conducted using the surface detection tool in Imaris, which allowed red puncta to be filtered by volume. A minimum volume of $24\mu m^3$ was calculated based on the minimum area of normal SPG nuclei determined by a prior study (Ryabova et al., 2021). This technique was deemed secondary because it generated results with limited comparability to already collected data quantifying the glial subtype-specific effects of tau expression in *Drosophila* (Kang, 2023).

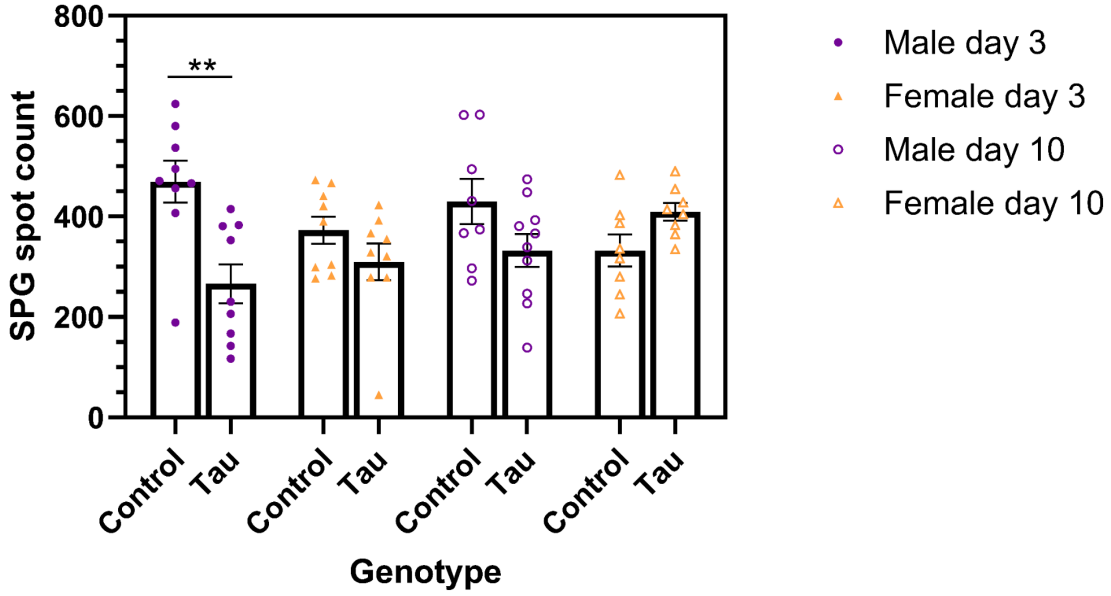

Figure 10. Human tau expression reduces quantity of SPG nuclei in 3-day-old male flies as measured by Imaris spot detection. SPG nuclei were quantified using the Imaris spot detection tool and compared across genotype, age, and sex of *D. melanogaster*. Data shown as mean +/- SEM. Statistical analysis was performed using a 3-way ANOVA with Šidák's multiple comparisons test in GraphPad Prism. **Adjusted P value ≤ 0.01. *n* = 8-10 flies per condition. Control genotype = *+/R54C07-GAL4,UAS-hisRFP*. Tau genotype = *UAS-tau/R54C07-GAL4,UAS-hisRFP*.

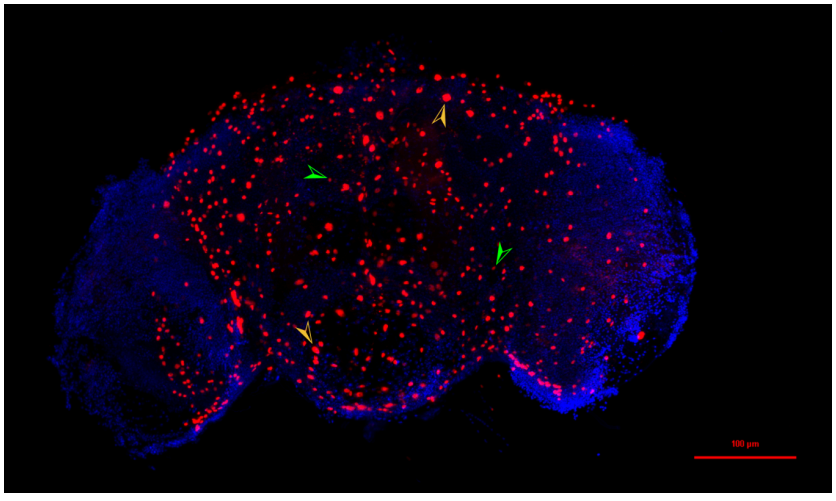

Figure 11. *R54C07* drives hisRFP expression in small, likely non-SPG nuclei. Maximum intensity projection showing the brain of a 10-day-old male fly (*D. melanogaster*) of the control genotype. Yellow arrowheads highlight large hisRFP-tagged nuclei likely belonging to SPG, while green arrowheads highlight very small hisRFP-tagged nuclei likely belonging to non-SPG cell types. Blue = DAPI. Red = hisRFP. Scale bar = 100µm. Control genotype = *+/R54C07-GAL4,UAS-hisRFP*.

The secondary quantification data were analyzed using a 3-way ANOVA, which identified genotype (P = 0.0015), genotype-age interaction (P = 0.0184), and genotype-sex interaction (P = 0.0021) as statistically significant sources of variation. Next, Šidák's multiple comparisons test was used to identify pairwise differences between groups differing by only one factor. After adjusting for multiple comparisons, the only significant pairwise difference was between 3-day-old male flies of the control genotype and those of the tau genotype (adjusted P value = 0.0004). The analyses conducted using the secondary quantification method further support the age- and sex-dependent effect of tau on SPG nuclear quantity as well as the toxic effect of tau on SPG in young male flies (Figure 12).

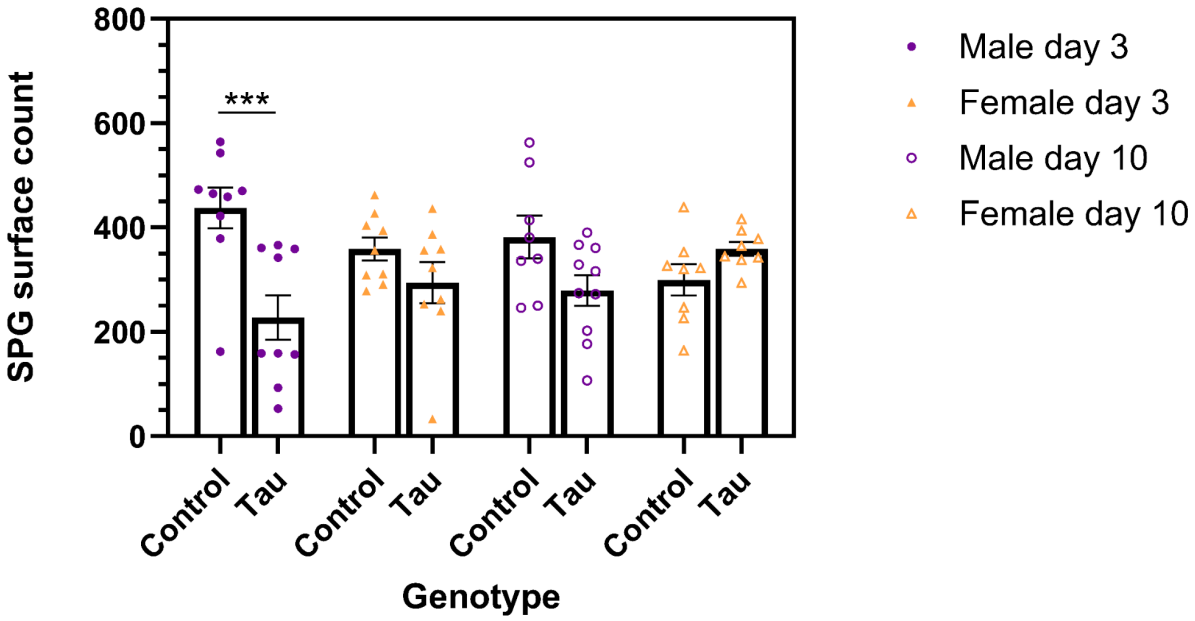

Figure 12. Human tau expression reduces quantity of SPG nuclei in 3-day-old male flies as measured by Imaris surface detection. SPG nuclei were quantified using the Imaris surface detection tool and compared across genotype, age, and sex of *D. melanogaster*. Data shown as mean +/- SEM. Statistical analysis was performed using a 3-way ANOVA with Šidák's multiple comparisons test in GraphPad Prism. ***Adjusted P value ≤ 0.001. *n* = 8-10 flies per condition. Control genotype = *+/R54C07-GAL4,UAS-hisRFP*. Tau genotype = *UAS-tau/R54C07-GAL4,UAS-hisRFP*.

2. SPG Nuclear Morphology Exhibits Genotype-Related Patterns

Prior research has demonstrated that human tau expression disrupts nuclear morphology in *Drosophila* cortical glia (Kang, 2023). To test whether the same is true for SPG, the volume

and sphericity of SPG nuclei in control and tau brains were calculated using the surface detection tool in Imaris. These values were averaged across all surfaces for each brain, and the resulting averages were used for statistical analysis.

The 3-way ANOVA for nuclear volume identified sex (P = 0.0005) and genotype-age interaction (P = 0.0417) as statistically significant sources of variation. In the context of the dataset, the interaction effect appears to describe a phenomenon in which human tau expression results in decreased SPG nuclear volume in young flies and increased SPG nuclear volume in older flies (Figure 13). However, after adjusting for multiple comparisons, Šidák's multiple comparisons test found no significant pairwise differences between groups differing by only one factor, limiting the specificity of interpretation.

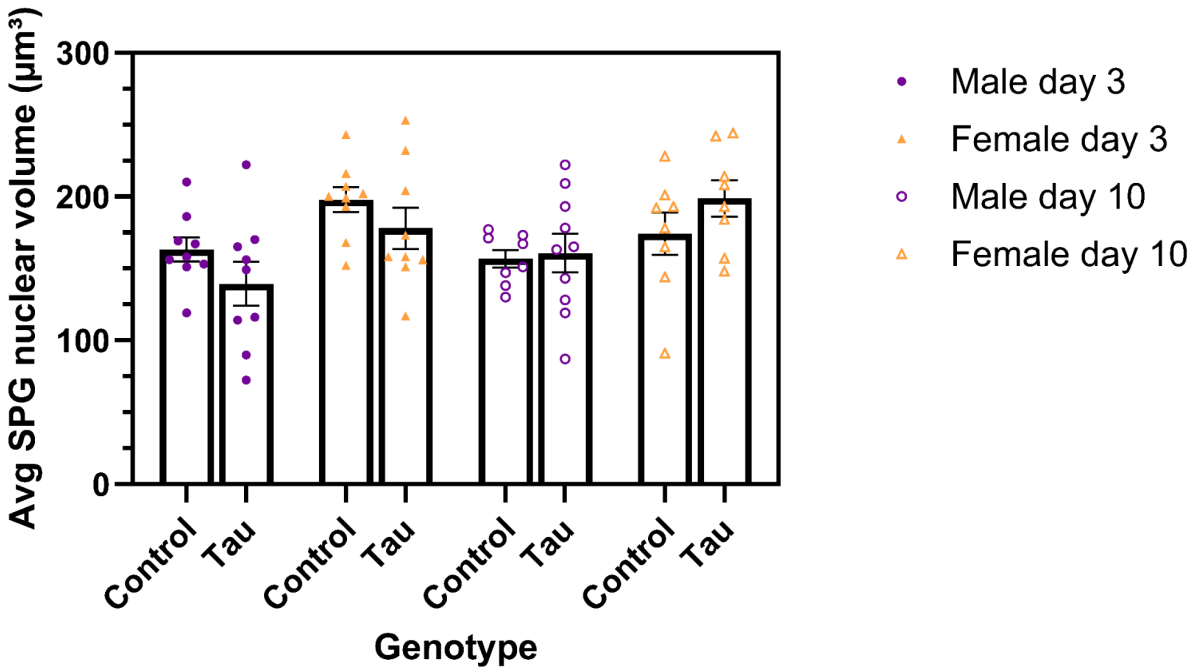

Figure 13. Average SPG nuclear volume by genotype, age, and sex. Average SPG nuclear volume in μm^3 was calculated using the Imaris surface detection tool and compared across genotype, age, and sex of *D. melanogaster*. Data shown as mean +/- SEM. Statistical analysis was performed using a 3-way ANOVA with Šidák's multiple comparisons test in GraphPad Prism, revealing no statistically significant pairwise differences. Control genotype = +/*R54C07-GAL4,UAS-hisRFP*. Tau genotype = *UAS-tau/R54C07-GAL4,UAS-hisRFP*.

Sphericity is a measure of roundedness calculated by comparing a shape's surface area to the surface area of a sphere of the same volume (St. Pierre et al., 2023). The 3-way ANOVA for nuclear sphericity identified genotype (P = 0.0010) and age (P = 0.0040) as significant sources of variation. In the context of the dataset, the main effect of genotype suggests that human tau expression results in decreased SPG nuclear sphericity (Figure 14). However, after adjusting for multiple comparisons, Šidák's multiple comparisons test again found no significant pairwise differences between groups differing by only one factor, limiting the specificity of interpretation.

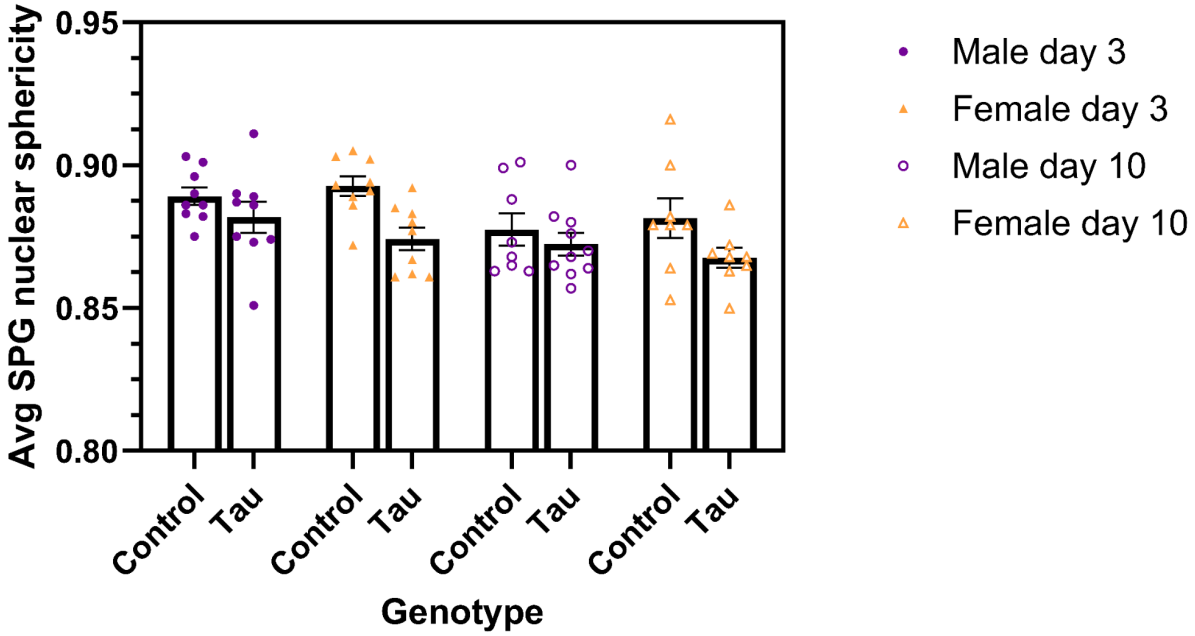

Figure 14. Average SPG nuclear sphericity by genotype, age, and sex. Average SPG nuclear sphericity was calculated using the Imaris surface detection tool and compared across genotype, age, and sex of *D. melanogaster*. Data shown as mean +/- SEM. Statistical analysis was performed using a 3-way ANOVA with Šidák's multiple comparisons test in GraphPad Prism, revealing no statistically significant pairwise differences. Control genotype = +/*R54C07-GAL4,UAS-hisRFP*. Tau genotype = *UAS-tau/R54C07-GAL4,UAS-hisRFP*.

3. Brain Dissection Protocol Induces Variable Damage to SPG Sheath

SPG are known to form a continuous sheath around the brain (Kremer et al., 2017). However, large patches of surface area lacking SPG nuclei were observed on several brains in the present study, including control brains. These empty patches were most prevalent on the optic lobes (Figure 15). To test whether this sheath was damaged during the dissection protocol, a line

of flies was generated to express both histone-bound RFP and membrane-tethered GFP in SPG. In these flies, GFP could be used to trace the full extension of SPG processes. The brains dissected revealed varying degrees of damage to the SPG sheath (Figure 16). In some cases, SPG nuclei are visible outside the boundaries of intact SPG sheath, indicating that it is possible to pull off SPG processes during dissection while leaving behind intact SPG nuclei. However, SPG nuclei tend to be sparse in regions where the sheath is absent, suggesting that the dissection process may artificially lower SPG cell count.

It was suspected that variable damage to the SPG sheath during dissection might inflate the within-group variability of SPG nuclear quantity. To explore this possibility, the variability of SPG spot count within each sex of 3-day-old control flies was quantified using the coefficient of variation. As a point of comparison, data from prior research were used to calculate the coefficients of variation for astrocyte quantity in 3-day-old male and female control flies (Kang, 2023). In both male and female flies, the coefficient of variation for SPG spot count was substantially higher than for astrocyte count (Table 4). In order to visualize this difference in variability, data were normalized to their means. That is, for each sex-glial subtype combination, the dataset was rescaled such that zero remained as zero but the mean was set as 100. Side-by-side boxplots of these normalized datasets highlight the wide spread of SPG spot counts compared to astrocyte counts (Figures 17 and 18). This suggests that when quantifying SPG nuclei, damage during dissection may introduce an additional source of within-group variability beyond the natural biological differences observed with any glial subtype.

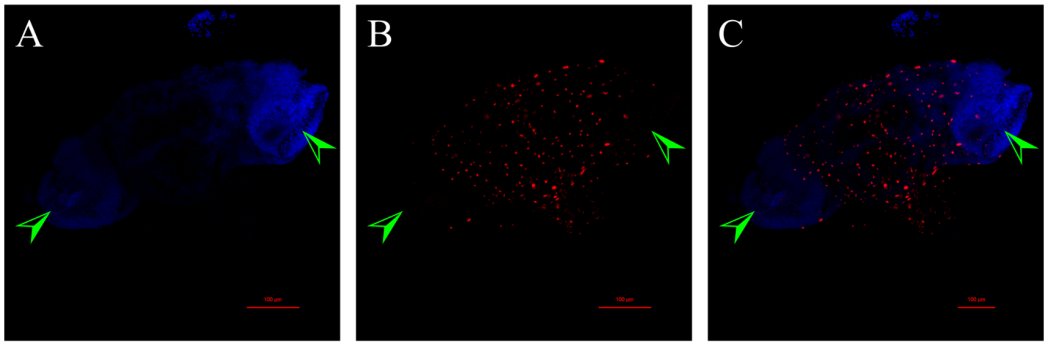

Figure 15. A representative control brain appears to be missing SPG nuclei. Maximum intensity projections showing the brain a 3-day-old male fly (*D. melanogaster*) of the control genotype. **(A)** DAPI, showing all cell nuclei in blue. **(B)** RFP, showing SPG nuclei in red. **(C)** Merge. Note the near absence of SPG nuclei in the optic lobes (green arrowheads). Scale bar = 100µm. Control genotype = *+/R54C07-GAL4,UAS-hisRFP*.

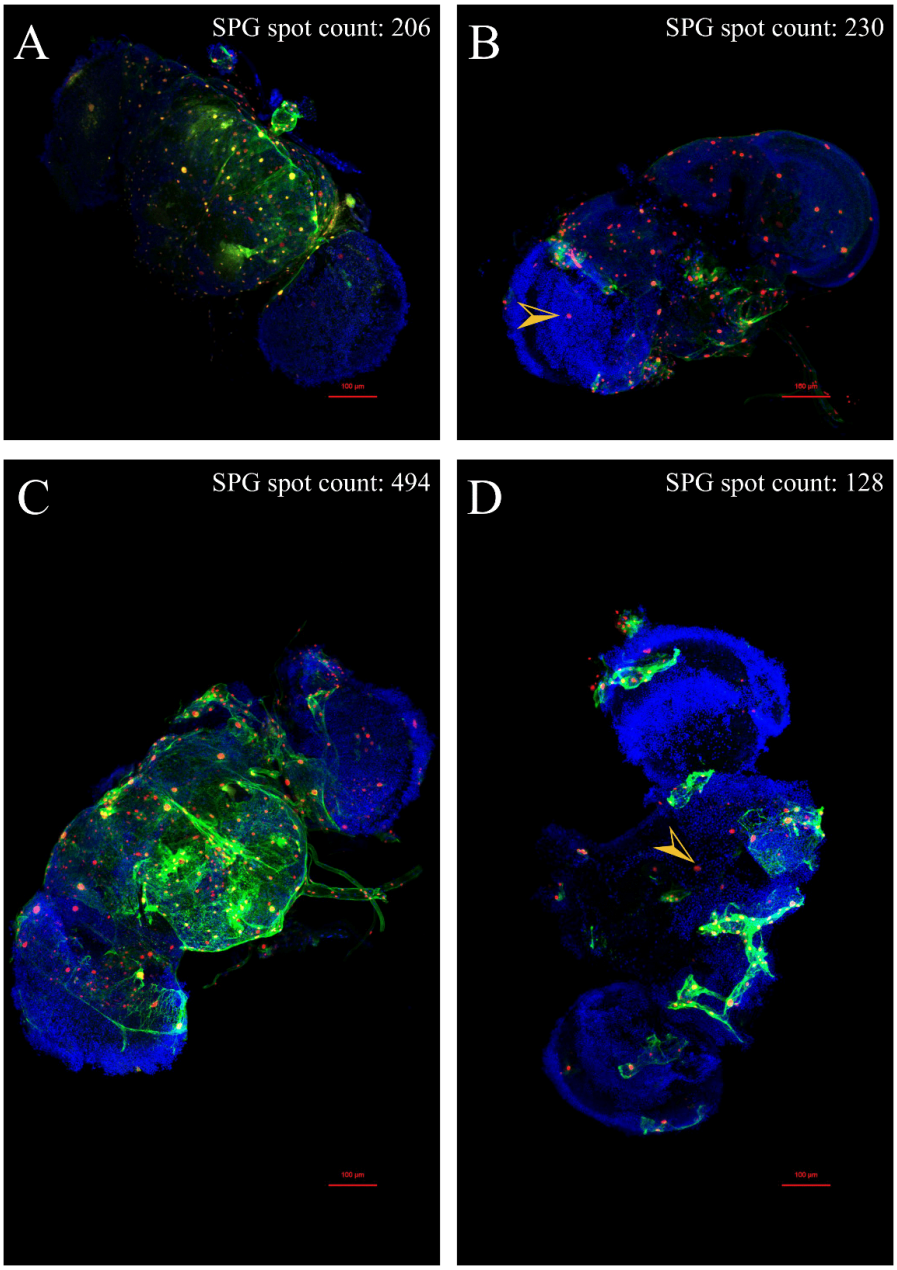

Figure 16. Brain dissection protocol induces variable damage to SPG sheath. Maximum intensity projections showing the brains of 3-day-old **(A-B)** male and **(C-D)** female flies (*D. melanogaster*) of the GFP genotype. Note varying degrees of SPG sheath integrity, from moderately intact in **(C)** to severely damaged in **(D)**. SPG spot count is indicated on each brain, appearing to rise and fall approximately in line with SPG sheath integrity. Also note the presence of hisRFP-tagged SPG nuclei appearing outside the boundaries of intact SPG sheath (yellow arrowheads). Blue = DAPI. Red = hisRFP. Green = membrane-tethered GFP. Scale bar = 100µm. GFP genotype = +/*UAS-mCD8::GFP*; *R54C07-GAL4,UAS-hisRFP*/+.

Table 4. SPG spot count is more variable than astrocyte count in young control flies. The coefficients of variation for SPG spot count were calculated in GraphPad Prism for 3-day-old male and female flies (*D. melanogaster*) of the control genotype. Additionally, using data from prior research, the coefficients of variation for astrocyte count were calculated for 3-day-old male and female *w-* control flies (Kang, 2023). *n* = 9 per sex for SPG spot count; 14 per sex for astrocyte count. Control genotype (mine) = *+/R54C07-GAL4,UAS-hisRFP*. *w-* control genotype (Kang) = *UAS-hisRFP,R86E01-GAL4/w-*.

Fly characteristics	**Coefficient of variation: SPG spot count**	**Coefficient of variation: Astrocyte count**
Control male day 3	26.53%	9.609%
Control female day 3	22.00%	8.572%

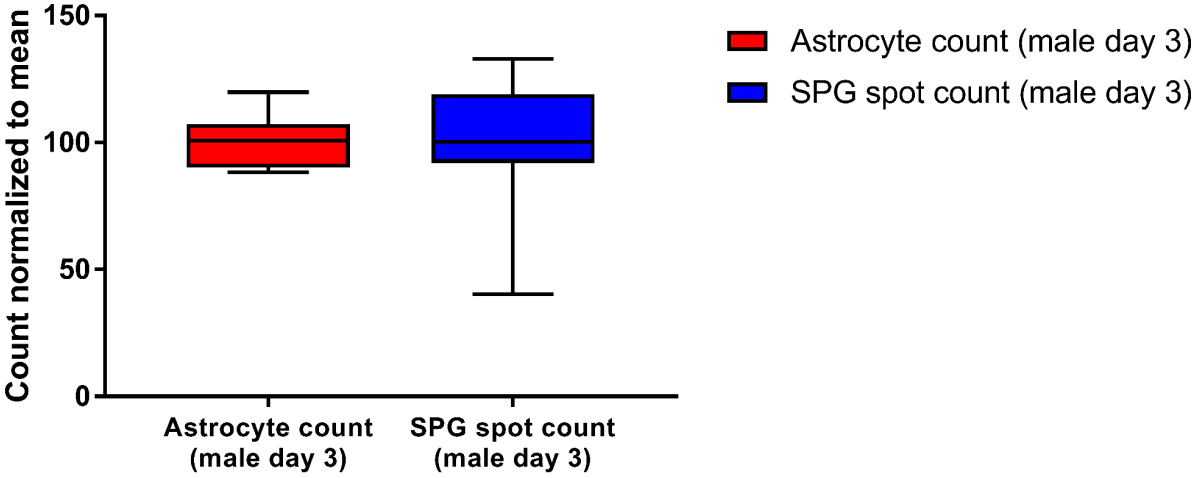

Figure 17. SPG spot count appears more variable than astrocyte count in young control flies (male). SPG spot count and astrocyte count were normalized to their respective means in 3-day-old control male flies (*D. melanogaster*), visualized in side-by-side box and whiskers plots (Kang, 2023). *n* = 9 for SPG spot count; 14 for astrocyte count. Control genotype (mine) = *+/R54C07-GAL4,UAS-hisRFP*. *w-* control genotype (Kang) = *UAS-hisRFP,R86E01-GAL4/w-*.

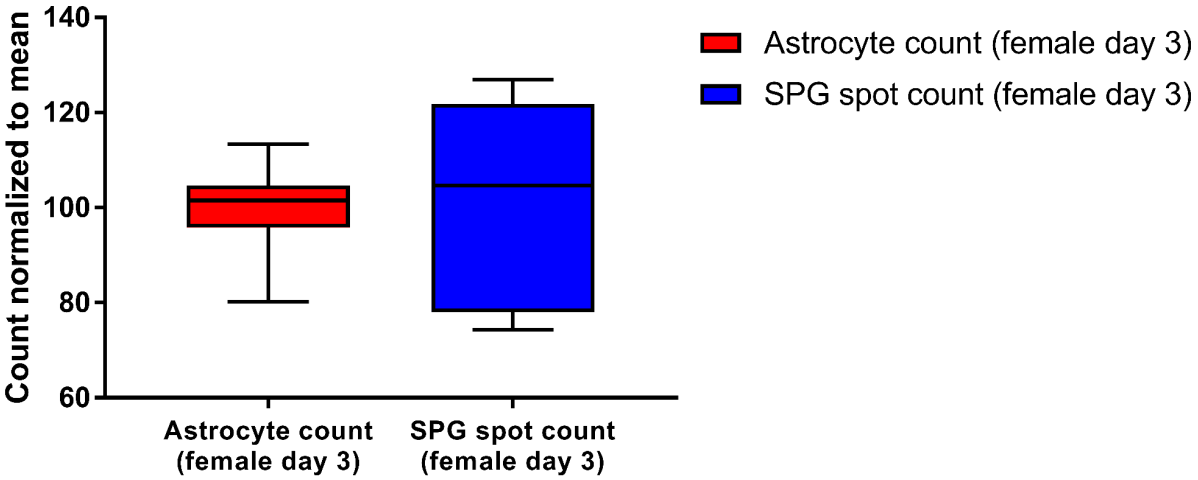

Figure 18. SPG spot count appears more variable than astrocyte count in young control flies (female). SPG spot count and astrocyte count were normalized to their respective means in 3-day-old control female flies (*D. melanogaster*), visualized in side-by-side box and whiskers plots (Kang, 2023). *n* = 9 for SPG spot count; 14 for astrocyte count. Control genotype (mine) = +/*R54C07-GAL4,UAS-hisRFP*. *w*- control genotype (Kang) = *UAS-hisRFP,R86E01-GAL4/w*-.

DISCUSSION

1. Summary of Findings

The present study compared SPG nuclei in flies with and without SPG-specific human tau expression. This revealed an age- and sex-dependent effect of tau on SPG nuclear quantity. In particular, human tau expression results in a significant reduction in SPG nuclei in 3-day-old male flies, an effect not observed in 10-day-old male flies, 3-day-old female flies, or 10-day-old female flies (Figures 10 and 12). Additionally, tau expression results in a general decline in SPG nuclear sphericity. While this trend is statistically significant in the dataset as a whole, it does not correspond to a significant effect in any one age-sex combination (Figure 14).

To get a qualitative understanding of the effect of the whole brain dissection protocol on SPG sheath integrity, this study analyzed the brains of flies expressing GFP along the membranes of SPG cells. This revealed that portions of the SPG sheath are inadvertently damaged to varying degrees during dissection (Figure 16). However, even when SPG cell membranes are pulled off the brain, intact SPG nuclei sometimes remain. Overall, this study illuminates the age- and sex-dependent toxicity of tau to SPG in *Drosophila melanogaster* while challenging the validity of whole brain dissection to facilitate the analysis of blood-brain barrier (BBB) cells in the fly.

2. Age-Limited Tau Toxicity to SPG in Male Flies

The results from this study indicate that human tau is toxic to SPG in young male flies (Figures 10 and 12). However, the mechanism of this toxicity is unclear. One possibility is that human tau expression triggers apoptosis in SPG. The ability of tau to induce apoptosis of *Drosophila* brain cells is supported by a study in which researchers used TUNEL staining and immunofluorescence against activated caspase to identify apoptosis as a mechanism of glial and neuronal cell death in flies expressing panglial human tau (Colodner & Feany, 2010). However, they noted that while tau expression significantly increases the number of apoptotic brain cells in 20-day-old and 30-day-old flies, this is not the case for 10-day-old flies. Thus, their research

does not provide clear support for apoptosis as a mechanism of tau-induced cell death in young flies such as the 3-day-old males in my study.

An alternative hypothesis to explain the observed toxicity is that human tau inhibits SPG nuclear division in young male flies. While SPG do not undergo full cellular division over the course of fly development, they do undergo nuclear division, resulting in multinucleate cells (Contreras et al., 2024; Unhavaithaya & Orr-Weaver, 2012). Nuclear division is a mitotic process, and tau has previously been shown to disrupt mitosis in *Drosophila*. For example, 4R tau expression in larval eye discs results in the formation of monopolar mitotic spindles instead of the normal bipolar configuration that pulls chromosomes apart in anticipation of cell division (Malmanche et al., 2017). A similar tau-induced perturbation could impair SPG nuclear division, resulting in a decreased quantity of SPG nuclei.

Whether via apoptosis, impaired nuclear division, or another mechanism, human tau exhibits toxicity to SPG in young male flies. However, by the time these flies reach ten days of age, this toxic effect is no longer observed. If tau initially triggers apoptosis, it is possible that SPG compensate for reduced numbers by increasing nuclear division. Alternatively, according to the impaired-division hypothesis, the observed age-specificity of toxicity would suggest that tau delays nuclear division rather than preventing it entirely. This is consistent with the work of Malmanche et al. (2017), who found that 4R tau delays mitotic progression in *Drosophila* neuronal progenitors. Either of these explanations assumes substantial nuclear division between Day 3 and Day 10. However, no significant difference was found in SPG nuclear quantity between these timepoints in tau-expressing male flies. Thus, the reason for the observed age-specificity of tau toxicity to male SPG remains elusive.

3. Making Sense of Sex Differences

In contrast to males, female flies display no significant difference in SPG nuclear quantity between the control and tau genotypes at either timepoint analyzed (Figures 10 and 12). This suggests that some aspect of female sex offers SPG resilience to human tau expression, or, viewed another way, that some aspect of male sex leaves SPG vulnerable to tau toxicity. One aspect of sex that might be at play is hormonal difference.

A recent human-focused review highlights the protective effects of estrogen on the BBB (Weber & Clyne, 2021). Research in rats reveals that estrogen decreases brain mitochondrial production of reactive oxygen species, which are associated with BBB impairment (Razmara et al., 2007; Weber & Clyne, 2021). Additionally, a study combining *in vivo* and *in vitro* methods found that estrogen protects against inflammation-driven BBB defects (Maggioli et al., 2016). Given the role of estrogens as female sex hormones, these results emphasize the potential of hormonal differences to drive sexual dimorphisms in BBB resilience (Cui et al., 2013).

It is debatable whether the impact of sex hormones on the BBB is relevant to flies. As late as 1998, it was widely believed that insects lacked sex hormones (De Loof & Huybrechts, 1998). However, this understanding has fallen out of fashion since growing research suggests that secreted factors acting as sex hormones play a role in driving sex differences in *Drosophila* (Sawala & Gould, 2018). For example, increased levels of the steroid hormone ecdysone in female flies compared to males contributes to sexually dimorphic intestinal stem cell activity (Ahmed et al., 2020). Thus, hormonal differences emerge as a plausible explanation for the sexually dimorphic response of SPG to human tau expression.

Alternatively, sex differences in SPG vulnerability to human tau toxicity may be due to differences in gene expression. In flies, the number of X chromosomes determines the expression of sex-specific transcription factors: DSX, which has a male isoform and a female isoform, and FRU, which is expressed exclusively in males (Arbeitman et al., 2016; Hoxha et al., 2013). These differentially expressed transcription factors in turn drive sex differences in gene expression. Prior research has confirmed that FRU and DSX are active in SPG: SPG-specific RNAi knockdown of either factor results in reduced courtship behavior in male *Drosophila* (Hoxha et al., 2013). Perhaps, in addition to regulating courtship behavior, FRU and DSX drive morphological or functional sex differences in SPG that affect cellular response to human tau expression.

In conclusion, differences in hormone levels or SPG-specific gene expression could drive the observed sex-specificity of tau toxicity. The gene expression hypothesis is most consistent with historical understandings of sex determination in flies, whereas the hormonal hypothesis aligns better with human studies. A combination of both factors could be at play.

4. Examining Morphological Changes

The general reduction in nuclear sphericity in tau-expressing flies indicates that human tau disrupts nuclear morphology in SPG (Figure 14). This is consistent with findings in *Drosophila* neurons. In flies with panneuronal expression of mutant human tau, invaginations of the nuclear envelope were observed along with reduced levels of Lamin, which belongs to a class of proteins involved in nuclear shape and strength (Houben et al., 2007; Frost et al., 2016). It is plausible that the same changes occur in SPG, resulting in decreased nuclear sphericity due to a warped nuclear envelope. If this is true, it is indicative of early-stage tau toxicity: Findings in *Drosophila* neurons suggest that Lamin dysfunction and nuclear envelope invagination occur upstream of apoptosis in tau-transgenic flies (Frost et al., 2016). Furthermore, a subsequent study revealed that tau-induced invaginations of the nuclear envelope likely drive aberrant RNA export (Cornelison et al., 2019). In sum, the reduced nuclear sphericity observed in tau-expressing flies in my study may represent pathological invaginations of the nuclear envelope, although further research is required to test this hypothesis.

5. Comparison to Other *Drosophila* Studies

Effect of Human Tau Expression by* Drosophila *Glial Subtype

In conjunction with prior research, this study supports the notion that the effect of human tau expression differs by *Drosophila* glial subtype. A Colodner Lab alum found that human tau is toxic to cortical glia across age and sex, while the protein increases the quantity of astrocyte-like glia in 3-day-old female flies (Kang, 2023). In light of my own findings, Kang and I have collectively outlined three responses to human tau expression specific to different glial subtypes: widespread cell loss (cortical glia), limited cell loss (subperineurial glia), and limited cell gain (astrocyte-like glia). In order to fully understand the variable impacts of human tau expression on *Drosophila* glia, it will be important to characterize the results of tau expression in the remaining glial subtypes. This will establish a series of distinct fly models of glial tauopathy that may be useful in studying different roles of glia in human tauopathy pathogenesis.

Vulnerability of SPG to Neurodegenerative Proteins

To the best of my knowledge, this is the first study to investigate the specific effect of tau on SPG. However, tau is not the only protein associated with neurodegenerative disease. Some neurodegenerative diseases are associated with the aggregation of proteins with expanded poly-glutamine (polyQ) stretches, whose impact on SPG has been previously characterized by a group of researchers based in Taiwan (Yeh et al., 2018). Using the *moody* driver, these researchers expressed a mutant polyQ-expanded protein (Atxn3-84Q-Myc) in SPG and pseudocartridge glia, which are a specialized type of SPG localized to the lamina (Kremer et al., 2017; Yeh et al., 2018). This resulted in a reversed electrophysiological response to light, severe blood-retina barrier dysfunction, and milder BBB dysfunction (Yeh et al., 2018). Thus, their study demonstrates the toxicity of polyQ-expanded proteins to *Drosophila* when expressed in SPG.

Collectively, my research and the study described above indicate that *Drosophila* SPG are vulnerable to multiple proteins associated with neurodegenerative disease. However, there are some methodological differences that must be considered. For one thing, Yeh et al. investigated the physiological implications of protein expression in SPG, while I focused on SPG nuclear quantity and morphology. Additionally, I looked for sex differences in the results of protein expression, whereas Yeh et al. do not specify the sex of flies used in their study. Finally, Yeh et al. emphasize the role of pseudocartridge glia in their analysis of the blood-retina barrier, whereas I removed the lamina during brain dissection, thus excluding pseudocartridge glia from analysis. It is evident that my results are not directly comparable to those of Yeh et al. Still, when viewed side by side, the two studies provide a strong case for the utility of *Drosophila* SPG in the investigation of protein abnormalities implicated in neurodegeneration.

6. Limitations and Future Directions

Damage to SPG During Dissection

This study highlights the difficulty of analyzing SPG using whole brain dissection. Because SPG reside toward the outside of the *Drosophila* brain, they are frequently damaged during the dissection process, which may artificially reduce the observed numbers of SPG nuclei (Figure 16). Since there is no obvious reason why the extent of this damage would differ by the

genotype, sex, or age of the fly, the significant difference in SPG nuclear quantity between 3-day-old male flies of the control genotype and those of the tau genotype retains validity. However, damage to the SPG sheath varies on a brain-by-brain basis, which seems to inflate within-group variability in SPG nuclear quantity (Table 4). This may mask real between-group differences, preventing them from reaching statistical significance. Thus, findings of statistical equivalence in SPG nuclear quantity do not necessarily preclude meaningful biological differences.

In order to uncover potential false equivalences laid out by this study, the experiment should be repeated with measures implemented to reduce the variability introduced by damage during dissection. One option would be to generate a GFP tau genotype of flies co expressing hisRFP, human tau, and membrane-tethered GFP in SPG. By replacing the tau genotype with this new GFP tau genotype, and the control genotype with the established GFP genotype, the integrity of the SPG sheath could be tracked across experimental conditions. Information from the GFP and DAPI channels could be used to calculate the percentage of each brain's surface covered by SPG, and any samples below a certain percentage could be excluded from analysis. Accordingly, data analysis would be limited to brains with reasonably intact SPG sheaths.

As an alternative to the above proposal, brain dissection could be foregone entirely in favor of paraffin sectioning. This is a technique by which fly heads can be fixed within a block of paraffin wax and then sliced into serial sections that collectively reveal the entirety of the brain (Sunderhaus & Kretzschmar, 2016). By eliminating the need to dissect the brain out from the cuticle surrounding the fly's head, this method would hopefully retain the entirety of the SPG sheath, albeit divided into several sections of brain tissue. In conclusion, methodological innovations are warranted to enable more reliable quantification of SPG nuclei.

Limited Range of Included Ages

In order to examine the effect of age on SPG response to human tau expression, this study included both 3-day-old flies and 10-day-old flies. However, these timepoints cover a fraction of the *Drosophila melanogaster* lifespan, which is about three months (Helfand & Rogina, 2003). To better characterize the impact of tau on SPG over time, this experiment should

be extended to 30-day-old flies. This would enhance comparability to previous studies that have included 30-day-old flies in analyses of the impact of glial tau expression (Colodner & Feany, 2010; Kang, 2023). Additionally, SPG in 30-day-old flies may reveal evidence of BBB degeneration, as occurs in elderly humans (Weber & Clyne, 2021). These considerations merit an extension of the present experiment to older flies.

Limited Scope of Analysis

A compelling argument for the importance of examining the effect of human tau expression on SPG is the logical connection between the roles of this glial subtype in *Drosophila* and the clinical manifestations of human tauopathies. SPG make up a substantial component of the *Drosophila* BBB and play a role in regulating sleep (Coll-Tané et al., 2021; Yildirim et al., 2019). Correspondingly, tauopathy patients often face BBB disruptions and sleep disturbances (Hokelekli et al., 2021; Michalicova et al., 2020; Moretti et al., 2005). In light of these connections, the observed impacts of human tau on SPG nuclear quantity and morphology provide limited support for a role of tauopathy-induced glial damage in driving abnormalities in BBB function and sleep behavior. I draw this conclusion tentatively not only because of obvious differences between humans and flies but also because this study's dependent variables—SPG nuclear quantity and morphology—do not directly address the functional consequences of tau expression.

Two follow-up tests could elucidate the potential impact of SPG-specific human tau expression on *Drosophila* brain function. Firstly, a dye-exclusion assay could be used to assess BBB integrity by measuring the degree to which fluorescent dye injected into each fly's body cavity is successfully excluded from its brain (Bhasiin et al., 2023). Secondly, sleep behavior could be examined by placing flies in an activity monitor that detects motion using infrared light beams, where sufficiently long periods of inactivity are classified as sleep (Coll-Tané et al., 2021). Carrying out these two assays on control and tau flies would go a long way towards characterizing the functional implications of SPG-specific human tau expression, which would in turn provide insight into the possible utility of this intervention to model clinical manifestations of tau pathology.

7. Conclusions and Implications

This study set out to determine how the presence of human tau in *Drosophila* SPG affects the quantity of these cells in the brain. The results reveal that human tau decreases the quantity of SPG nuclei in 3-day-old male flies, an effect not observed in other age-sex combinations (Figures 10 and 12). In the case of SPG, nuclear quantity is not numerically identical to cell count due to the presence of multinucleate SPG (Unhavaithaya & Orr-Weaver, 2012). However, it is a functionally comparable measure of toxic or proliferative effects resulting from cell type-specific protein expression. Thus, the data show that human tau is toxic to the SPG of young male flies.

Careful observation of confocal images collected during the early stages of the study gave rise to two additional questions. Firstly, how does the presence of human tau in *Drosophila* SPG affect the morphology of SPG nuclei? Statistical analysis reveals that human tau generally decreases SPG nuclear sphericity (Figure 14). This is indicative of a tau-induced disruption to nuclear morphology that may occur regardless of age or sex. Secondly, are SPG damaged during whole brain dissection? According to exploratory qualitative analysis, the brain dissection protocol does induce variable damage to the SPG sheath (Figure 16). This suggests that whole brain dissection may be an inappropriate method to analyze the cells of the *Drosophila* BBB.

The present study fits into a larger effort within the Colodner Lab to characterize the effect of human tau expression on *Drosophila* glia by glial subtype as well as fly age and sex (Kang, 2023). Using a convenient, genetically tractable model organism, we hope to create a robust database elucidating potential mechanisms of glial tau toxicity. This foundation of knowledge will be useful to future researchers working towards improved treatment options for human tauopathies. For example, pharmacological interventions could be tested for their ability to modify responses to human tau expression in *Drosophila* glial subtypes. Additionally, mammalian researchers could comb through our data to identify the most promising areas of research to pursue further in rodent studies. These future directions are speculative because the precise outcome of our work cannot be predicted, as is the nature of basic scientific research. However, by combining stubborn hope with meticulous scientific rigor, we just might give the tauopathy patients of tomorrow a better shot at happiness and health.

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