ABSTRACT

The protein tau is involved in the pathology of neurodegeneration through development into aggregates by abnormal hyperphosphorylation events. Tau is mostly expressed in neurons, but tau pathology in glial cells is seen as a hallmark of many neurodegenerative diseases. Traumatic brain injury (TBI) is known to enhance this formation of tau aggregates and relative toxicity from its expression in neurons and glia. While there are emerging discoveries surrounding the relationship between tau and TBI, the time point/exposure level in which TBI can incite robust aggregation and toxicity has not been explored in a cell-type specific disease model. In this study, we used *Drosophila melanogaster* as a model of tau overexpression in astrocytes, a major glial cell of the brain. We then exposed flies to various amounts of TBI at various time points within their lifespan. We found that there was no difference in the presence of tau aggregates at day 10, but there was a significantly higher mortality index at the same time point in flies that were hit on four of the 10 days compared to flies hit on day 9 and flies that were not hit at all. We found that flies hit on day 9 had significantly higher tau pathology at day 30 than flies hit later in life. Additionally, we saw that the females hit day 9 had significantly higher tau pathology at day 30 compared to males with the same TBI exposure. We also saw a decreased lifespan up to day 60 in flies hit multiple times compared to hit once or not at all. These data suggests that toxicity is not linked to aggregate presence, and the tau toxicity associated with the increase in mortality for flies who experienced multiple hits should be explored.

Impact of Traumatic Brain Injury on Astrocytic Tau Pathology in *Drosophila melanogaster*

by

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For Mom, Gran, & Grandpa

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INTRODUCTION

1. Astrocytes

1.1 Glial Cells

In the brain, there are two cell types: neurons and glia. While neurons are well-known for their ability to communicate and incite many essential life functions, glial cells are a subtype of brain cells that perform regulatory functions to maintain the integrity of the brain (Figure 1). These cells were found in the mid-1800s by scientists like Rudolf Virchow, Theodor Schwann and Camilo Golgi; Virchow named these cells 'glia', which comes from the Greek word for glue (Kettenmann & Verkhratsky, 2008; Ndubaku & de Bellard, 2008). This name was given originally because these cells were thought to act as 'brain glue', filling in the holes where neuronal tissue was absent. This led to the belief that glial cells have a passive role in brain activity. Still today, this concept has resulted in less research dedicated to glial cells compared to neurons (Allen & Barres, 2009).

A main reason that glial cells have been ignored in research is due to their inability to produce action potentials like their neuronal counterparts (Stevens, 2003). While they don't stimulate neurons, they are abundant in the brain. In humans, there are equal numbers of glial cells and neurons in the whole brain (Azevedo et al., 2009). In addition to their abundance, neurons rely on glial cells to survive and communicate properly. As more research has emerged regarding the active and crucial role that glia provide to support neuronal function, there are more groups spending time examining this cell type. Through this increase in research, it has been found that these cells are essential for brain activity and actively contribute to it (Kremer et al., 2017). This demonstrates the importance of glia for survival. The increase in research of glia has also allowed for discoveries that better the understanding of neurological disorders. For example, scientists were made aware of the basis of the pathology driving multiple sclerosis, a disease involving autoimmunity of myelin, through research surrounding the glial cells involved in myelination (Barres, 2008). Like this disease, investigation of glia enhances understanding of general brain function and neurological disorders.

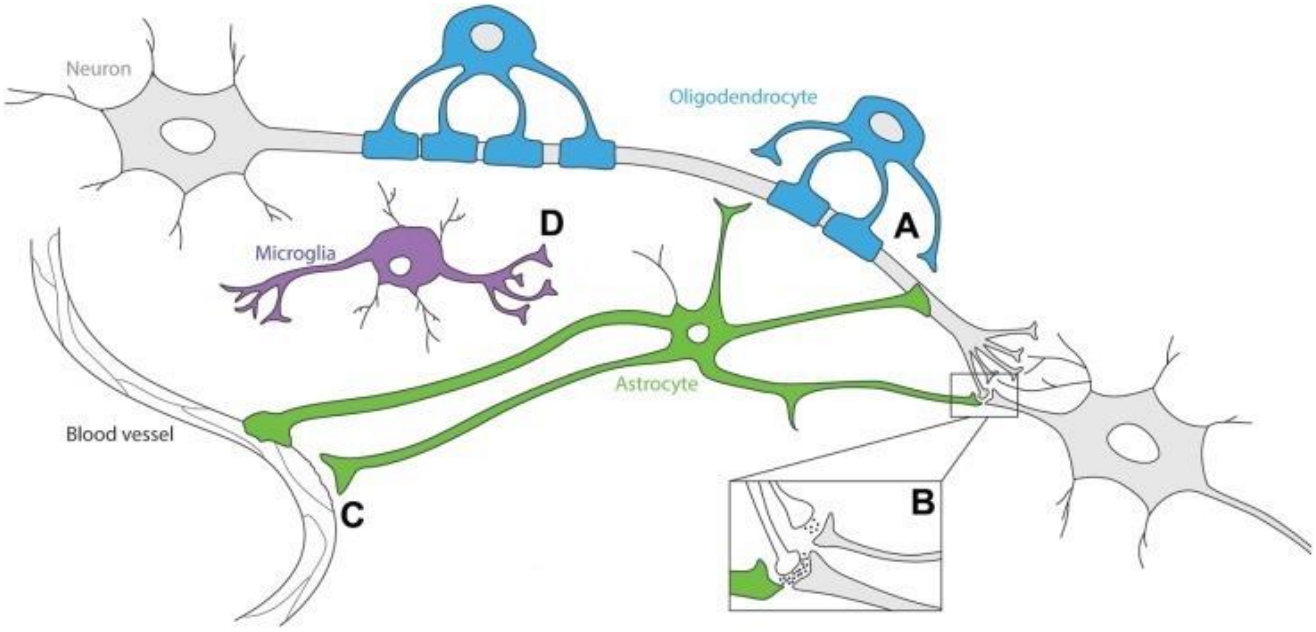

Figure 1. Glial cells have many functions throughout the brain. Astrocytes (green), microglia (purple), and oligodendrocytes (blue) are shown interacting with neurons and, for astrocytes, blood vessels as well. Oligodendrocytes myelinate neurons at multiple points. Microglia are the macrophages of the brain and are essential for any immune responses. Astrocytes support neuronal communication and wrap around blood vessels to regulate the blood brain barrier. Adapted from Kahlson & Colodner, 2015.

Glial cell types can be divided into categories based on their role in the nervous system: maintenance (astrocytes), immune response (microglia), and myelination (oligodendrocytes). This is indicated by their diverse structures (Figure 1). One cell type that is particularly important for normal brain function is astrocytes. Astrocytes fall into the category of maintenance due to their diversified abilities to support the brain. Because of their many roles in the central nervous system (CNS), astrocytes are organized into categories that describe their structure and location in the brain. Astrocytes can be interlaminar, protoplasmic, varicose projection, or fibrous (Vasile et al., 2017). These categories come with many differences among the types of astrocytes. First, the depth that astrocytic subtypes reside in the cortex is diverse (Figure 2). Morphological distinctions between each type of astrocyte exist as well. Intralaminar and varicose astrocytes

have long projections while protoplasmic and fibrous astrocytes are wider (Figure 2). The most abundant type of astrocyte is protoplasmic, and this is thought to be due to its important role in the CNS. One protoplasmic astrocyte can be responsible for supporting 5 blood vessels, 8 somas, and numerous synapses. The exact number of constituents per protoplasmic astrocyte differs based on neuron/blood vessel density per layer (Oberheim et al., 2006). Overall, there are varieties of astrocytes that have specific differences in the CNS.

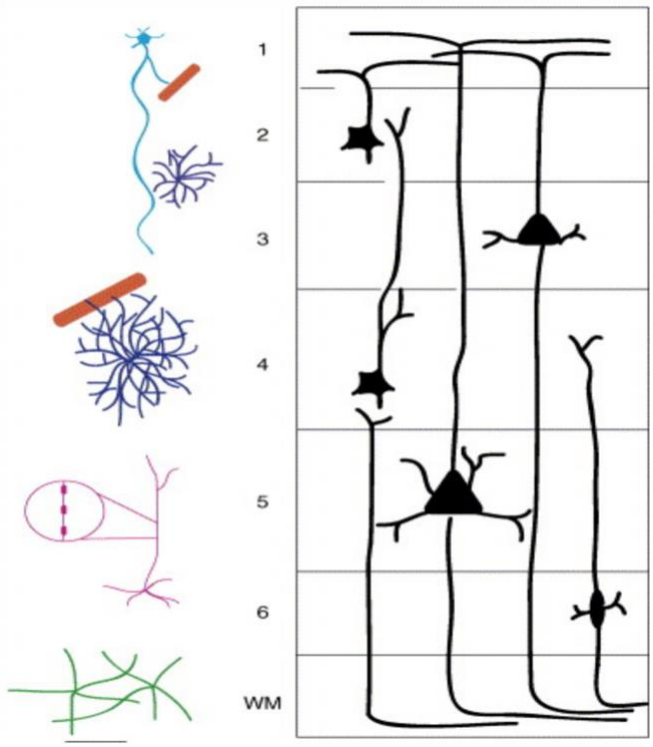

Figure 2. Astrocyte subtypes are morphologically and geographically distinct in the human CNS. Interlaminar (light blue), protoplasmic (purple), varicose projection (pink), and fibrous (green) astrocytes are shown with their respective morphologies and depths of the CNS. Layer 1 through white matter (WM) denotes the layers of the cortex. Interlaminar and protoplasmic astrocytes are shown to interact with neurons and blood vessels (in red). Varicose projection astrocytes are shown with enlarged swells. Scale bar is 100 μm. Adapted from Oberheim *et al.,* 2006.

1.2 Astrocytic Function and Role

Astrocytes are an abundant glial sub-type in the CNS with two main responsibilities: blood brain barrier (BBB) regulation and synaptic communication. Given these two avenues, astrocytes have complex roles to ensure the BBB is functioning properly or impairment of neurons' ability to send and receive action potentials. These responsibilities are in addition to the

role of maintaining neuronal health. For instance, neuronal death that is common in neurodegenerative diseases (NDDs) is associated with a loss of astrocytes (Schipper, 1996). This is partly due to the oxidative stress that occurs in neurons when astrocytes can't modulate the amount of antioxidant that is present in neurons (Drukarch et al., 1998). This is not unique to NDDs because astrocytic dysfunction is involved in the majority of neurological disorders (Molofsky et al., 2012), underscoring how crucial resident astrocytes are in contributing to proper function in the CNS.

Astrocytes are major regulators of BBB. Through this role, one responsibility astrocytes are known for is modulating the amount of blood flow when necessary to maintain homeostasis (Sofroniew & Vinters, 2010). They are able to do this by producing molecules that can initiate dilation of the blood vessels. This is useful because it allows astrocytes to respond to differing neuronal activity and regulate blood flow accordingly (Sofroniew & Vinters, 2010). Astrocytes are also known to maintain BBB integrity. The mechanism behind this function is unknown. Structurally, astrocytic support to the BBB is through the formation of gap junctions (Figure 3). However, the actual seal that prevents blood from leaking into the cerebrum comes from tight junctions in endothelial cells (Dunton et al., 2021; Sofroniew & Vinters, 2010). Gap junctions are channels that connect cells together. Astrocytes are able to create gap junctions through the expression of connexin proteins, which make up these channels that enable these cells to connect (Vasile et al., 2017). These junctions can observe changes in the BBB and communicate to the rest of the astrocyte to attend to those needs. Astrocytes have also shown to be able to induce endothelial cells to become more specialized to support the needs of the neurovascular unit (Abbott et al., 2006). Ultimately, the BBB is highly regulated by astrocytes.

Additionally, astrocytes are heavily involved in synaptic transmission between neurons. This starts with astrocytic ability to influence the formation of functional synaptic connections. When cultured neurons were cocultured with astrocytes, they were able to create working synaptic connections with other cells significantly more than neurons cultured in the absence of astrocytes (Zhang et al., 2016). This demonstrates how astrocytes are highly involved in synapse formation. This involvement relies on astrocytic ability to induce signals for formation (Bolton & Eroglu, 2009; Kucukdereli et al., 2011). Astrocytes are also highly involved in neurotransmitter

reuptake, which prevents excessive activation of neurons and recycles neurotransmitters for energy production (Vasile et al., 2017). These cells are particularly known for their ability to reuptake neurotransmitters like GABA and glutamate (Sofroniew & Vinters, 2010; Vasile et al., 2017). With many ways to regulate neural communication, astrocytes are a major player for the physiology of neuronal communication.

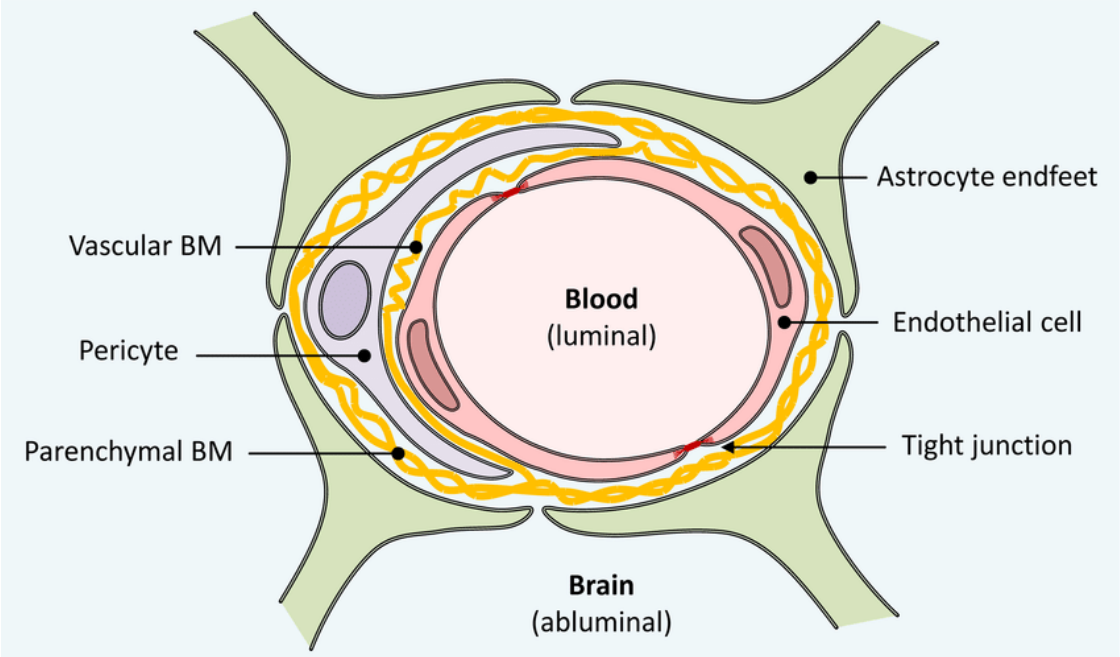

Figure 3. Astrocytic endfeet engulf blood vessels in the CNS. Endothelial cells line blood vessels and are sealed with tight junction proteins. Astrocytic endfeet encase pericytes, the vascular basement membrane (BM), and the parenchymal BM. This seal created by endothelial cells allows for blood to flow within the vasculature of the brain without leaking onto CNS tissue. Adapted from Neumaier *et al.,* 2021.

2. Tau

2.1 Structure and Function

On the 17th chromosome, the *microtubule associated protein tau* (*MAPT*) gene encodes the protein tau, which is expressed mostly in neurons of the CNS. Tau was discovered in 1975 by a research group from Princeton University (Weingarten et al., 1975). Since then, there has been a lot of focus on understanding this protein's role in health and disease. One result of this is the identification of the 6 isoforms of tau. These isoforms differ by either 3R or 4R repeat domains on the C-terminus and by insert domains in the N-terminus of either 0N, 1N, or 2N (Bachmann et al., 2021) (Figure 4). The N-terminal inserts both include 29 more amino acid residues while the C-terminus domain repeats either 31 or 32 respectively for 3R and 4R (Zempel & Mandelkow, 2014). In a healthy brain, splicing is dependent on development; the shortest tau isoforms are found in fetal brains (Johnson & Stoothoff, 2004). Splicing is also influenced by brain region as different isoforms reside in different parts of the brain (Park et al., 2016). This demonstrates that tau's structure is reliant on alternative splicing.

Regardless of isoform, the native protein structure of tau is not compact and flexible (Mandelkow & Mandelkow, 2012). Tau lacks a complex tertiary structure, which leads to a native unfolded structure, which is string-like. This structure allows tau to stabilize microtubules along the axon of neurons (Holtzman et al., 2016). This function is dependent on the three functional domains of tau: C-terminal assembly domain, microtubule binding domain, and N-terminal projection domain (Zempel & Mandelkow, 2014). The projection domain is proline-rich and responsible for positioning tau to bind correctly to microtubules along axons (Barbier et al., 2019; Chen et al., 1992). This occurs through electrostatic repulsion between the amino acids within this domain and the ionic contents of the environment (Mukhopadhyay & Hoh, 2001). Additionally, these proteins have shown a propensity to engage with signaling molecules of the cytoskeleton (Avila et al., 2004; Hirokawa et al., 1988). However, this domain does not bind tau to microtubules (Zempel & Mandelkow, 2014).The microtubule binding domain's name describes its function, which is the binding of tau proteins to microtubules when axon stability is necessary. This binding happens along the entire axon and is independent of the transport of nutrients along axons, which explains why tau does not interrupt motor proteins (Barbier et al., 2019). The C-terminal also binds to microtubules and is central for the assembly of tubulin into

microtubules (Zempel & Mandelkow, 2014). When working together, these domains allow for tau to stabilize microtubules.

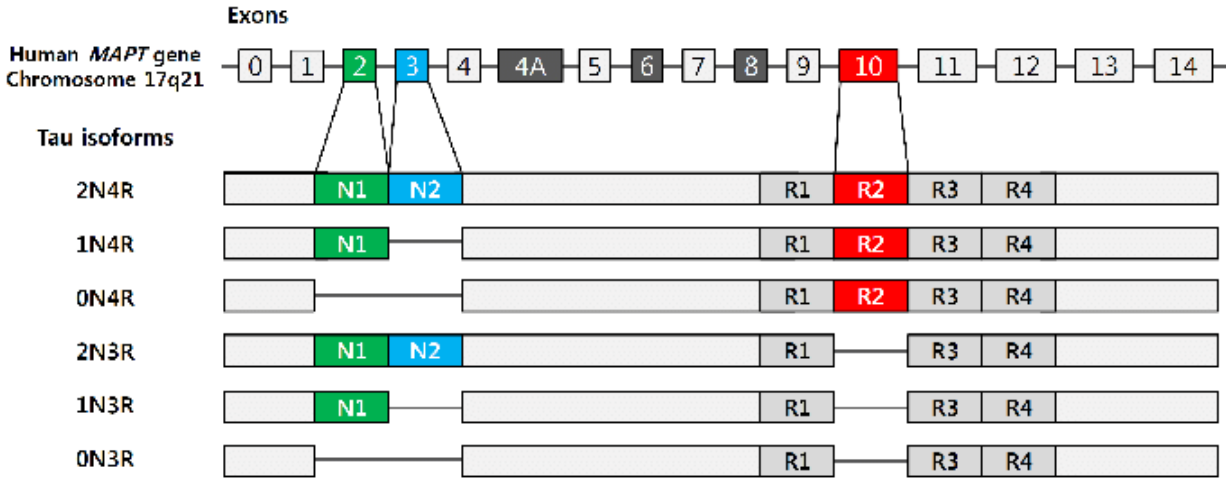

Figure 4. The six isoforms of tau. There are 6 versions of tau that can be synthesized from alternative splicing. On the N-terminus, there can be 1 or 2 inserts and 1 extra repeat domain in the C-terminus region. N-inserts are denoted as 0N, 1N or 2N to represent the number of inserts present, and the repeat domains are written as 3R or 4R based on the number of repeat domains present. This creates isoforms called 2N4R, 1N4R, 0N4R, 2N3R, 1N3R, 0N3R. Adapted from Park *et al*., 2016.

2.2 Hyperphosphorylation of Tau

Tau is known to be highly phosphorylated in health and disease. This is apparent through the fact that 20% of the longest tau isoform has the capacity to be phosphorylated (Goedert et al., 1989; Johnson & Stoothoff, 2004). While the mechanism of misfolding/tangling is still unknown, pathological tau is characterized by unusual phosphorylation events along on the amino acid side chains of the protein (Cowan & Mudher, 2013). The ability for tau to be inappropriately phosphorylated relies on the fact that phosphorylation of tau regulates its ability to stabilize microtubules (Lindwall & Cole, 1984). In physiological conditions, phosphorylation reduced tau's ability to bind to microtubules, which evokes depolymerization of the cytoskeleton. This

has been shown through phosphorylation on specific sites of tau decreasing the protein's binding affinity to microtubules (Johnson & Stoothoff, 2004). An example of an epitope that is important for regulating the dynamics of microtubules is threonine231(Thr231). Thr231 phosphorylation causes tau to disassociate from microtubules, which can induce changes in the structure of the cytoskeleton it is attempting to stabilize. This has been shown through observations of Thr231-phosphorylated tau only being present in the soluble fraction of cell lysates (Johnson & Stoothoff, 2004). Through this, phosphorylation on tau is a major regulator of its physiological function.

In addition to epitopes being related to tau's native function, there are abnormal phosphorylation sites that are indicative of unique forms of tau tangles, which are associated with particular diseases. Overall, there are 85 known phosphorylation sites with more than half being associated with Alzheimer's disease (AD) (Noble et al., 2013). Two key examples of disease-associated tangles are the AT100 and serine422 (Ser422) phosphorylation. For example, absent in healthy tissue, the double phosphorylation site AT100 epitope (Thr212/Ser214) is associated with tau tangles in AD and chronic traumatic encephalopathy (CTE) (Katsumoto et al., 2019; Zheng-Fischhöfer et al., 1998). Another important epitope is Ser422. This phosphorylation site is an indicator of intracellular neurofibrillary tangles (NFTs), which are the hallmark form of tau aggregation (Augustinack et al., 2002). Ser422 is also unrecognizable in healthy adult tissue, which makes it a specific marker for pathological phosphorylation of tau in many different tauopathies (Bussière et al., 1999). This comes from its association with paired helical filaments (PHFs) that are the foundation for NFTs. Residues like Thr212, Ser214, and Ser422 demonstrate pathological versions of phosphorylated tau.

As there are many tau hyperphosphorylation sites, there are several kinases that are responsible for phosphorylating and dephosphorylating physiological and pathological versions of tau. Glycogen synthase kinase 3β (GSK3β) has shown to be involved in both. In health, Thr231 is a primed GSK3β site, which means a different epitope, in this case serine235, must be phosphorylated by GSK3β first (Goedert et al., 1994; Johnson & Stoothoff, 2004). Because Thr231 is implicated in microtubule stabilization, GSK3β can be seen as a mediator of this role. As well as contributing to native tau function, GSK3β has also shown to be implicated in the

hyperphosphorylation of tau in AD, which can be increased by the presence of amyloid-ß (Aß) plaques (Johnson & Stoothoff, 2004). Particularly, GSK3β has shown to be involved in the phosphorylation of the Thr212 site of the AT100 epitope described above (Zheng-Fischhöfer et al., 1998). In addition to GSK3β, cyclin-dependent kinase 5 (Cdk5) also has shown to be heavily involved in the phosphorylation of tau. This kinase has shown to inhibit and increase tau phosphorylation in different environments (Johnson & Stoothoff, 2004). These contradictory results leave scientists examining the actual role of Cdk5. The examination of tau-associated kinases is crucial to understanding mechanisms of microtubule stability and tau aggregation.

Similarly to kinases, there are known phosphatases for tau that are implicated in pathology. Primary phosphatase 2A (PP2A) is a major phosphatase in regards to tau (Hu et al., 2021). PP2A activity decreased in brains of AD patients, which contributes to the irreversibility of hyperphosphorylated tau (Hu et al., 2021). Cdc25 phosphatase is another phosphatase that is relevant to tau pathology. This enzyme is involved in regulating the cell cycle and has been found in post-mortem analyses of AD brains (Oliveira et al., 2023). In *Drosophila*, overexpression of its homolog gene has shown to decrease vacuolization and cell death markers (Oliveira et al., 2023). This suggests that this phosphatase could potentially be disrupted by tau hyperphosphorylation and may be a potential target for therapeutics. Continued investigation regarding the phosphatases and kinases relevant to tau hyperphosphorylation uncover mechanisms that contribute or inhibit disease processes.

2.3 Tau Aggregation

Hyperphosphorylation is an important aspect of tau aggregation because it allows the protein to change its binding affinity. Once hyperphosphorylated, the protein becomes associated with its own microtubule binding domain and loses its unfolded structure (Goedert et al., 1994). This results in tau aggregates that are linked with differing capacities to progress disease or maintain health, which is dependent on the hyperphosphorylation site. Another element of tau aggregation is the different amounts of individual tau proteins that engage with each other within a single aggregate. Once hyperphosphorylated, a monomer of tau can dimerize with another tau protein, which can eventually oligomerize with many other tau proteins. These oligomers have the ability to turn into a PHF, several of which form NFTs. These stages of aggregation have been established but the underlying mechanisms of formation are still being studied.

The levels of protein structure are one avenue shown to be involved in the mechanisms of aggregation. When assessing PHFs, the same hexapeptide from the 3rd repeat domain of native tau was present (Von Bergen et al., 2005). This conservation between primary peptide structure suggests that certain amino acid sequences may be important for aggregation. Additionally, it was shown that the regions of soluble, unaggregated tau that exhibit a random coil structure while the same regions on PHFs have a beta sheet structure, indicating that there is a transition from random coil to beta sheet at some point in the course of aggregation (Von Bergen et al., 2005). The switch from secondary structures suggest that aggregate forms of tau have different properties and function, which may contribute to their relative toxicity. Furthermore, the quaternary structure of tau aggregates varies, which highlights distinct conformations between aggregates of the same isoform. For instance, 4R tau aggregates can take the conformation of 'snake', 'twister', or 'jagged' (Hernández et al., 2022). More inquires on the specific qualities of these aggregates will uncover their role in health and disease.

Although many hypothesize that this aggregation is toxic to neurons, there is evidence for it being nontoxic as well (Cowan & Mudher, 2013). For example, human fetal brain tissue has shown to contain tau aggregates that are not capable of spreading throughout the brain (Hu et al., 2021). This may indicate that aggregated forms of tau throughout the brain may be an underlying requirement for toxicity. No matter the reason, the purpose of these fetal aggregates is still unknown, but some hypothesize that these aggregates allows for axonal growth in the developing brain (Hu et al., 2021). Therefore, understanding aggregation involves studying tau at many levels.

2.3.1 Propagation of Aggregates Through Tau Seeding

Tau seeding is the propensity for already aggregated tau to induce native tau to become misfolded and aggregate (Gibbons et al., 2019). This concept comes originally from prion disease, which is a neurological disease where prion proteins seed and spread within the tissues of the brain to contribute to neuronal death. While prion proteins from one organism can be transmitted to another as a mechanism of disease spreading, tau proteins have not shown the ability to be transmitted between individuals through bodily fluids like prions are (Irwin et al., 2013). Therefore, tau can be regarded as a 'prion-like pathogen' (Gibbons et al., 2019). Tau seeding is a hallmark of tau pathology because it is one known way for aggregates to spread

throughout the brain. While the trigger for tau aggregation is unknown, this is one way in which tau can become tangled.

The mechanism through which a seed is able to induce the hyperphosphorylation and misfolding of a native tau protein is still unknown. However, seed competent aggregates are tangles that have the ability to induce native tau proteins to misfold. Not all aggregates have this ability, so understanding which ones do is important in understanding tau's propensity to spread throughout the brain. One known factor that contributes to the development of seed-competency starts with the structural level of aggregates. Monomeric tau has not shown the ability to perform as a seed (Goedert & Spillantini, 2017). This is important to distinguish because monomeric tau can enter other cells but has not been shown to encourage aggregation of native tau proteins. While it is known that monomeric tau is not seed competent, there are conflicting studies on how many tau proteins are necessary for seeding. However, there is consistent evidence demonstrating that larger oligomers are seed competent (Mudher et al., 2017). Specific hyperphosphorylation sites are also important in inducing seed-competent aggregates (Hu et al., 2021). Aggregates positive for AT100 hyperphosphorylations are seed-competent among many others (Mudher et al., 2017). Unrelated to phosphorylation sites, specific residues are also important modulators of seed competency. For example, the 4R tau isoform cannot act as a seed when residues 275-280 and 306-311 are absent (Falcon et al., 2015). These examples illustrate that there are many avenues that can influence seed competence.

In addition to understanding what can influence the ability for tau to seed, many groups are evaluating what contributes to promoting seeding. Some studies show that the biochemical environment present increases the ability for tau fibrils to seed. For example, dystrophic neurites associated with Aβ plaques have shown to be ideal environments for tau seeding in AD (He et al., 2018). Jain *et al.* 2023 showed that increasing the activity of TREM2, a receptor on microglial cells highly involved in AD-associated neurodegeneration, increases tau seeding in mice (Jain et al., 2023). This demonstrates that mechanisms independent of tau also can play a role in tau seeding.

2.4 Glial Tau

The role of glial tau in the progression or pathobiology of NDDs is still unknown. While glial cells do not express the same amount of tau as neurons in normal states, glial tau

aggregation is a common occurrence in many tauopathies, which mostly occurs in oligodendrocytes and astrocytes (Kahlson & Colodner, 2015). Transmission of glial tau aggregates can occur in oligodendrocytes independent of neuronal tau expression, but astrocytic tau expression alone cannot do this; therefore, astrocytic tau pathology relies on neuronal tau (Narasimhan et al., 2020). Even though glial tau expression is very low in non-diseased brains, the ratio between glial and neuronal tau pathology is not consistent among NDDs, with some diseases having more glial tau pathology than neuronal (Kahlson & Colodner, 2015). Many groups artificially overexpress tau in glial cells as a way to model glial tau pathology and learn about the consequences of their aggregation in respective glial subtypes or in all glial cells. With this overexpression system, pan-glial tau expression has shown to shorten lifespan of *Drosophila* (Colodner & Feany, 2010). Additionally, overexpression of tau in murine glia contributes to neuronal and glial cell death (Forman et al., 2005; Narasimhan et al., 2020). While overexpression of tau in glia is not a facet of human disease, glial cell death is a common occurrence in NDDs (Narasimhan et al., 2020). Therefore, evaluating glial tau pathology through artificial overexpression of tau in many models complements its effects in human disease.

2.4.1 Astrocytic Tau Aggregation

While astrocytes express tau at low levels relative to neurons in normal conditions, astrocytic tau aggregates are common in many NDDs (Figure 5). Interestingly, astrocytic tau expression does not increase in diseases like AD, so it is assumed that a large portion of tau aggregation comes from endogenous sources (Chiarini et al., 2017). Neurons that die are thought to release their tangles into the extracellular matrix (ECM), which are referred to as ‘ghost tangles’. These NFTs have the ability to enter astrocytes via their processes (Fleeman & Proctor, 2021). The mechanisms of this intake of tau are unknown. However, recent studies have found that tau fibrils can enter astrocytes through the integrin αV/β1 receptor (Wang & Ye, 2021). Thus, mechanisms on ghost tangle integration into astrocytes are being uncovered by research like this.

Once tau has entered an astrocyte, astrocytic tau pathology is displayed in many morphologies including tufted astrocytes (TA), astrocytic plaques (AP), ramified astrocytes (RA), thorn-shaped astrocytes (TSA), globular astroglial inclusions (GAIs), and granular fuzzy astrocytes (GFA) (Kovacs, 2020). These morphologies differ in the brain region where tau

accumulates and can be specific to certain diseases (Figure 5). GFAs and TSAs are both found in aging-related tau astrogliopathy (ARTAG). However, TAs and RAs are involved in diseases like Pick's disease (PiD) and progressive supranuclear palsy (PSP). While both are present in ARTAG, TSA aggregates are found in the cell body and processes of astrocytes. This differs from GFA aggregates, which are mostly located in the astrocytic branches and right outside of the cells' nucleus (Kovacs, 2020). The mechanisms that allow for the development of the diverse array of astrocytic aggregates are not known but having knowledge regarding which type of aggregates are present in particular diseases is relevant to postmortem diagnosis.

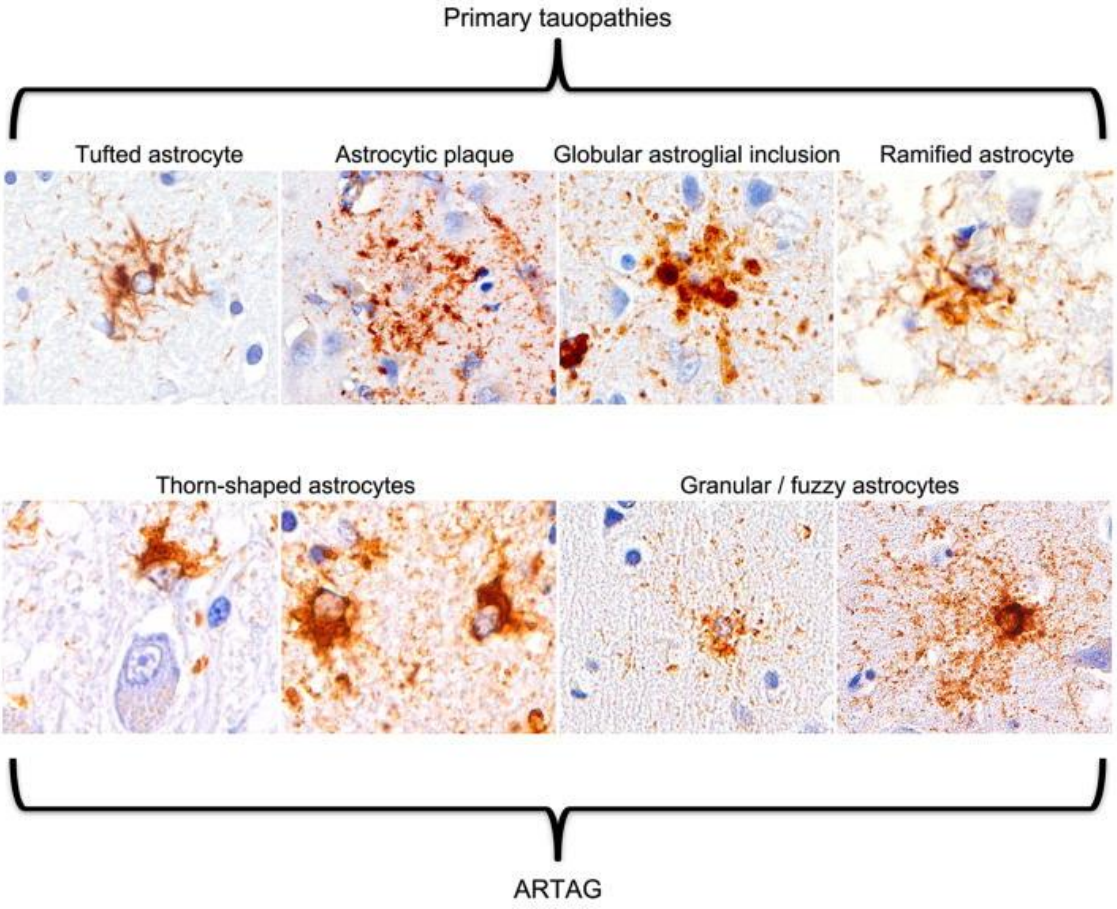

Figure 5. Astrocytic tau aggregates manifest in diverse ways. AT8+ postmortem human astrocytic tau aggregates are shown labeled with their respective category of aggregate. TAs, APs, GAIs, and RAs are associated with primary tauopathies. However, TSAs and GFAs are distinct to ARTAG, which, on its own, is an aspect of primary tauopathies' cytomorphology. Adapted from Kovacs *et al.,* 2016.

2.4.1.1 Consequences of Astrocytic Tau Pathology

While neuronal tau aggregation is associated with neuronal death, astrocytic tau aggregation has been shown to disrupt several of their own functions in the CNS, rather than inducing cell loss. The effects of astrocytic tau aggregation are thought to be due to altered gene expression, as expressing 4R tau in murine astrocytes evokes a neurotoxic genetic signature within the cells (Ezerskiy et al., 2022). The change from homeostatic to neurotoxic has shown to

deter their ability to function, which results in infiltration of the BBB and decreased glutamate regulation (Ezerskiy et al., 2022; Kahlson & Colodner, 2015). One known mechanism that induces this astrocytic dysfunction is a change in the structure of astrocytes, which is related to the change in gene expression. This structural shift increases neuroinflammation because the introduction of the new structure is accompanied by an increased expression of cytokines (Leyns & Holtzman, 2017). Neuroinflammation has shown to decrease astrocytic regulation of glutamate and to insult the integrity of the BBB because this disrupts the aspects of the cellular architecture that usually prevent BBB leakage (Fleeman & Proctor, 2021; Tilleux & Hermans, 2007). Through deterring multiple responsibilities of astrocytes, astrocytic tau aggregation disrupts homeostasis within the CNS.

As well as inciting astrocytic dysfunction, astrocytic tau aggregation hinders neurons from performing their native functions. These impairments on astrocytic function have shown to have broad effects on neuronal mechanisms for survival. One process that is deterred by this is a neuron's ability to support its axonal and synaptic components. In a rat model of astrocytic tau pathology, kinesin function was altered, limiting the amount of vesicles transported to the axon terminal (Yoshiyama et al., 2003). This impairs the neuron's ability to transport and receive necessary nutrients to the end of the cell's axon, which could result in neuronal dysfunction or death. Additionally, the astrocyte-expressed cytokines that induce neuroinflammation take a toll on neuronal function. Presence of the cytokine TNF-α has been shown to increase neuronal tau aggregation when present in culture (Fleeman & Proctor, 2021). Additionally, the overall health of neurons is disrupted by exposure to pro-inflammatory cytokines (Fleeman & Proctor, 2021). Through these processes, astrocytic tau aggregation reduces neuronal function and longevity.

3. Neurodegenerative Diseases

3.1 Overview of Neurodegeneration

Neurodegenerative diseases are illnesses that are known to shrink the brain due to neuronal loss. This physical loss of brain mass can result in symptoms such as dementia, insomnia, and impaired motor control. The symptomatology is dependent on the region in the brain and the type of neuron that is known to degenerate. In many NDDs, specific proteins aggregate in certain parts of the brain and elicit disease-specific pathologies. There are many distinct protein aggregates that are involved in different NDDs, which result in symptoms unique to the disease. For example, one symptom of Parkinson's disease (PD), which involves aggregation of α-synuclein proteins, is hand tremors. Another instance of this is in cerebral amyloid angiopathy patients who experience seizures that are linked to Aβ plaques interfering with BBB integrity. There is currently no cure for any NDDs, and most diseases don't have effective treatments that target disease mechanisms, but instead rely on therapeutics that target the symptomatology.

Another aspect that differentiates NDDs from each other is the type of cell they target. Neurons are at the forefront of pathology in NDDs, and each disease targets subgroups of neurons based on their particular type and location. This specific cell dysfunction/degeneration allows for a diverse array of symptoms associated with each disease. PD, for example, is a condition that results in a loss of control over motor function. These symptoms are related to the degeneration of dopaminergic neurons in the substantia nigra region of the midbrain, and decreased presence of dopamine has been shown to manifest in symptoms like tremors (Kalia & Lang, 2015). Other NDDs affect neurons that utilize neurotransmitters like acetylcholine and GABA (Young, 2009). Therefore, specific types of signals are depleted in NDDs, which results in a manifestation of abnormal behavioral symptoms. This is directly linked to the loss of neurons that are capable of sending and receiving these signals. In addition to the death of specific neurons, neurons with different functions or locations eventually degenerate as disease progresses. Likewise, glial cells are also impacted by these diseases. Glial cells have been shown to decrease in numbers in many NDDs and are functionally deterred by protein aggregation (Kurosinski et al., 2002). NDDs elicit both neuronal and glial cell death, which is relative to each disease.

3.2 Tauopathies

Tauopathies are a major class of NDDs characterized by the aggregation of the protein tau. Some of these diseases include AD, PiD, and PSP (Figure 6). Tauopathies range from two types: primary and secondary. Primary tauopathies are diseases where tau pathology is the main source of pathology, whereas secondary tauopathies feature tau pathology, but it is not the main or only biomolecule associated with disease (Hu et al., 2021). PiD and PSP are primary tauopathies while AD is a secondary tauopathy due to the concomitant presence of Aβ plaques. This is one of many ways to classify tauopathies.

To understand tauopathies, many groups are investigating aspects of tau pathology that may contribute to the toxicity of these diseases. One avenue of research is reliant on the role of tau aggregation in the degenerating brain. This is due to a large amount of evidence that connects the presence of tau aggregation to overall loss of neurons. This belief relies on the finding that the number of NFTs present in human AD brains is positively correlated with the level of cognitive decline (Arriagada et al., 1992). The correlation between these two aspects of AD suggests a role for aggregates in neuronal dysfunction due to the association between cognitive decline and loss of neurons (Terry et al., 1991). Another aspect of tau pathology that is being studied is how different tau isoforms generate varying presence of aggregate and levels of toxicity. This is relevant to tauopathies because different isoforms of tau are associated with different tauopathies. Some tauopathies display tau pathology that is derived from more than one isoform. This is seen in diseases like CTE and AD because both have 3R and 4R tau aggregates (Cox et al., 2016). Other tauopathies have one isoform of tau to aggregate. PSP and PiD demonstrate this because PSP has 4R tau pathology while PiD has 3R tau (Cox et al., 2016). Understanding the mechanisms of pathology in different tauopathies helps highlights what parts of the protein are contributing to the disease.

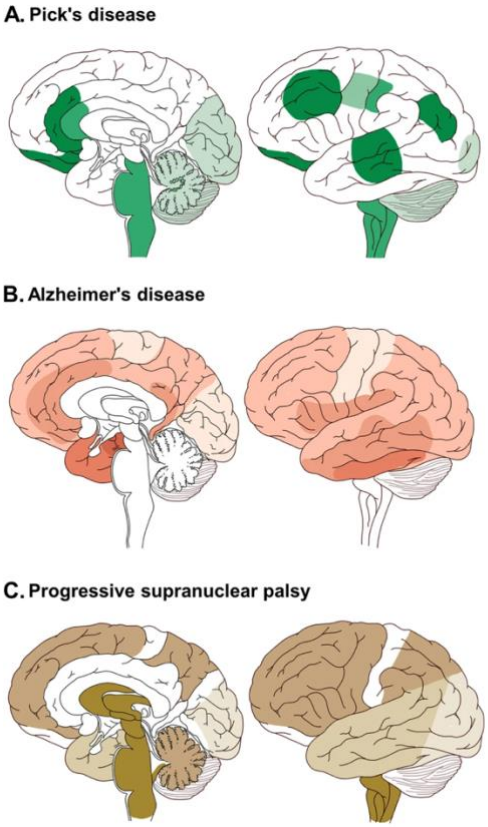

Figure 6. Tau pathology progression in several tauopathies. Tau pathology is described by region in three different tauopathies. PiD is shown in green (A), AD in pink (B), and PSP in brown (C). Darker shades of the respective colors demonstrate how early in the disease the tau pathology is present, so these regions have tau pathology at the start of the illness. Lighter areas are where tau pathology eventually spreads to. Adapted from Zhang *et al*., 2022.

3.2.1 Reactive Astrocytes

A common symptom of tauopathies is reactive astrogliosis or reactive astrocytes. Astrocytes are considered reactive once they change their homeostatic genetic signature, which influences their morphology and function. This is usually in response to disease or injury in the brain (Escartin et al., 2021). This event causes them to activate molecular processes distinct from their homeostatic processes that develop based on the severity and type of injury (Sofroniew, 2015b). The particular changes that occur are diverse and specific to the event that caused the pathology in the CNS (Sofroniew, 2015a). Reactive astrogliosis occurs in many contexts, so it is

important to identify common features among these different events. Highlighting these shared aspects helps identify these cells, regardless of what caused the switch from homeostatic to reactive.

The genetic signature of astrocytes changes once it transforms into a reactive cell. This change in genetics ultimately leads to changes in the protein present. This is one mechanism researchers have taken advantage of to identify reactive astrocytes in many contexts. The most widely used protein marker is the glial fibrillary acidic protein (GFAP) (Eng et al., 2000). This is due to it being robustly present in post-mortem AD brains at the time when reactive astrogliosis was first discovered (Liddelow & Barres, 2017). Even though reactive astrocytes are measured using GFAP localization in the research of many other diseases/injuries, the presence of it in AD brains is what brought this marker into the spotlight. The GFAP protein is known to be a part of the cytoskeleton and can increase the length of astrocytic processes in the CNS (Figure 7) (Yang & Wang, 2015). While it is in widespread use to identify reactive astrocytes, some researchers believe that it is not the best indicator of this astrogliosis (Escartin et al., 2021; Liddelow & Barres, 2017). This is due to the lack of GFAP presence in models of traumatic injury and the high GFAP presence in developing astrocytes unaffected by injury or disease (Escartin et al., 2021). To continue the use of this marker and maintain credibility of labelling reactive astrocytes, some recommend the use of multiple markers that will encapsulate the diversity of reactive astrogliosis.

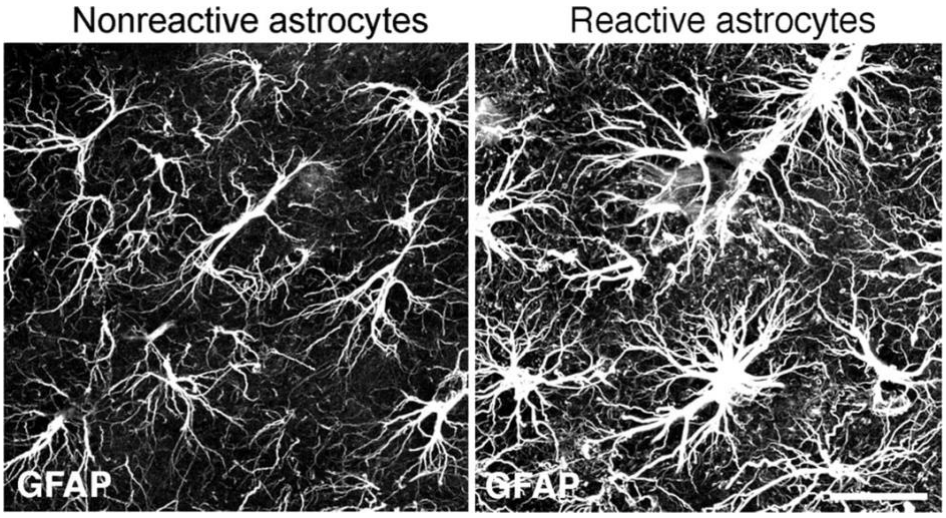

Figure 7. Reactive astrocytes express higher levels of GFAP. The presence of GFAP is shown in nonreactive astrocytes and reactive astrocyte using immunohistochemistry. The reactive astrocytes were collect from the dentate gyrus of mice four-days post unilateral lesion in the entorhinal cortex. Nonreactive astrocytes were obtained from the injured side of the dentate gyrus. Adapted from Wilhelmsson *et al*., 2006.

While reactive astrocytes are present in the CNS for various reasons, tauopathies are known to have robust reactive astrogliosis. All tauopathies elicit the switch from homeostatic astrocytes to reactive. However, tau aggregation within astrocytes is not met with reactive astrogliosis (Togo & Dickson, 2002). So, astrogliosis likely comes from tau pathology derived from neuronal tau expression. One reason this appears to be true is because reactive astrocytes respond to any neuronal damage (Ferrer, 2018). This also demonstrates why there is a high presence of reactive astrocytes in tauopathies because tauopathies feature large amounts of neuronal damage and death. Overall, tauopathies are prone to switch astrocytes from homeostatic to reactive.

Robust astrogliosis in tauopathies doesn't serve its evolutionary purpose and is known to be dysfunctional in some cases. Appropriate activity of reactive astrocytes is known to support and repair injured tissue, but defective reactive astrocytes can be toxic and cause worsened pathology (Sofroniew, 2020). Specifically, the presence of reactive astrocytes in tauopathies has

shown to contribute to the pathological symptoms as well as be a response to fight pathology. When assessing post-mortem tissue, one group identified a larger presence of GFAP+ astrocytes in the superior temporal sulcus in AD patients with dementia symptoms compared to AD patients without dementia symptoms (Perez-Nievas et al., 2013). Similarly, the level of GFAP present in the PS19 mouse model of tauopathy positively correlated with behavior deficits (Patel et al., 2022). Although the mechanism is unknown, the presence of reactive astrocytes is implicated in worsening symptoms generally present in NDDs.

4. Traumatic Brain Injury

4.1 Primary and Secondary Injuries

Traumatic brain injury (TBI) is an injury that causes damage to the brain and is a common occurrence that impacts many people, with an average of 1.5 million cases per year. While this is a common injury, the treatments available for TBI victims are not effective across all patients (Michinaga & Koyama, 2021). Because injuries from one individual to the other are diverse, categories exist to acknowledge the different types of damage that these injuries elicit. Damage is categorized as either a primary or secondary injury. The primary injury of a TBI event is the physical impact that contacts the head, which may result in bleeding or bruising. Secondary injuries are the pathologies that occur after the injury that might be present throughout the rest of the patient's lifespan (Saatman et al., 2008). Secondary injuries incite many pathologies in the CNS like neuroinflammation and cell death, but can also induce synaptogenesis to create new connects where the loss/damage to neurons occurred (Michinaga & Koyama, 2021). Having these categories gives attention to both tiers of the injury that occurred, so that they are both diagnosed and treated properly.

The level of secondary injuries can differ based on how often an individual was exposed to TBI and when the injury occurred in context to brain development. Although it is a common conception that brain plasticity is able to support children who experience TBI, many research groups have found that the earlier in life a TBI event occurs will result in more difficult/unsuccessful recoveries (Anderson et al., 2005; Andruszkow et al., 2014). One group found that infants who had mild TBI had worse recovery outcomes than children with severe TBI events when comparing brain function at the same age (Anderson et al., 2005). These findings are supported by the belief that biological cues related to development are disrupted by TBI, which deters the individual from recovery. In addition to the timing of injury, the amount of TBI also plays a role in how the severe the secondary injuries are. Single TBI exposures have the ability to induce pathology like brain atrophy, tau pathology, and Aβ plaques (Johnson et al., 2011; Smith et al., 2013). However, more exposure to TBI results in an increased risk for more severe secondary injuries compared to single exposure to TBI (Smith et al., 2013). There is still a lot to be learned about the pathological consequences influenced by the frequency of TBI events. This gap in knowledge is partly due to the difficulty of studying chronic or single TBI events.

This is because it is difficult to get a consistent group of subjects who have experienced single or chronic TBI in a similar way. Exploring experimental models of chronic TBI in comparison to single injuries will illustrate the differences between these timepoints.

4.2 Pathological Consequences of Traumatic Brain Injury

4.2.1 Traumatic Brain Injury Deters Astrocytic Function and Limits Recovery

Astrocytic response is a part of recovery to any kind of disruption to the CNS, including TBI. Astrocytes are key in sensing damage and recruiting proper cells and biomolecules to repair the damage they are sensing. Astrocytes perform this function through the use of cell surface receptors that recognize damage-associated molecular patterns (DAMPs) (Yuan & Wu, 2022). This demonstrates that TBI can evoke an immune response because these receptors are involved in recruiting transcription factors that initiate gene expression of cytokines and chemokines, which increases neuroinflammation (Shi et al., 2018). Additionally, single and repetitive exposures to TBI has shown to increase GFAP+ astrocytes in several areas of the mouse brain, indicating the presence of reactive astrocytes (Ojo et al., 2013). Therefore, as a means of recovery, the function of astrocytes is modified by exposure to TBI at many levels.

In addition to trying to repair the injury, astrocytic function is disrupted by TBI. When an injury occurs, astrocytes quickly become reactive, as mentioned above, and increase the expression of structural and cytokine genes (Chen & Swanson, 2003). This initiates gliosis, recruiting microglia and macrophages, and increases inflammation. While astrocytes can use this function to protect and repair the brain, too much inflammation and gliosis can be harmful. The effects of this involve disruption of the BBB and surrounding tissues (Michinaga & Koyama, 2021). TBI-affected astrocytes also show an inability to control neuronal excitability through a lack of glutamate regulation. The inability to regulate glutamate allows neurons to increase Ca^{2+} signally, which induces oxidative stress due to the product of increased calcium activity (Yuan & Wu, 2022). Through these mechanisms, astrocytes are no longer able to attend to injuries and promote recovery because TBI has disrupted their function.

4.2.2 Traumatic Brain Injury Exacerbates Tau Pathology

Tau pathology is especially enhanced by TBI. Robust amounts of NFTs have shown to be present after a single TBI occurrence in postmortem human brains (Johnson et al., 2011). Additionally, one study found that there were significantly higher levels of total phosphorylated

tau from individuals who had severe TBI compared to those who experienced moderate TBI (Yang et al., 2017). Using the same subjects, this group found correlations between individuals who experienced severe TBI and lower phosphatase activity in comparison to individuals who experienced moderate TBI. They also found a similar correlation between the severe TBI and higher kinase activity (Yang et al., 2017). These findings highlight a potential mechanism where tau phosphorylation increases as an individual experiences a more severe injury, and the phosphatases that could potentially revert the hyperphosphorylations are stunted.

In addition to general tau pathology, tau seeding increases when TBI is present. TBI is hypothesized to be a trigger for the formation of seed-competent tau tangles, which leads to a widespread presence of tau aggregation in the CNS (Edwards et al., 2017). Zanier *et al.,* 2018 tested this by exposing mice to a single, severe TBI event and collecting the brain homogenates from these mice. They then inoculated mice who experienced no injury with the brain homogenate and found that NFTs were present (Zanier et al., 2018). They also found that doing this procedure using brain homogenates from mice who were not exposed to TBI elicited a significantly lower NFT burden than the mice who received the TBI homogenates (Zanier et al., 2018). This demonstrates the ability for tau tangles associated with TBI to spread within the brain and potentially incite more pathology in regions that were previously unaffected.

Previous studies have shown that TBI also increases the hyperphosphorylation of tau proteins in glial cells and promotes glial tau toxicity (Byrns et al., 2021). There is less research regarding TBI's influence on glial tau, but some groups have characterized this relationship. Specifically, TBI has shown to incite tau aggregation in astrocytes in mice (Kahriman et al., 2021). Analyses of postmortem human tissue have also confirmed that TBI elicits astrocytic tau pathology (Arena et al., 2020). This demonstrates the ability of TBI to enhance tau pathology in cell types other than neurons.

4.2.3 Traumatic Brain Injury Enhances Neurodegeneration

TBI is a risk factor for many NDDs. This is especially true regarding the risk of developing tauopathies as well. Through this, there is a large emphasis on the risk TBI brings to develop AD and CTE, but there are many other NDDs that are incited by TBI. For instance, PD and amyotrophic lateral sclerosis (ALS) risk increases in the presence of TBI (Acosta et al., 2015; Gupta & Sen, 2016). Exact mechanisms that lead to increased risk for many NDDs are not

yet known, but it is suspected to be linked to the creation of reactive oxygen species that can act as mediators for activation of NDDs (Cruz-Haces et al., 2017). In addition to risk increase, the amount of NDD-specific protein aggregates increases in people who are exposed to TBI. In AD, Aβ plaques are shown to be present within four hours of a TBI event and can even show up 2.5 years later (Finnie & Blumbergs, 2002). PD-associated α-synuclein pathology was shown to increase in the presence of TBI (Acosta et al., 2015). Overall, TBI is a major regulator of the presence of NDDs and their associated pathologies.

4.2.3.1 Chronic Traumatic Encephalopathy

First known as 'punch drunk syndrome' and then 'dementia pugilistica', CTE is a tauopathy that is highly associated with exposure to traumatic brain injury. CTE has only been diagnosed in people who have experienced TBI (Mckee et al., 2016). This is unique for NDDs because it shows a very strong correlation between environmental risk and neurodegeneration, which means there is opportunity for prevention. Additionally, genetics don't seem to be involved in the acquisition of CTE, which is a stark difference from any of NDDs. This makes CTE an avenue for understanding mechanism that initiate neurodegeneration because there are no genetic processes that initiate this disease. Understanding and noticing differences between the brains of non-TBI patients versus those who have experienced TBI could illuminate neurodegenerative generators in the CNS. It is not known what amount of TBI is required to introduce the disease, as the amount of exposure to TBI that will elicit CTE varies. This is demonstrated though the diversity among age and stage of CTE (Bieniek et al., 2021).

4.2.3.1.1 Vulnerable Populations

While this disease does not have known genetic risk factors, there are specific populations that are at high risk for CTE. Athletes are particularly at risk of TBI and, therefore, also at risk from suffering from CTE at a higher rate than non-athletes (McKee et al., 2009). Currently, there is an emphasis on its prevalence on professional and collegiate American football players. Since CTE was first described, the National Football League has received a lot of concern from families of players and fans regarding the vulnerability of professional footballers to this degenerative disease. The NFL and affiliated teams have used these critiques to justify monetary donations to research institutions dedicated to understanding the mechanisms of this disease and ways to prevent and treat this specific tauopathy. More understanding on how

specific sports increase risk for CTE will hopefully bring forth regulations to protect athletes from acquiring this disease.

In addition to professional athletes, there are other reasons for someone to become at risk for this disease. This is due to the potential for TBI to occur in many scenarios. Specific examples of at-risk populations include those who are exposed to explosives and people who face violence. Blast injuries are those that occur in individuals who are present when a bomb or explosive weapon is set off (Goldstein et al., 2012). These occurrences result in mild TBI to those who are present in the surrounding environment relative to the blast. Those who are exposed to blast-related TBI have also shown to have similar cognitive symptoms as CTE patients (Goldstein et al., 2012). Another group of people who are susceptible to CTE are those who experience violent attacks. Violence-based TBI can occur in many scenarios. Domestic abuse victims are beginning to be diagnosed with this disease, as well. Since 1990, CTE has been identified in multiple individuals who were victims of domestic abuse (Danielsen et al., 2021; Roberts et al., 1990). Therefore, there are many avenues in which someone could acquire CTE.

4.2.3.1.2 Diagnosis Requirements

CTE has its own pathological hallmarks among other tauopathies. The key feature of CTE pathology is the aggregation of 3R and 4R tau aggregation surrounding blood vessels in both neurons and astrocytes. This aspect of CTE brings attention to how the BBB is affected, since tau aggregation is known to reduce BBB integrity. Additionally, tau aggregation around the BBB has shown to induce neuroinflammation (Figure 8), which, can disrupt the efficacy of the BBB in many ways (Michalicova et al., 2020). Another hallmark of CTE pathology is astrocytic tau tangles at the sulcal depths of the brain (Mckee et al., 2015). This means that there is a concentrated presence of astrocytic tau aggregates at the deepest grooves in the sulci in the brain. Knowing the pathological hallmarks of CTE help doctors recognize this disease in post-mortem tissue.

The diagnosis requirements for CTE are still influx due to the novelty of the disease. As scientists are continuing to understand this disease, it is important to be able to distinguish it from other common tauopathies. More than a third of those diagnosed with CTE have showed to also meet the requirements to be diagnosed with other NDDs like Lewy Body Disease, AD, and frontotemporal lobar degeneration (DeKosky & Asken, 2017). Previously, it was required for

astrocytic tau tangles to be present around blood vessels in addition to NFTs (Mckee et al., 2016). However, astrocytic tau tangles are no longer required for diagnosis. This is due to the difficulty of distinguishing between CTE and ARTAG (Bieniek et al., 2021). Currently, to diagnose CTE, NFTs are the only type of tau tangle required for diagnosis. Although astrocytic tau aggregates are no longer required for diagnosis, their role in disease is still being explored due to their large presence in many CTE cases. Another aspect of CTE diagnosis is the staging of the disease. There were originally 4 stages of CTE that could be assigned by autopsy. The staging of CTE was adjusted into a simpler system that uses descriptions of low and high CTE. This change was encouraged by the results of a study where physicians could agree on CTE diagnosis but had a difference in opinion when attempting to stage the level of CTE that each patient had (Bieniek et al., 2021). Overall, the diagnostic tools for CTE will improve as a broader understanding of the disease is established.

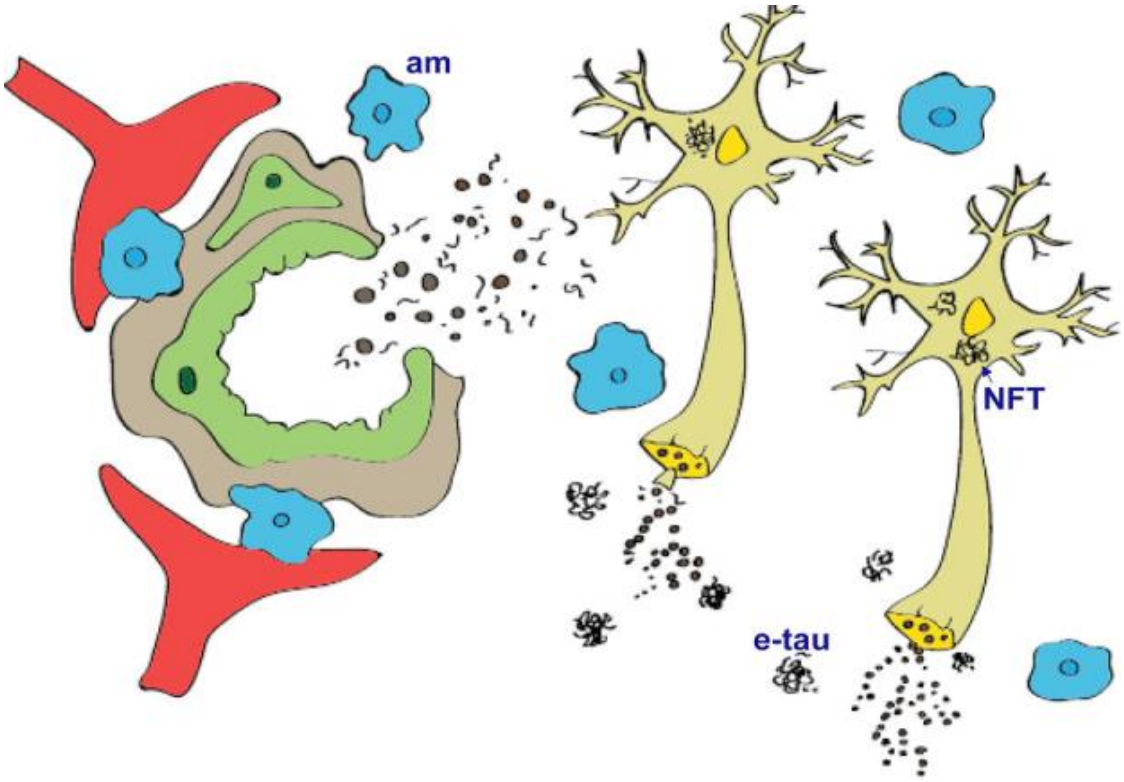

Figure 8. NFTs around blood vessels result in BBB infiltration. The BBB is made up of astrocytes (red) that encapsulate tight junctions (grey) which seal endothelial cells (green) together. When extracellular tau (e-tau) tangles activate microglia (am), an increased presence of cytokines and chemokines induces inflammation that can cause a disruption of the BBB. Adapted from Michalicova *et al.,* 2020.

5. *Drosophila melanogaster*

5.1 *Drosophila melanogaster* as a Model for Biomedical Research

Drosophila melanogaster, commonly known as the fruit fly, has been used in biomedical research for a long time. Throughout their use as model organisms, fruit flies have been used to understand and uncover mechanisms of neurogenesis, learning and memory, and synaptic communication (Bellen et al., 2010). In addition to this, there is a long history of using fruit flies to study neurodegenerative diseases (Ma et al., 2022). This is due to their ability to be genetically manipulated and because they have homolog genes that complement approximately 75% of the human genome (Cowan et al., 2011). The similarities between humans and fruit flies enable scientists to make use of these invertebrates as a means to learn more about the biology of humans in health and disease.

While comparable to humans, the *Drosophila* nervous system has a unique structure (Figure 9A). This is important to mention because it informs the way scientists can make conclusions about general mammalian biology through *Drosophila* research. First, fruit flies have hemolymph as an alternative to blood. They do not use it to transport oxygen, but instead to only transport nutrients throughout their body. Within their brain and throughout their body, an invasive network of trachea transport oxygen within the tissue. This tracheal network in the CNS is similar in structure to the vasculature within the human CNS. Hemolymph also coats the *Drosophila* brain instead of CSF in animal brains. Morphologically, the actual structure of the brain is wider than the human homolog but is organized in a similar way. The neuronal cell bodies are sectioned into the outer cortex regions while the axons stretch into the center of the brain, which called the neuropil region (Kremer et al., 2017). Two optical lobes extend on both sides of the head. While abundant, the differences between the *Drosophila* and human CNS do not prevent scientists from using these as a model to understand the brain in health and disease.

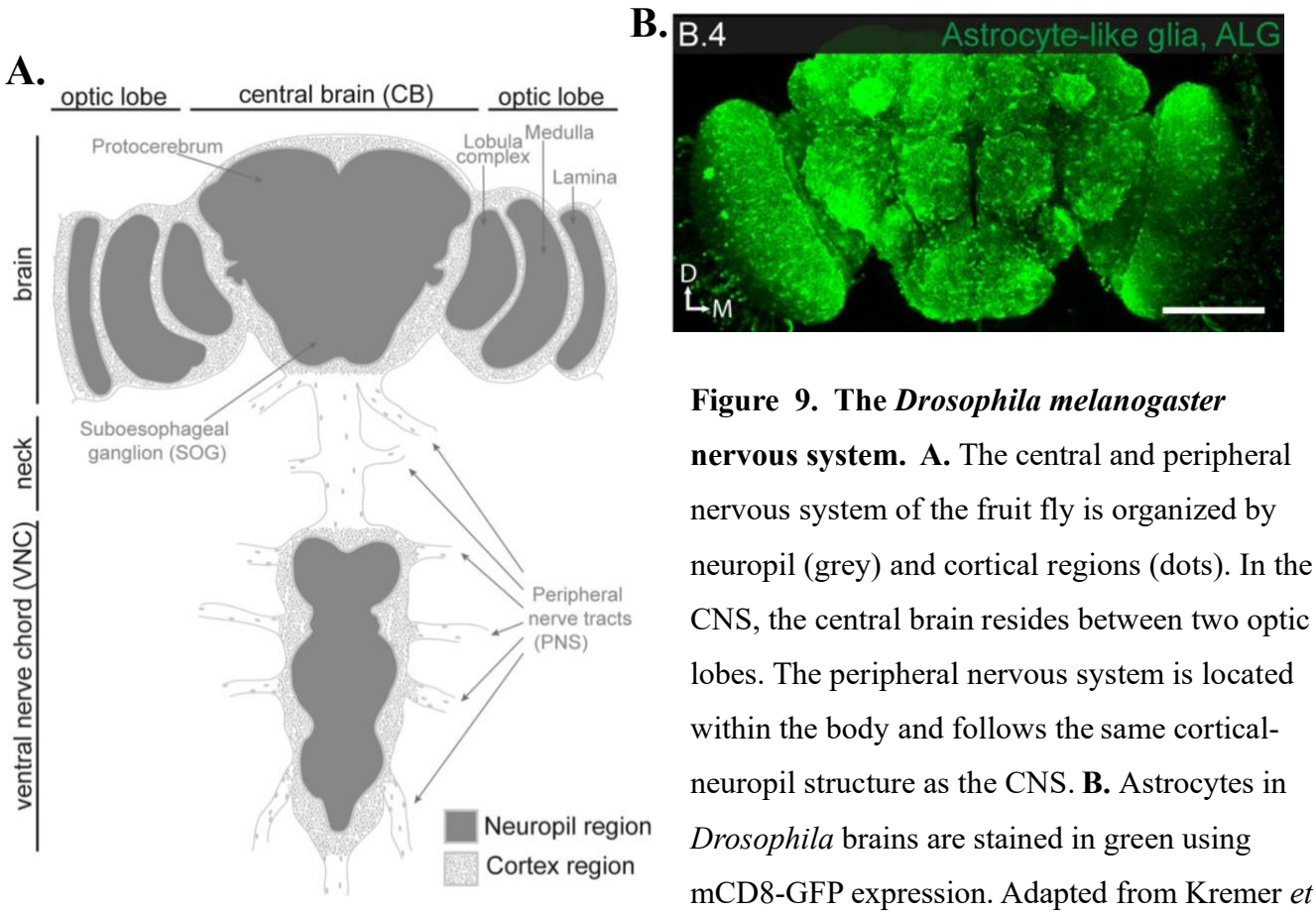

Figure 9. The *Drosophila melanogaster* nervous system. A. The central and peripheral nervous system of the fruit fly is organized by neuropil (grey) and cortical regions (dots). In the CNS, the central brain resides between two optic lobes. The peripheral nervous system is located within the body and follows the same cortical-neuropil structure as the CNS. **B.** Astrocytes in *Drosophila* brains are stained in green using mCD8-GFP expression. Adapted from Kremer *et al.,* 2017.

5.2 Astrocytes of *Drosophila melanogaster*

In terms of glial cells, fruit flies have six glial subtypes that complement human glial subtypes (Cowan et al., 2011). This has allowed for their use as a model for understanding glial cells and their function, following the first discoveries of glia's existence (Ndubaku & de Bellard, 2008). This use of the *Drosophila* model to understand glial function and roles have encouraged the discoveries of many glial functions. Such discoveries include exposing glia's ability to prune axons in development and identifying the necessary signals for brain cells to differentiate into either neurons or glia (Molofsky et al., 2012).These discoveries are only some among many others.

Drosophila astrocytes have many similarities to mammalian astrocytes. However, differences between the two are still present and relevant to any study using these invertebrate cells. Structurally, all fruit fly astrocytes are most similar to protoplasmic astrocytes. This highlights the first difference between *Drosophila* and human astrocytes, which is the lack of diversity among types of astrocytes present in the fly (Freeman, 2015). Additionally, the location of where astrocytes reside is different in the fly compared to the human (Figure 9B). Fly astrocytes are present in the neuropil region, but some also reside in the lamina (Kremer et al., 2017). Contrastingly, human astrocytes extend all over the brain. The function of astrocytes in fruit flies also are similar to their mammalian counterparts. For example, both species' astrocytes have shown to be involved in neuronal homeostasis (Freeman, 2015). These similarities allow for scientist to use *Drosophila* to understand mammalian biology in health and disease.

5.3 *Drosophila* as a Model of Tau Pathology

Fruit flies allow scientists to model both neuronal and glial tau pathologies. Even though fruit flies express their own version of the tau protein, it is common in tauopathy research to express/overexpress human tau in specific fruit fly cell types (Cowan et al., 2011). Using human tau creates a model that is more physiological to the tau aggregation that occurs in human disease. This allows for similar processes that may occur in reaction to human tau in the human brain. For example, neuronal degeneration enhanced by tau pathology also occurs in fruit flies (Iijima-Ando & Iijima, 2010). Through these similarities, use of this model has been used to understand pathological processes of tau pathology like phosphorylation activity (Marsh & Thompson, 2006). Interestingly, fruit flies that express human tau in neurons do not form NFTs as seen in diseases like AD and CTE (Wittmann et al., 2001). Some groups believe that this feature of the fly tauopathy model reveals that there is a role in neurodegeneration for smaller aggregates called oligomers (Lee et al., 2005). In contrast, these advanced aggregates, named 'glial fibrillary tangles', are present when human tau is overexpressed in all glial cells (Colodner & Feany, 2010). This contradiction between the presence of advanced neuronal and glial aggregates leaves a lot of questions unanswered about the *Drosophila* model of tauopathy. However, this model is still valuable because it serves as a vessel for discoveries surrounding the toxicity of human tau aggregates.

5.4 *Drosophila* Models of Traumatic Brain Injury

Drosophila melanogaster have been used for many years to model TBI. One important structural similarity between flies and mammals that allows for *Drosophila* to model TBI is because the fly brain is protected by cuticle, which can be compared to the skull of humans (Aggarwal et al., 2022). This commonality allows for the injury produced through experimental mechanism to mimic human experience because there are the similar organs protecting actual brain tissue. This similarity and the fact that *Drosophila* are small organisms that reproduce quickly encouraged the development of many TBI paradigms specific to *Drosophila* that can be used to model many types of injuries (Figure 10). Additionally, these models try to replicate specific types of TBI as a means of addressing the diverse ways TBI may occur in humans. For example, there is a blast wave simulator that tries to encapsulate the injuries that come from exposure to explosives (Figure 10D) (Aggarwal et al., 2022). In addition to the type of injury, researchers have the ability to control how specific the injury is to the head. One research group developed a machine that elicits injury only on to the head (Saikumar et al., 2020). In this model, they are also able to control the severity of the injury in addition to the specificity (Figure 10B). Models like these give researchers tools that can be used to inform clinician understanding of TBI primary and secondary injuries. Groups have used these models to identify genes regulated by TBI and how specific genes can increase/decrease lifespan of the fly (Byrns et al., 2021; Swanson et al., 2020). Because many scientific findings have come from use of these models, fruit flies are regarded as an appropriate model organism for experimental TBI research.

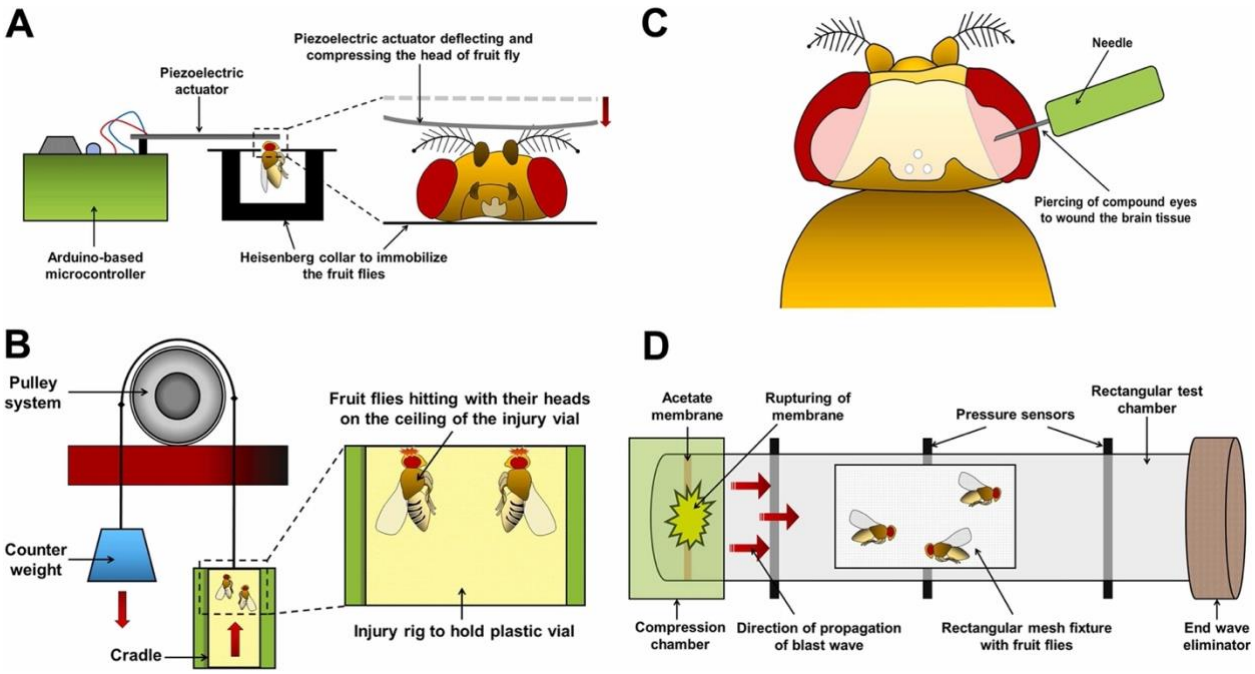

Figure 10. Several paradigms exist to expose *Drosophila* to TBI. A. The piezoelectric actuator device is used to elicit a head specific TBI event through tapping the head of a single fruit fly, which compresses its brain. **B.** Using a pulley system, the head-first impact model causes flies to be hit on top of their heads because the lack of gravity present when ejected upward. **C.** Penetrative TBI is evoked through directly disturbing the brain by piercing it with a needle. **D.** In the blast wave simulator, pressure causes the acetate membrane to break, and the pressure causes flies to bang around their contained space. Adapted from Aggarwal *et al.,* 2022.

5.4.1 High Impact Trauma Model of Traumatic Brain Injury

Katzenberger *et al.,* 2013 developed the high impact trauma (HIT) device, which was the first model developed to induce TBI on *Drosophila*. Because this model causes a non-specific injury, it is thought to replicate TBI that may be due to car accidents or sports injuries (Aggarwal et al., 2022). To induce TBI, flies are placed in empty vials, secured with a cotton plug, that is attached to a metal spring (Figure 11). The vial is pulled back to the angle of deflection, which can be adjusted to support what each study is exploring. As the vial is released, it bangs against a polyurethane pad. In the first records of HIT use, a dose-dependent decrease in lifespan was observed, validating the reproducibility of the HIT model (Katzenberger et al., 2013). This

technique is especially relevant in studying TBI's relationship with NDDs because common pathologies of a degenerating brain were found to be present in flies who were exposed to TBI (Katzenberger et al., 2013). As this technique is widely used in modeling TBI with *Drosophila*, the HIT device was utilized in this project as well.

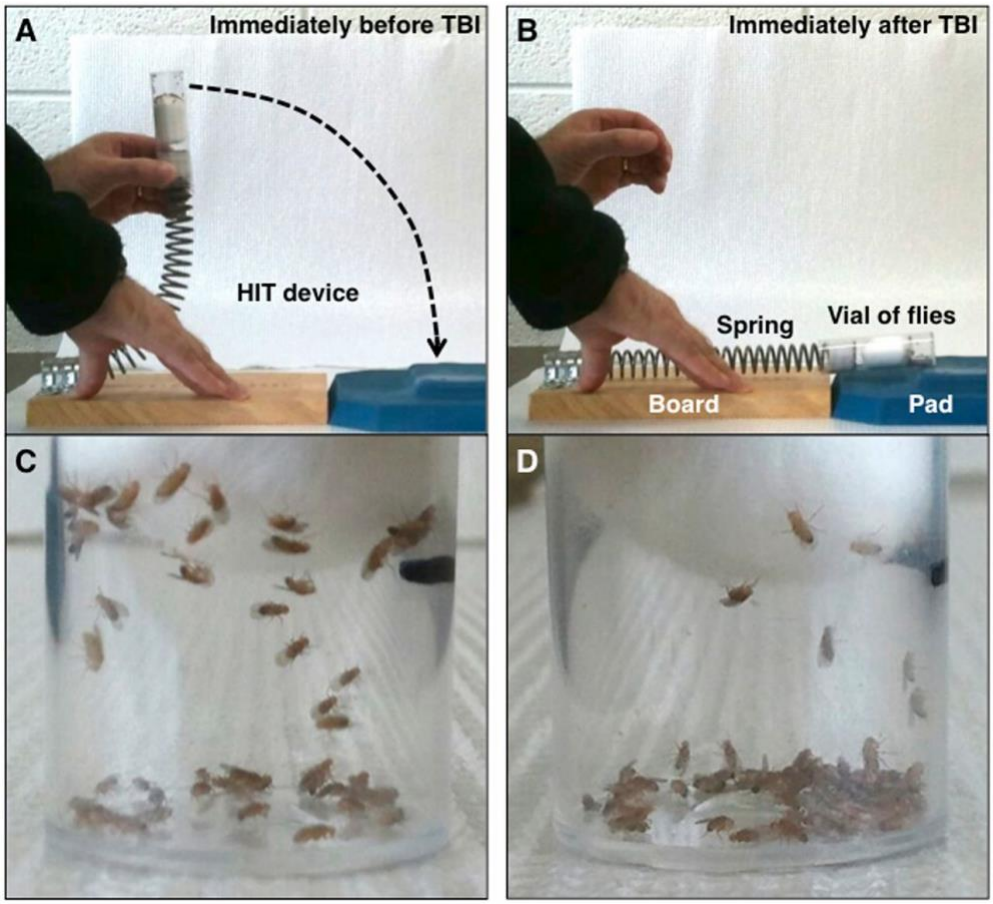

Figure 11. HIT device exposes flies to TBI. The TBI flies are exposed to using the HIT mechanism disrupts their behavior. Flies are seen flying and moving within their vial before TBI and are concentrated at the bottom of the vial after injury. Adapted from Katzenberger *et al.,* 2013.

6. Aim of Study

6.1 Purpose and Importance

The purpose of my thesis is to assess how TBI affects tau aggregation in astrocytes. Because these pathologies all have their own detrimental effects independent of one another, it is important to examine them in unison. In addition to this, there are currently few analyses published on TBI's effect on glial tau pathology, with even less on astrocytic tau pathology. This gap in knowledge should be filled because it could uncover specific pathways for neurodegenerative pathologies that may be associated with glial dysfunction.

6.2 Summary of Study

For my thesis, we examined the specific effects of different TBI paradigms on tau aggregation that comes from an overexpression of human tau in astrocytes. We used a *Drosophila* model of astrocytic tau pathology and induced TBI at different frequencies and timelines. We hypothesized that earlier and more chronic models of TBI will have the highest presence of tau aggregation and related toxicity compared to astrocytic tau flies that experience late or no TBI. This is due to TBI's ability to interrupt development due to secondary injuries. In addition to this, we hypothesized that the presence of TBI in addition to an overexpression of human tau will be more toxic compared to TBI in flies that don't express human tau. To address these questions, we performed experiments that assess how TBI influences the presence of astrocytic-tau tangles and the toxicity of astrocytic tau in fruit flies. Immunostaining allowed for the visualization of tau aggregates with differing hyperphosphorylation sites. Performing a lifespan study up to day 60 and mortality index at day 10 highlighted how the interaction between astrocytic tau and TBI influences homeostasis in the whole organism. These analyses combined illuminate the specific aggregation patterns of tau proteins and how those aggregates impact the ability for *Drosophila* to perform mandatory bodily functions that keep them alive.

MATERIALS AND METHODS

1. *Drosophila melanogaster* and the GAL4/UAS System

To create a model that expressed human 0N4R tau specifically in astrocytes, we utilized the GAL4/UAS system in the common fruit fly, *Drosophila melanogaster* (Figure 12). GAL4 is a transcription factor native to yeast, which is known to bind to and activate transcription at a specific Upstream Activation Site or UAS (Duffy, 2002). Scientists can make transgenic flies with GAL4 attached to genetic markers of specific tissues, which allows for protein expression in the specified locations/cell types. The genetic code for the protein of interest is attached to a UAS that is used to make transgenic flies. Our lab receives flies that have already been generated with either GAL4 or UAS construct in their genome. Using these stocks, we can cross a fly with a GAL4 construct to a fly with a UAS construct, which creates progeny that express a gene of interest in a tissue-specific manner (Figure 12). In the case of our project, attaching the GAL4 gene to an astrocyte-specific gene promoter allows for the GAL4/UAS system to only be activated in astrocytes.

1.1 Stocks and Controls

Due to stock availability, we used a stock of *astrocyte-GAL4* flies that contained *UAS-histone red fluorescent protein* (hisRFP) on the same chromosome as the GAL4 construct, though the hisRFP was not utilized for analysis in this project. *Astrocyte-GAL4, UAS-hisRFP* flies were crossed with *UAS-tau* flies, to express human tau in the astrocytes of *Drosophila* (Table 1). The controls for this project include crosses of *astrocyte-GAL4, UAS-hisRFP* with *white minus* (*W-*) flies and *astrocyte-GAL4, UAS-hisRFP* with *UAS-lacZ* (Table 1). *W-* flies are a stock that have a mutation that causes white pigment in the eyes instead of red. This negative control is used widely in research using fruit flies because it is the stock of flies used to create either GAL4 or UAS construct. Both constructs contain the *W+* gene, which provides a red-orange pigment in the eye. So, scientist can phenotypically check if the genetic insertion was successful by observing a red-orange eye in the flies that were injected (Duffy, 2002). Using *UAS-lacZ* as a protein control allows for *lacZ*, an enzyme that breaks down lactose, to be expressed. This control helps differentiate the effects of protein overexpression in general compared to the specific effects that are associated with human tau expression. All crosses

occurred at 25 degrees in plastic vials filled with corn meal-based food on a 12-hour light/dark schedule. Once crossed, the samples used for analysis were also kept in the same conditions.

To denote any crosses using the GAL4/UAS expression system, '>' will indicate the GAL4/UAS system. The cell type where the gene is expressed will be written before the '>', and the gene that is being expressed will be written after the '>'. For example, *astrocyte-GAL4, UAS-hisRFP/UAS-tau* will be written as *astrocyte>tau*. Because crosses with W- flies do not involve any gene expression, they will be denoted as *astrocyte-GAL4/-*.

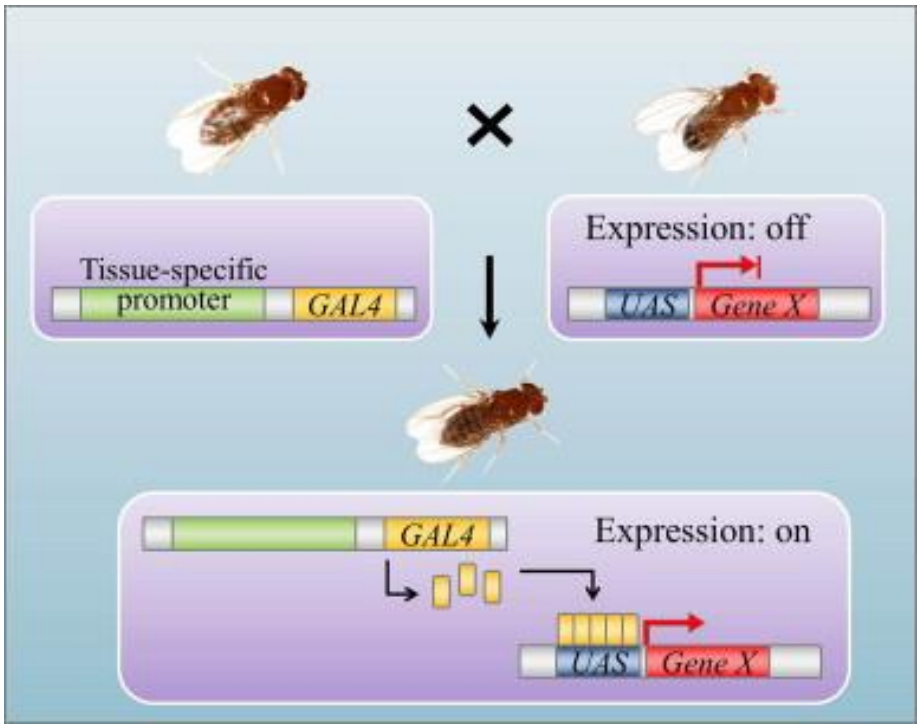

Figure 12. Using the GAL4/UAS system with *Drosophila melanogaster.* Virgin females with a tissue specific GAL4 construct are crossed to male flies with the UAS constructs attached to a gene sequence that, when crossed with the virgin female, will lead to tissue specific gene expression in the progeny. Adapted from Cho *et al.,* 2014.

Table 1. Fruit fly models created using the GAL4/UAS promoter driver system.

Stock	Virgin Female	Male	Progeny used in experiments
W-	$\frac{+}{+},\frac{+}{+},\frac{Astrocyte-GAL4,UAS-hisRFP}{Astrocyte-GAL4,UAS-hisRFP}$	$\frac{-}{-},\frac{+}{+},\frac{+}{+}$	$\frac{-}{+},\frac{+}{+},\frac{Astrocyte-GAL4,UAS-hisRFP}{+}$
LacZ	$\frac{+}{+},\frac{+}{+},\frac{Astrocyte-GAL4,UAS-hisRFP}{Astrocyte-GAL4,UAS-hisRFP}$	$\frac{+}{+},\frac{+}{+},\frac{UAS-LacZ}{UAS-LacZ}$	$\frac{+}{+},\frac{+}{+},\frac{Astrocyte-GAL4,UAS-hisRFP}{UAS-LacZ}$
Tau	$\frac{+}{+},\frac{+}{+},\frac{Astrocyte-GAL4,UAS-hisRFP}{Astrocyte-GAL4,UAS-hisRFP}$	$\frac{+}{+},\frac{+}{+},\frac{UAS-Tau}{Tm3sb}$	$\frac{+}{+},\frac{+}{+},\frac{Astrocyte-GAL4,UAS-hisRFP}{UAS-Tau}$

2. Induction of Traumatic Brain Injury

TBI was induced using the high impact trauma device (Figure 13) (Katzenberger et al., 2013). This is a whole-body impact paradigm, which means that injury occurred throughout the body and not only to the head. On the designated day or days, flies were placed in an empty plastic vial attached to a metal spring. The vial was plugged and positioned at a 90-degree angle relative to a flat table. Flies underwent either single or multi-hit paradigms (Figure 14). A single hit is one session of 4 strikes against a polyurethane mat with a 5-minute break between each strike. Multiple hits are single hit sessions that take place multiple days over the span of a week (Figure 14). Both multiple hit paradigms gave the subjects a rest day after a session of a single hit. Therefore, there were no paradigms where TBI occurred two days in a row, three days in a row, etc. If used for histological analysis, flies were fixed in 10% formalin on either day 10 or day 30 and sent to Pioneer Valley Life Sciences Institute to be processed for paraffin sectioning.

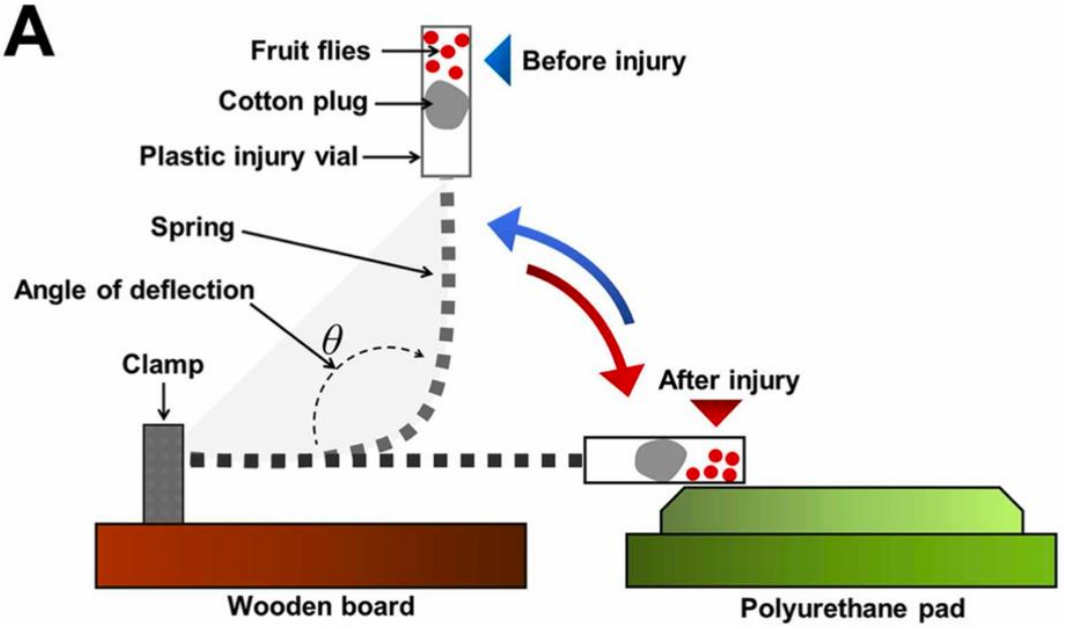

Figure 13. HIT paradigm. Flies are loaded into an empty plastic vial and attached to a spring. The spring is pulled back to 90 degrees and lands on a polyurthane pad. Adapted from Aggarwal *et al.,* 2022.

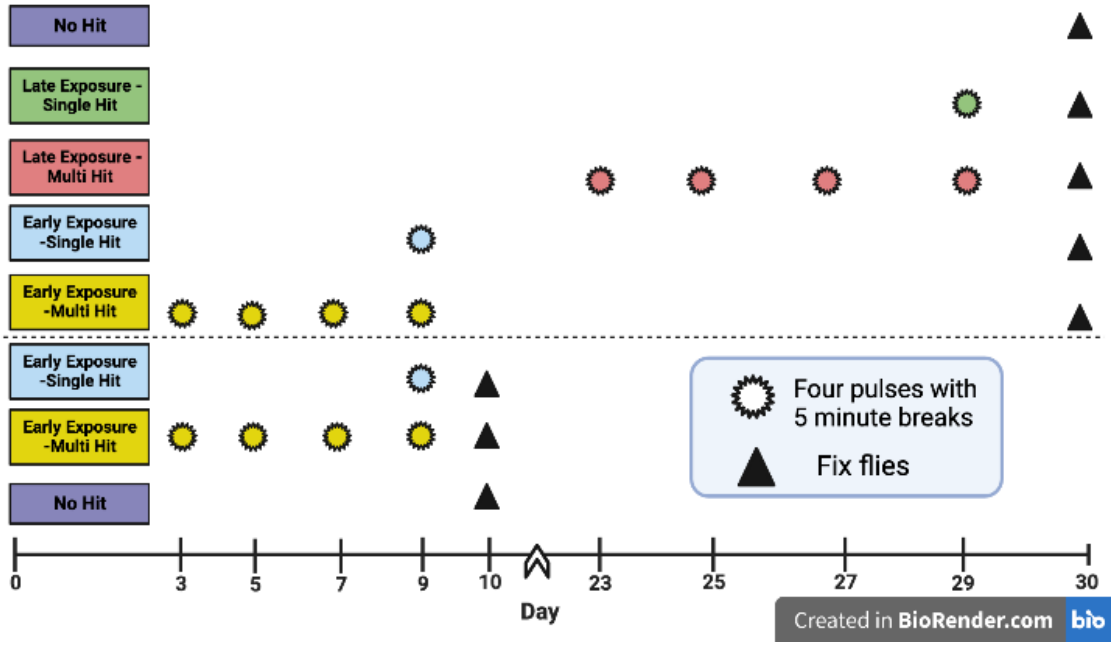

Figure 14. TBI induction paradigms and their corresponding timelines. TBI was administered early or late in life. Injury occurred once, multiple times, or not at all. Two age points were examined for protein assays: day 10 and day 30, which were the days when the flies were fixed. Flies exposed to early TBI were aged to both day 10 and day 30. The groups of flies that were exposed to early TBI were hit on days 3, 5, 7, 9 or just day 9. Flies exposed to late TBI were aged to day 30 and hit on days 23, 25, 27, 29 or just day 29. Groups that experienced no TBI, called 'no hit' groups, were also aged to either day 10 or day 30.

3. Paraffin Sectioning and Immunostaining

We utilized paraffin sectioning and antibody staining as a way to visualize hyperphosphorylated tau tangles present in brain tissue of the flies (Figure 15). After fixing and processing the flies for paraffin sectioning, we removed the head from the body and embedded it into a paraffin wax. Once hardened, we sectioned the tissue at 4μm using a Lecia RM 2135 manual rotary microtome. Sections were transferred onto precleaned Fisher Brand *Superfrost Plus* microscope slides via a water bath at 37 degrees. Slides were incubated overnight at 37 degrees and were processed through a histoclear-ethanol (EtOH) series at room temperature to remove the paraffin wax from the slides. This series includes 2 histoclear incubations for 5 minutes each followed by 2 washes of 100% EtOH, 1 wash of 95% EtOH, and 1 wash of 70% EtOH for 2 minutes per wash. After, slides incubated in 2 washes in deionized water (dH_2O) for 5 minutes per wash.

Once deparaffinized, the slides were ready to be processed for antibody staining. Antigen retrieval occurred through slide incubation in 1X sodium citrate, pH 6.6 for 15 minutes in the microwave. Slides were then allowed to cool before continuing to the next steps. Once cooled to room temperature, slides were briefly washed in 1X phosphate buffered saline (PBS) and 1X PBS with 0.3% TritonX-100 (PBST). To prevent the nonspecific binding of antibodies, slides were blocked with 0.5% milk powder in 1X PBST for 30 minutes at room temperature. After blocking, slides were incubated in primary antibody diluted in blocking solution that binds to the protein of interest overnight at room temperature (Table 2). After incubating overnight, the slides were washed 3 times in 1X PBST for 10 minutes per wash. The next day, slides incubated in secondary antibody diluted in blocking solution for 2 hours.

When performing a stain, it is important to choose the antibody carefully, keeping several factors in mind. The primary antibody that was chosen had to have an epitope for the protein of interest and was produced in a mammalian species (Table 2). The protein of interest was the protein we sought to visualize through the immunostaining. The secondary antibody that was chosen was based on two factors. The first consideration was the type of stain that was being performed. For a fluorescent stain, the secondary antibody was chosen if it was tagged with a fluorescent protein. When we performed a stain using immunohistochemistry, we used a secondary antibody that was appropriate for the reaction that would eventually take place, which

would produce a precipitate at the site where the protein of interest bound to the primary antibody. The second factor that was taken into consideration was the species of the secondary antibody. To make sure the primary antibody would be recognized, the species of the secondary antibody had to be different than the species of antibody used in the primary incubation. However, that antibody must be able to recognize antibodies of the species used in the primary antibody.

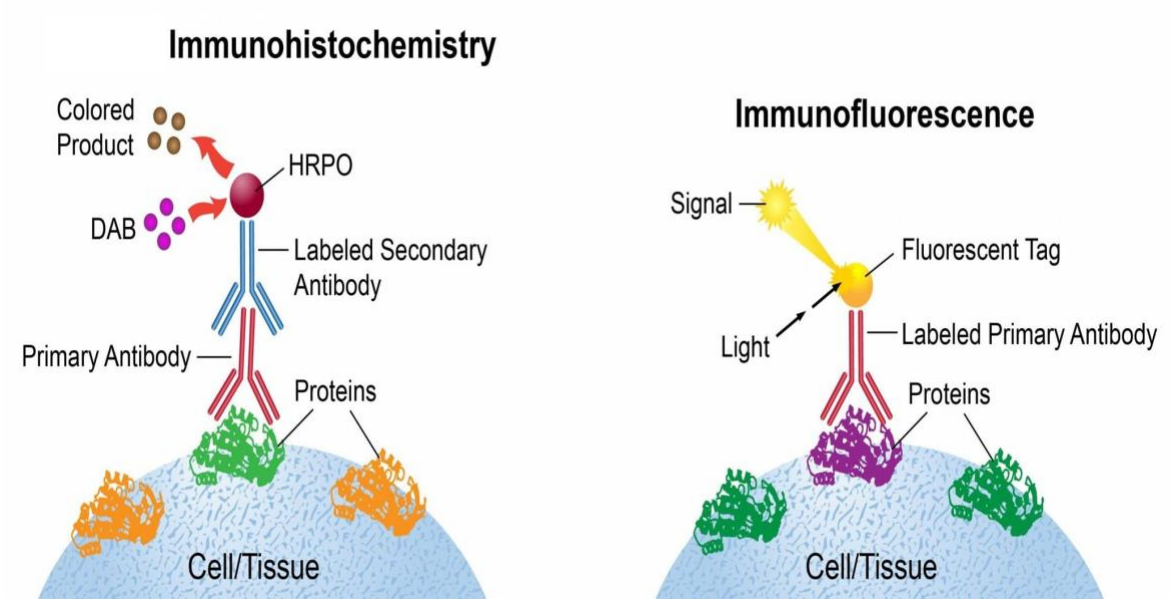

Figure 15. Two methods of immunostaining: immunohistochemistry and immunofluorescence. Immunohistochemistry involves multiple chemical tags to antibodies that interact to create a brown precipitate where the primary antibody bound to the protein of interest. Immunofluorescence utilizes fluorescent proteins found in marine species and binds them to secondary antibodies, so that there is fluorescence at the location of the protein of interest. Adapted from Millipore Sigma, 2023.

3.1 Immunofluorescence

Immunofluorescent staining was used to visualize our desired proteins through the presence of fluorescent proteins (Figure 15). The entire immunofluorescent protocol is done in the dark. Slides were kept covered by cardboard and, when able, were kept in a dark drawer. After the 1X PBST washes, the immunofluorescent secondary antibody incubated on the slides at room temperature for 2 hours (Table 3). After incubation, the slides were washed 2 times in 1X PBST and 1 time in 1X PBS. After, slides were mounted with VWR Microscope cover glasses and Vectashield aqueous mounting media with DAPI, which stained the nuclei of cells. To seal the aqueous mounting media, slides were sealed with clear nail polish after 5 minutes of drying. Slides were stored at 4 degrees in a dark slide box until ready to be imaged.

3.2 Immunohistochemistry

In the case of immunohistochemical experiments, 3,3′-diaminobenzidine (DAB) staining was used as a way to identify proteins of interest (Figure 15). After going through 3 washes of PBST, slides incubated in biotinylated secondary antibody for 2 hours at room temperature (Table 3). Once this incubation was over, slides were washed twice in 1X PBST and once in 1X PBS for 10 minutes per wash. They then incubated for 2 hours at room temperature in avidin biotin complex (ABC) reagent in 1X PBS to induce the binding of a horseradish peroxidase (HRP) to the biotin tag on the secondary antibody. This prepared the slides for the DAB reaction that would introduce DAB to the HRP tag. This interaction results in a brown precipitate that marks the location of where the primary antibody bound to the protein of interest (Figure 15). Again, following the ABC incubation, slides were washed twice in 1X PBST and once in 1X PBS. Slides then were introduced to ~300uL of DAB reagent diluted in tap water for approximately 1.5 minutes to allow for the reaction to take place. Once the reaction has taken place, slides are transferred directly to tap water to end the reaction. Once all slides had been exposed to DAB and moved to tap water, they were transported to dH_2O. After this wash, they were counterstained with 0.1% cresyl violet in dH_2O for 3 minutes. They were then briefly washed 4 times in dH_2O. On the last dH_2O wash, they incubated for 3 minutes. Then, they were dehydrated by going through an EtOH to histoclear series. In this series, the slides spent 2 minutes in one 70% EtOH, one 95% EtOH, and two 100% EtOH. Afterward, they incubated in 2 washes of histoclear for 5 minutes each. Following the second incubation, the slides were

mounted with Kleermount in Xylene and VWR Microscope cover glasses. Slides are stored in a dark slide box at room temperature until ready to image.

Table 2. Primary antibodies and respective dilutions

Antibody	Dilution	Company	Recognition
Rabbit α C-tau	1:200	Dako	C-terminal end of native tau proteins
Mouse α AT100	1:500	Thermo Fisher	Tau tangles with a double phosphorylation site at residues Thr212/Ser214
Rabbit α pS422	1:500	Fisher Scientific	Tau tangles with have phosphorylation site at residue S422

Table 3. Secondary antibodies and respective dilutions

Antibody	Dilution	Company	Recognition	Tag
Goat α Mouse Biotinylated	1:200	Thermo Fisher	Any mouse antibodies from primary incubation	Biotinylated tag
Goat α Rabbit Biotinylated	1:200	Thermo Fisher	Any rabbit antibodies from primary incubation	Biotinylated tag
Goat α Mouse Alexa 594	1:200	Thermo Fisher	Any mouse antibodies from primary incubation	Fluorescent tag at 594 λ
Goat α Mouse Alexa 488	1:200	Thermo Fisher	Any mouse antibodies from primary incubation	Fluorescent tag at 488 λ

4. Microscopy

4.1 Confocal Microscopy

Fluorescent images were taken on a Nikon Eclipse Ti2 confocal microscope at 20x and 40x. Three channels were used: TRITC, DAPI, and GFP. Each tag illuminated biomolecules stained with fluorescent tags that correlated with specific wavelengths. DAPI stained cells were illuminated around λ 405nm, EGFP stains around λ 490nm, and TRITC around λ 561nm. To take images of the slides, NIS-Elements AR 5.21.03 (copy right 1991-2020) software was used.

4.2 Brightfield Microscopy

Slides stained using the immunohistochemistry method were images using brightfield microscopy. Images were taken on an Olympus BX51 at 10x. Transition Brightfield microscopy was utilized to image slides at 10x magnification. Koehler illumination was utilized when imaging slides (Koehler, 1894). To take images of the slides, Olympus cellSens Standard 2.1 (copyright 2008-2018) software was used.

5. Toxicity Assessments

5.1 Mortality Index$_{10}$

Flies were kept at 25 degrees throughout the entire study and were only anesthetized using carbon dioxide on day 1 of life when they were sorted into their designated hit group. Flies were sorted into groups where they were exposed to TBI on alternating days between days 3-9, day 9, or not hit depending on their assigned group. The experimental group was *astrocyte>tau* and the controls were *astrocyte-GAL4/-* and *astrocyte>lacZ*. Every 2-3 days, vials were checked for flies who had died. The deaths were recorded by date and sex. After they were assessed for dead flies, the living flies that remained were transferred to a fresh vial with food. At day 10, a mortality index (MI_{10}) was calculated per vial of each genotype and hit exposure. The formula that was used to calculate this value and convert it into a percent was:

$$\text{Mortality Index}_{10} = \frac{\text{Number of dead flies after 10 days}}{\text{Total number of flies on day 1}} \times 100$$

5.2 Lifespan Analysis

Flies were kept at 25 degrees throughout the entire study and were only anesthetized using carbon dioxide on day 1 of life when they were sorted into their designated hit group. Flies were sorted into groups where they were exposed to TBI on alternating days between days 3-9, day 9, or not hit depending on their assigned group. The experimental group was *astrocyte>tau* and the controls were *astrocyte-GAL4/-* and *astrocyte>lacZ*. Every 2-3 days, vials were checked for flies who had died. The deaths were recorded by date and sex. After they were assessed for dead flies, the living flies that remained were transferred to a fresh vial with food. This continued until day 60. After each vial reached day 60, statistical analysis was done to determine differences in lifespan based on TBI exposure and genotype (Chapter 6.3).

6. Statistical analysis

All statistical analysis was done using GraphPad Prism 7.05 software (GraphPad Software Inc., San Diego, CA, USA). All graphs representing data were also made using this software.

6.1 Tau Expression Analysis

Five consecutive coronal sections were imaged by laser scanning confocal microscopy and used for analysis. We chose sections where the medulla was transected at its widest to ensure that the same area of the brain was being used for each sample. Slides stained with antibodies that indicated total tau presence were analyzed for the amount of total tau present. Images were converted into 8-bit images analyzed with ImageJ. Hemibrains were then traced, and regions of interest (ROIs) were created based on these tracings. The integrated density within the ROI was recorded and averaged among the 5 images per sample to create a single value per fly sample. The same threshold was used for all images within each experiment, which was a minimum of 39 and a maximum of 255. These averages were compared through a one-way ANOVA. We used Tukey's post hoc test for multiple comparisons.

6.2 Quantification of Tau Aggregation

Five consecutive coronal sections were imaged by brightfield microscopy and used for analysis. We chose sections where the medulla was transected at its widest to ensure that the same area of the brain was being used for each sample. Slides stained with antibodies that indicated tau phosphorylation at different epitopes were analyzed for the amount of tau aggregates present. Aggregates were counted on each hemibrain, and the 5 values were added up as the total number of tangles per fly. Only brown aggregates were counted, while irregular black precipitates were ignored. During the imaging and counting process, TBI condition and sex were blinded from the individual counting until all the samples within a data set were recorded. After counting all samples, slides were unblinded and organized into their respective groups by TBI exposure and sex. A one-way ANOVA was used to analyze each TBI condition regardless of sex. A two-way ANOVA was employed to compare the groups based on TBI condition and sex. We used Tukey's post hoc test for multiple comparisons for both types of ANOVAs.

6.2 Mortality Index$_{10}$

The mortality index equation (Chapter 5.1) was used on every vial within each genotype and TBI exposure to create multiple data points per condition and genotype. Using these data points, a two-way ANOVA was utilized to compare the average MI_{10} within genotypes and TBI condition. We used Tukey's post hoc test for multiple comparisons.

6.3 Lifespan Analysis

To generate the lifespan of flies by genotype and TBI exposure, a Kaplan-Meier survival curve was created (Kaplan & Meier, 1958). This test examines significant differences amongst the length of lifespan of different groups. The multiple comparisons test that generated p-values was Mantel-Cox test. To compare each group to each other and determine if there was statistical significance, we performed pair-wise analyses. In these analyses, we corrected for multiple comparisons using the Bonferroni method (Bonferroni, 1936). When they are more than 2 comparisons in a data set, the p-value must be corrected because each time a separate analysis occurs, the statistical test loses power. To identify true statistical significance, we used this formula:

$$\alpha_{\text{Bonferroni}} = \frac{\alpha_{\text{family}}}{K}$$

The Bonferroni α is the new p-value threshold that indicates statistical significance. The family α is the lowest significance value that we want our data to reach to be significant. For this value, we chose 0.05. K is the number of groups being compared. The K we used was 3 because there were 3 groups compared at a time. This gave us this value:

$$\boxed{0.016} = \frac{0.05}{3}$$

While all significant values reported were less than 0.016, we still denoted $p<0.016$ as **, $p<0.001$ as ***, and $p<0.0001$ as ****.

RESULTS

In our study, we aimed to understand the effects of TBI on human 0N4R tau overexpression in *Drosophila* astrocytes. We analyzed the presence of aggregates of two phospho-epitopes, AT100 and S422, in day 10 flies that experienced early TBI. Additionally, we evaluated AT100+ aggregates at day 30 in flies exposed to TBI early and late in life. After establishing aggregate levels, we assessed the toxicity of TBI's interaction with astrocytic tau expression by performing a mortality index$_{10}$ and lifespan analysis to day 60. Before we could perform any of these experiments, we had to ensure that flies in each group expressed the correct genes.

Tau is selectively expressed in astrocytes of flies with the *astrocyte>tau* genotype

We assessed the levels of total human tau present in adult *Drosophila* on the second day of life using immunofluorescent staining (Figure 16). Significantly lower levels of fluorescence intensity were present in both positive and negative controls in comparison to the experimental tau transgenic flies (Figure 16B). The flies that were the progeny of the *astrocyte>tau* cross had higher levels of tau with various morphologies (Figure 16A). The experimental genotype desired was *astrocyte >tau*, the negative control genotype was *astrocyte-GAL4/-,* and the control for protein overexpression was *astrocyte>lacZ*. Our negative control was a line of flies that had the *astrocyte-GAL4, UAS-hisRFP* complex but no other protein expression. This created a control that was closer to wild type yet still relevant to the other genotypes. The protein overexpression control expresses the gene *lacZ*, which encodes the enzyme ß-galactosidase that cleaves lactose into glucose. Expressing a protein that is not related to neurodegeneration allows for the identification of the effects of tau independent of the effects that may be due to the artificial expression of a foreign protein. These results show that we correctly generated the genotypes necessary to perform the remaining analyses.

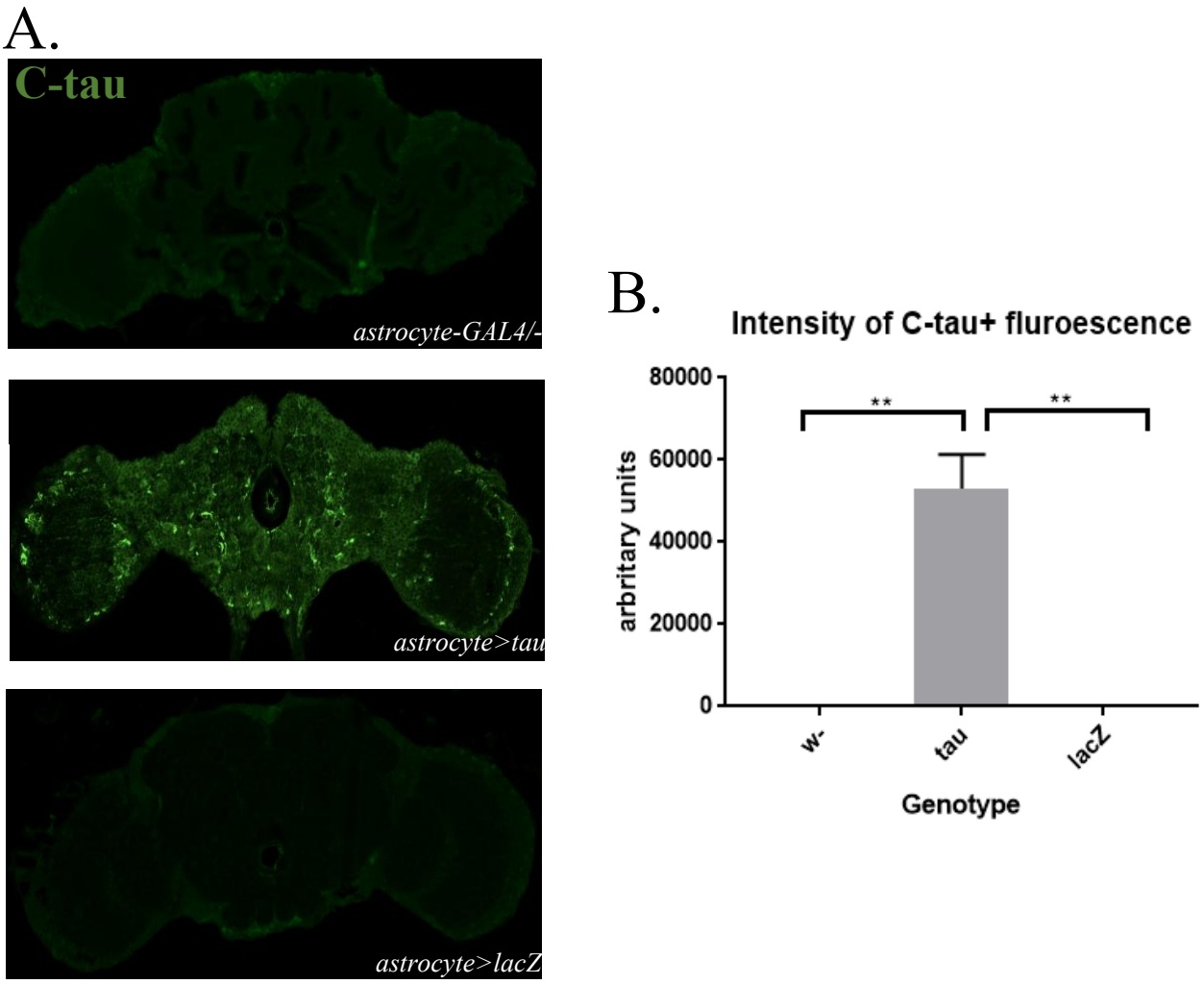

Figure 16. Tau is only present in flies that have both *astrocyte-GAL4, UAS-hisRFP* and *UAS-tau* transgenic constructs. A. Representative immunofluorescence images of coronal brain sections stained with C-terminal tau (c-tau) and imaged in the EGFP channel using laser-scanning confocal microscopy. **B.** Quantification of the level of fluorescent intensity per hemibrain. A one-way ANOVA with Tukey's post hoc test was used to establish the statistical significance. Values plotted are the average for each group and error bars indicate SEM. **= P<0.01. Genotype: *astrocyte-GAL4/-* (n=3); *astrocyte-GAL4, UAS-hisRFP/UAS-lacZ* (n=2); *astrocyte-GAL4, UAS-hisRFP/UAS-tau* (n=3).

Tau aggregation is not affected by TBI at day 10

To determine levels of tau aggregates at day 10, we performed immunohistochemistry to evaluate the presence of AT100+ and S422+ aggregates. We hypothesized that tau aggregates would be more present in flies that were exposed to TBI more than once during a 10-day period. Flies were exposed to either no TBI, a single hit on day 9, or were hit on days 3, 5, 7, and 9 (Hit D3-9). On day 10, flies were processed for immunohistochemistry.

We first analyzed AT100+ aggregates and found that there were no significant differences of aggregate presence found between any TBI paradigms (Figure 17A). This can be seen through the similarity between reported averages of no hit and multiple hit flies (Figure 17A, C). When comparing sex, there were no significant differences found between the sexes of the same group or of the same sexes of different groups (Figure 17B).

To explore whether TBI could affect tau aggregate formation, as defined by a different hyperphosphorylation epitope on tau, we assessed tau aggregation using an antibody against the S422 phospho-epitope. We chose to explore tangles with phosphorylation on the S422 residue because these aggregates are markers of AD and are associated with neurodegeneration. When counting S422+ tangles in *astrocyte>tau* flies not hit, hit on day 9, or every other day between days 3-9, we found no significant difference between groups (Figure 18). This can be seen through the similar averages between the no hit and single hit groups (Figure 18A, C). These results are consistent with the AT100 staining analysis on day 10. Additionally, there were no significant differences in males and females between or within each condition (Figure 18B). These results are consistent with the AT100 sex-analysis on day 10.

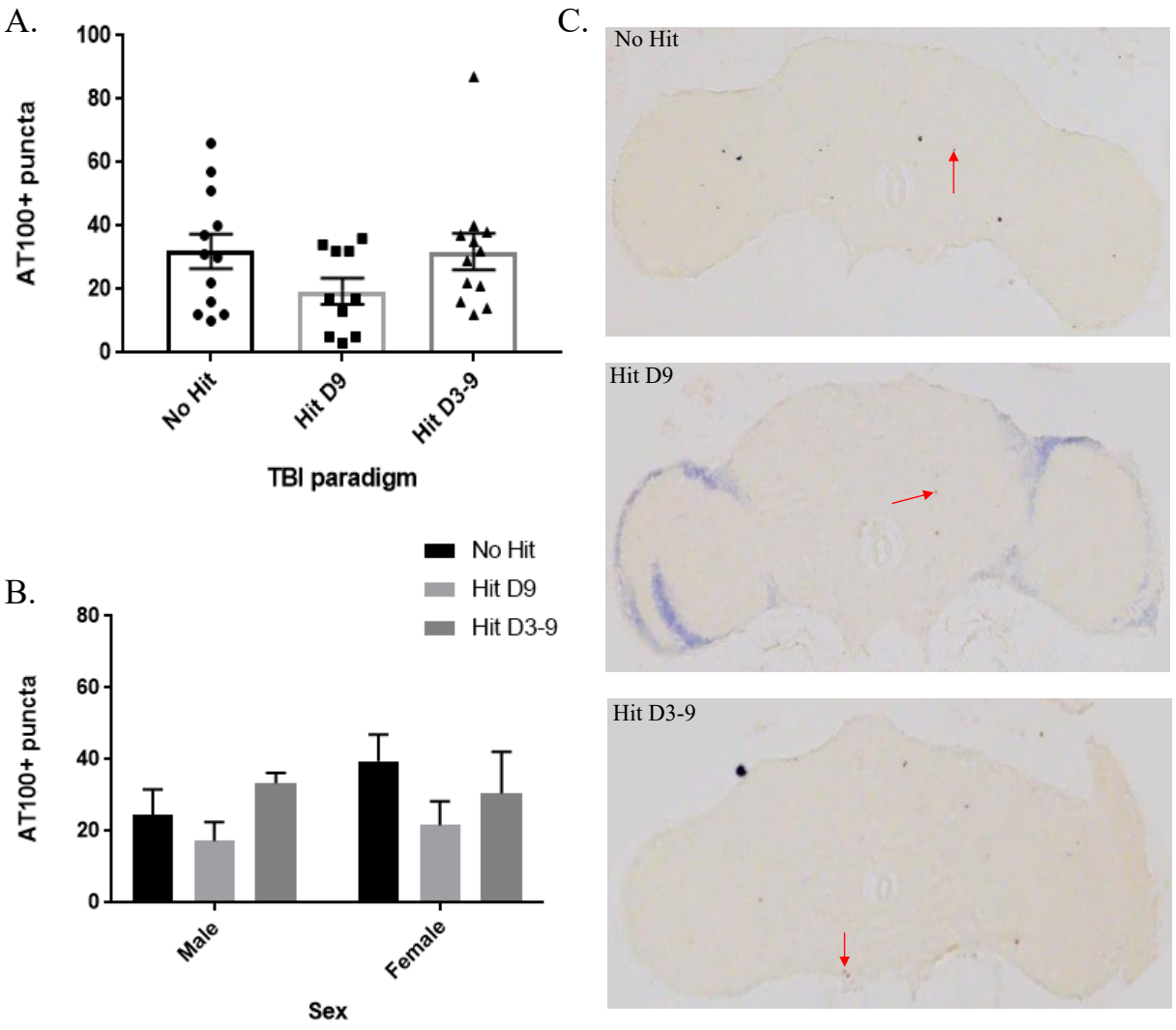

Figure 17. AT100+ tau tangles are not affected by TBI at day 10. A. Average of AT100+ puncta in five hemibrains per flies that were either not hit, hit on day 9, or hit day 3, 5, 7, 9. Flies were fixed on day 30 and analyzed thereafter. A one-way ANOVA was used to establish statistical significance. P=0.1883 (n=12; 10; 12). **B.** Average of AT100+ puncta on day 10 separated by hit group and sex. A two-way ANOVA was utilized to test for statistical significance. P=0.4832 (Males: n=6; 5; 6, Females: n=6; 5; 6). **C.** Representative images of AT100 staining of coronal sections of *Drosophila* brains. Genotype: *astrocyte>tau.* Error bars represent SEM.

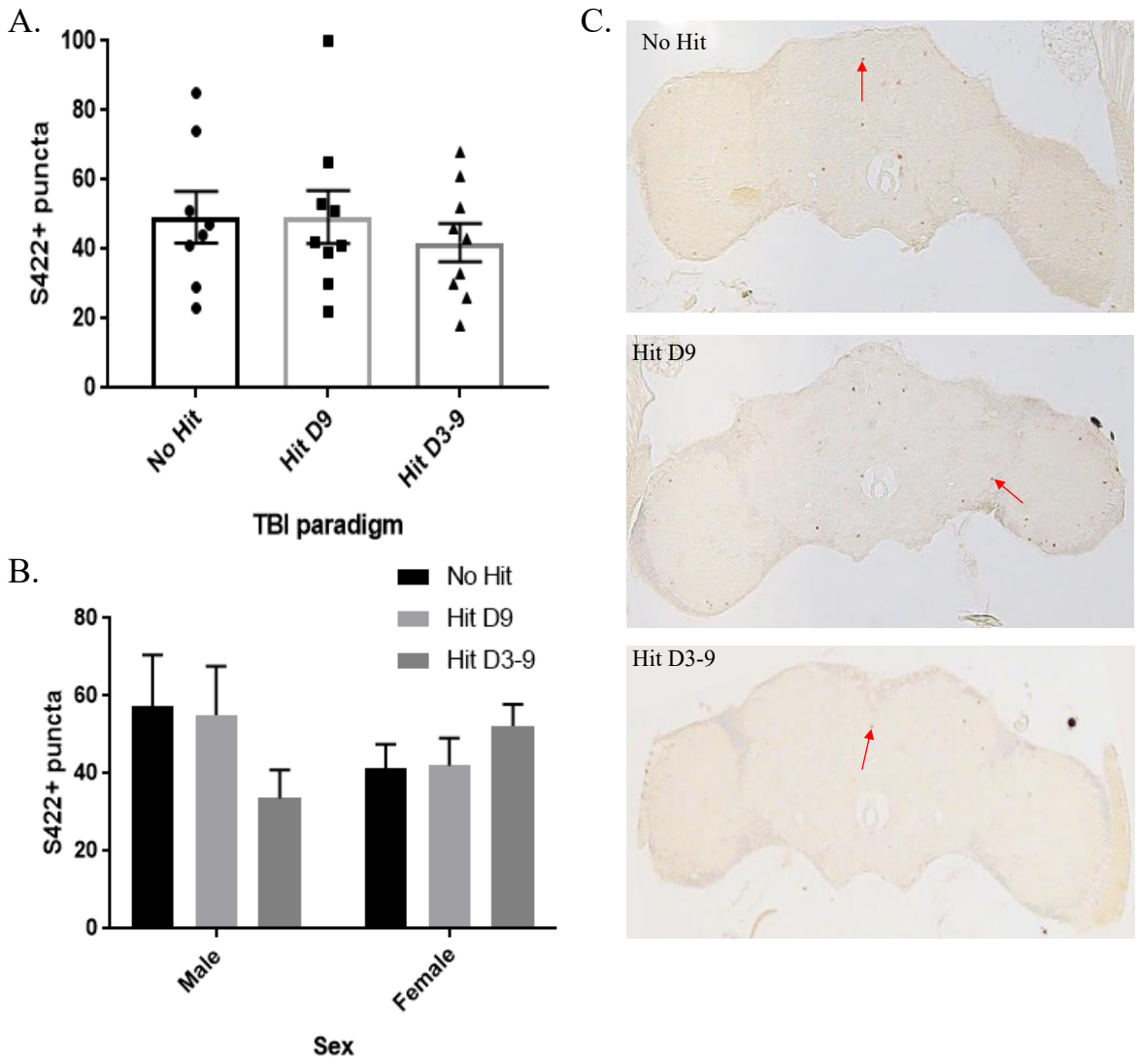

Figure 18. S422+ tau tangles are not influenced by TBI at day 10. A. Average of S422+ puncta in five hemibrains per flies that were either not hit, hit on day 9, or hit day 3, 5, 7, 9. Flies were fixed on day 10 and analyzed thereafter. A one-way ANOVA was used to establish statistical significance. P=0. 6874 (n=8; 9; 9). **B.** Average of S422+ puncta on day 10 separated by hit group and sex. A two-way ANOVA was utilized to test for statistical significance. P=0.1553 (Males: n=4; 5; 5, Females: n=4; 4; 4). **C.** Representative images of S422 staining of coronal sections of *Drosophila* brains. Genotype: *astrocyte>tau.* Error bars represent SEM.

AT100+ tau aggregation at day 30 is higher in *astrocyte>tau* flies that experienced TBI early in life

While we found no differences in aggregate presence at day 10, we decided to evaluate aggregation later in life using one of the same phospho-epitopes, AT100. For this analysis, we assessed tau aggregation in day 30 flies that were either hit on day 9, hit on day 29, hit on alternating days between day 23-29, or not hit at all.

Overall, flies hit on day 9 displayed the most AT100 tau aggregation on day 30 (Figure 19). In comparison to later hits, a single TBI event on day 9 had significantly more aggregates than both hit day 23-29 and hit day 29 (Figure 19A). However, there were no significant differences between the no hit group and any of the other hit groups (Figure 19A). We also wanted to identify if there were any sex differences between groups on day 30. This helps identify if one group experienced increased aggregation based on sex. Consequently, we found that females hit on day 9 were significantly higher than several groups (Figure 19B). Firstly, females hit day 9 had higher numbers of AT100+ aggregates than females hit day 23, 25, 27, 29 and females that were hit day 29. There was also a trend of higher aggregate presence amongst females who were hit day 9 compared to those who were not hit ($p=0.0691$). When comparing sexes of the same hit, the hit day 9 females had significantly more aggregates compared to males who experienced the same TBI exposure (Figure 19B).

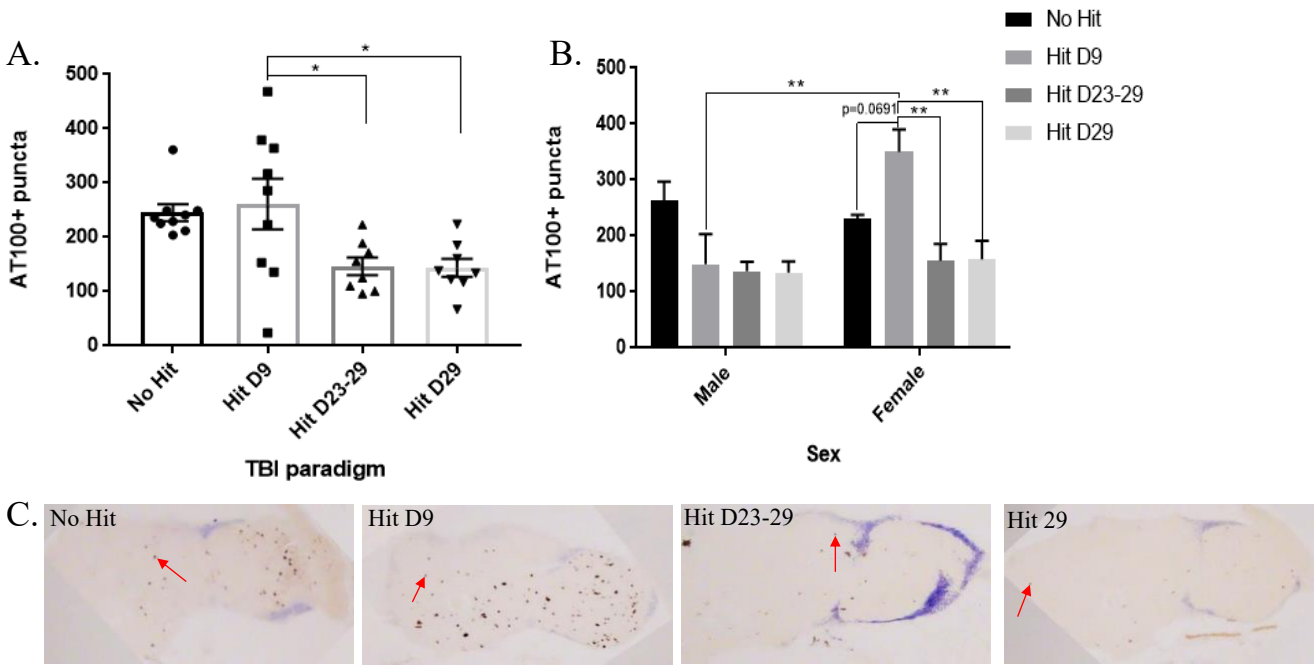

Figure 19. AT100+ tau tangles are influenced by TBI at day 30. A. Average of AT100+ puncta in five hemibrains per flies that were either not hit, hit on day 9, hit day 23, 25, 27, 29, or hit on day 29. Flies were fixed on day 30 and analyzed thereafter. A one-way ANOVA with Tukey's post hoc test was used to establish statistical significance. *=P<0.05. (n=9; 9; 8; 8). **B.** Average of AT100+ puncta on day 30 separated by sex. A two-way ANOVA with Tukey's post hoc test was utilized to test for statistical significance. **=P<0.01. (Males: n= 4; 3; 4; 5, Females: n= 5; 5; 4; 3). **C.** Representative images of AT100 staining of coronal sections of *Drosophila* hemibrains. Genotype: *astrocyte>tau.* Error bars represent SEM.

Flies that overexpress tau in astrocytes have a higher mortality index$_{10}$ when exposed to multiple TBI events

While examinations of aggregated tau proteins are indicators of tau pathology, assessing general toxicity can be thought of as a way to understand the overall effects of tau and TBI on the whole organism. To determine this toxicity within the first 10 days of life, we calculated a MI_{10} where we compared the total number of flies on day 1 to the number of flies dead by day 10. Flies were exposed to either no hit, a single hit on day 9, or were hit on days 3, 5, 7, 9. Vials were checked for dead flies every 3 days, and, once dead, their date of death was recorded. For each vial, the number of dead flies was divided by the total number of flies on day 1.

This assay demonstrated that *astrocyte>tau* flies that were hit 4 times within 10 days had the most relative toxicity within this time period (Figure 20). The MI_{10} for this group of flies was significantly higher than *astrocyte>tau* flies who experienced no TBI or had a single exposure to TBI (Figure 20). In both controls, TBI did not affect the MI_{10}. There were no significant differences within any of the control groups (Figure 20). There were no significant differences between groups with different genotypes. Therefore, *astrocyte>tau* flies were the most susceptible to early lethality associated with the multiple-hit paradigm.

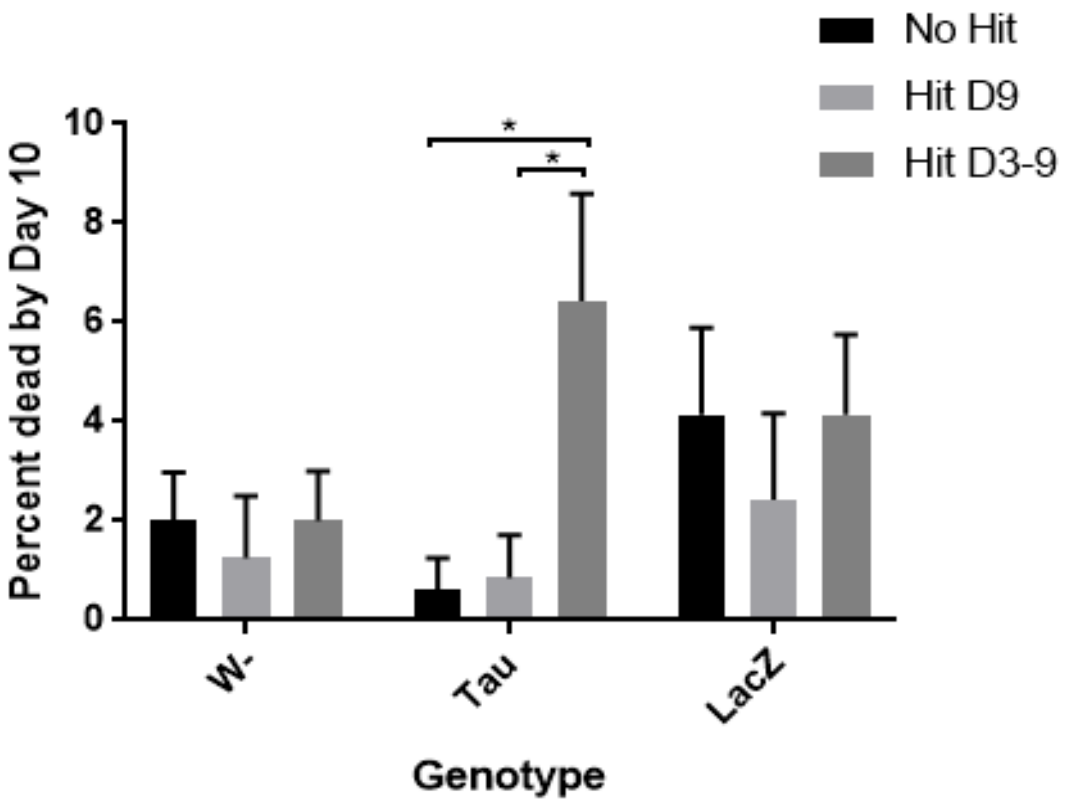

Figure 20. Flies with astrocytic tau expression who experience chronic TBI have a higher mortality index$_{10}$. The MI_{10} was found for flies hit on day 9, days 3, 5, 7, and 9, or not hit. Averages of MI_{10} are indicated by each bar. A two-way ANOVA with a Tukey's post hoc test was used to calculate statistical significance. *= $P<0.05$. Genotypes: *astrocyte-GAL4 /-* (No hit n=161; Hit D9 n=157; Hit D3-9 n=153); *astrocyte>lacZ* (No hit n=165; Hit D9 n=161; Hit D3-9 n=159); *astrocyte>tau* (No hit n=165; Hit D9 n=131; Hit D3-9 n=143). Error bars represent SEM.

TBI's interaction with astrocytic tau overexpression decreases lifespan within 60 days

We wanted to observe how viability was affected by TBI in the context of astrocytic tau expression in *Drosophila*. To determine this, we performed a lifespan study that went to day 60. In this analysis, we examined 3 types of TBI exposure: no exposure, single exposure on day 9, and multiple exposures on days 3, 5, 7, 9. Using these groups, we counted the number of dead flies every 3 days.

We found that lifespan significantly decreases in a TBI dose-dependent manner in *astrocyte-GAL4/-* and *astrocyte>tau* flies (Figure 21C, D). Flies that experienced no TBI lived longest, while one TBI event shortened lifespan, and multiple TBI exposures shortened lifespan the most. The *astrocyte>lacZ* flies had the shortest lifespan, but this decrease was not significant (Figure 21E). Therefore, the lifespan of *astrocyte>lacZ* flies is not affected by TBI exposure. Unexpectedly, when comparing genotypes within the same hit group, we found that *astrocyte>tau* flies had significantly longer lifespans than either control genotype for both TBI condition and the no hit control (Figure 21 F-H). Overall, TBI decreases lifespan, especially in flies who express human tau in their astrocytes.

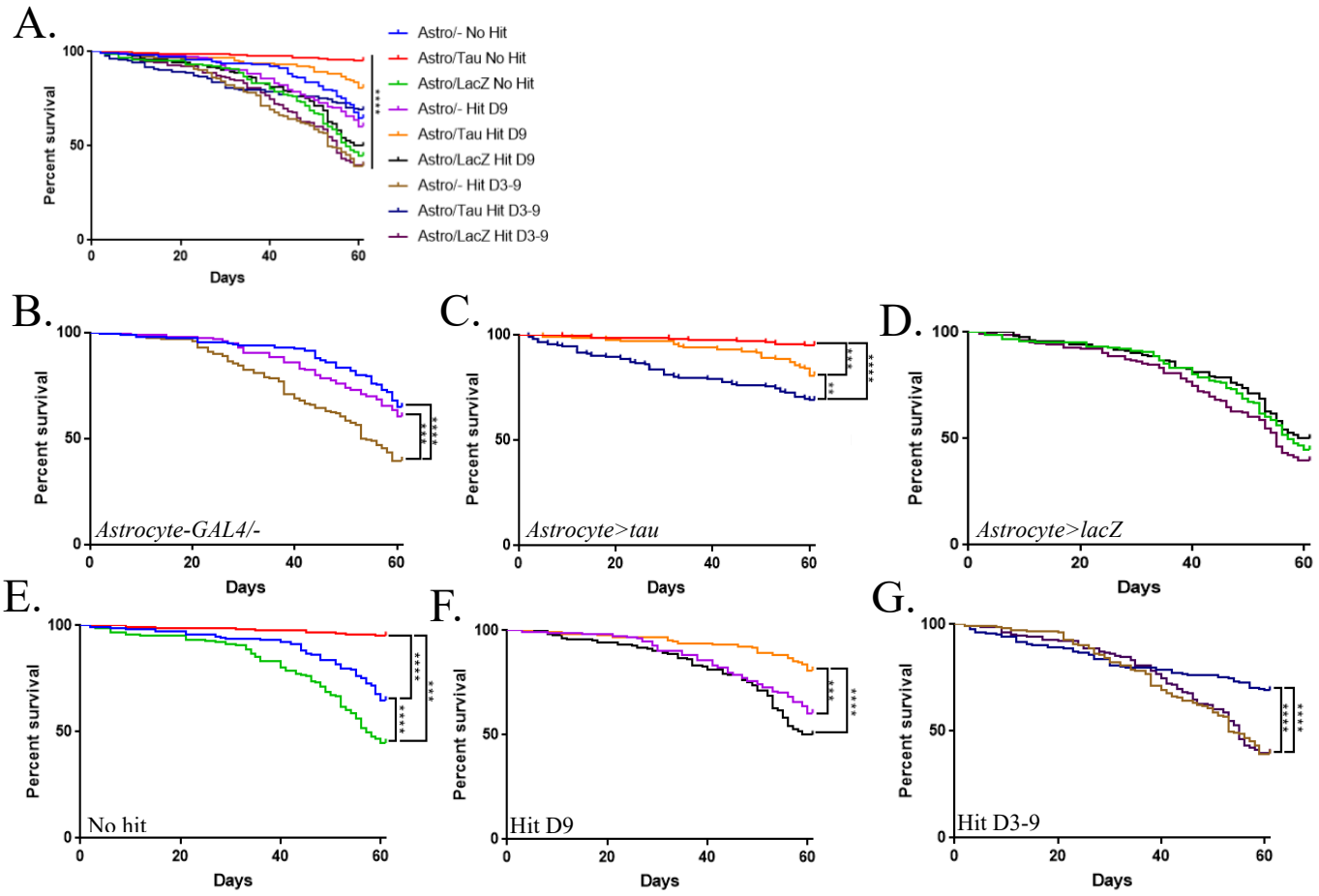

Figure 21. 60-day lifespan decreases in flies who have astrocytic tau and experience chronic TBI. A. Key for distinguishing what colors are representative of each group's genotype and hit. **B.** The lifespan of all groups compared to each other is shown. A Kaplan-Meier survival test was used to find the significance of this graph and the smaller comparisons as well. P-values were provided by Mantel-Cox test. **=P<0.01; ***=P<0.001; ****=P<0.0001. P-values were corrected for pairwise comparisons. **C.** The comparison between *astrocyte-GAL4 /-* flies of different TBI exposures. **D.** The comparison between *astrocyte>tau* flies of different TBI exposures. **E.** The comparison between *astrocyte>lacZ* flies of different TBI exposures. **F.** The comparison between flies of all 3 genotypes who had no exposure to TBI. **G.** The comparison between flies of all 3 genotypes who had exposure to TBI on day 9. **H.** The comparison between

flies of all 3 genotypes who exposure to TBI on days 3, 5, 7, and 9. Genotypes: *astrocyte-GAL4/-* (No hit n=161; Hit D9 n=157; Hit D3-9 n=153); *astrocyte>lacZ* (No hit n=165; Hit D9 n=161; Hit D3-9 n=159); *astrocyte>tau* (No hit n=165; Hit D9 n=131; Hit D3-9 n=143).

DISCUSSION

In this study, we assessed how different exposures to TBI could affect astrocytic tau aggregation and generate toxicity using *Drosophila melanogaster* as a model. We hypothesized that chronic TBI that occurred early in adulthood would elicit the highest presence of tau aggregation, and this type of TBI exposure would produce the most toxicity as well. Ultimately, we found that our hypothesis was partially supported, and we uncovered interesting aspects of how TBI interacts with astrocytic tau expression.

To assess the effects of TBI on tau aggregation, we started with aggregation assays because some studies show that TBI encourages tau to become hyperphosphorylated and eventually aggregate with other tau proteins. To do this, we looked at two specific types of phospho-epitopes, AT100 and S422, which were chosen based off of their prevalence in human tauopathies and their ability to be measured in the *Drosophila* model of tauopathy. After counting aggregates and performing statistical analyses, we found that there was no difference in S422+ and AT100+ aggregates at day 10 of life. These data sets also had no significant differences among sex for astrocytic tau aggregation in response to TBI. To determine if TBI affected AT100+ tau aggregation later in life, we assessed flies aged to day 30 for AT100+ puncta to see if this aggregation differs at that timepoint within their lifespan. In contrast to the day 10 aggregates assays, the TBI conditions that we assessed were no hit, hit day 9, hit on alternating days between day 23-29, and hit day 29. From this analysis, we found that there were significantly more aggregates in *astrocyte>tau* flies hit on day 9 compared to flies of the same genotype that were hit on day 23-29 or just hit on day 29. This confirmed our hypothesis that earlier TBI events would lead to more tau aggregation. While there are limited findings on how different timing of TBI can influence tau aggregation, there are reports on how TBI at younger ages elicits worse recovery outcomes compared to individuals who are exposed to the same amount of TBI later in life. One group found that severe TBI had worse long term cognitive effects when it occurs in young children compared to older children (Anderson et al., 2005). These differences highlight that development at a certain point allow for better recoveries from TBI. These behavioral results are consistent with our finding that tau aggregation is worsened if TBI at the same frequency, a single TBI event in our case, occurs early (day 9) compared to later

in life (day 29). Therefore, more analyses comparing the levels of tau aggregation in different timings of injuries will help emphasize this result. Because flies who experienced TBI chronically at the beginning of their life were not included in this assessment, we cannot know if the chronic aspect of our hypothesis was confirmed.

In addition to assessing the total groups, we wanted to explore sex differences due to the fact that females experience tau pathology at higher rates, specifically in AD (Andersen et al., 1999). We found that the females hit on day 9 were prone to high levels of AT100+ tau aggregation at day 30. They had significantly more aggregates than males that received the same exposure to TBI. They also had significantly more aggregates than females who were hit on day 29 or on days 23, 25, 27, and 29. These results indicate that aggregate differences may only be able to be detected later in life when studying astrocytic tau expression and early TBI.

As well as identifying sex differences, both data sets confirm that aggregation does not change within 10 days of TBI. In the day 10 analyses, there were no significant differences among either the single hit or multiple hit flies. Similarly, in the day 30 assessment of AT100+ aggregates, there were no significant differences between the flies hit within 10 days of day 30 (hit day 29 or hit days 23, 25, 27, 29). This suggests that more than 10 days, and possibly shorter than 30 days, is necessary to uncover an effect of TBI on tau aggregation, since we saw significant differences at day 30 in flies hit on day 9. Zanier *et al.,* 2018 also explored how the same injury elicited different amount of tau aggregates when assessed a different timepoints. In this study, they exposed wild-type mice to TBI then assessed the number of tau aggregates 3 months-post TBI and 12 months-post TBI. In their results, they found significantly higher levels of tau aggregates in mice 12-months post TBI compared to the no hit control but not at 3 months post injury (Zanier et al., 2018). These results in unison with our day 30 aggregate analysis illustrate that aggregate presence is modified after considerable amounts of time post injury. This delayed response in tau aggregation suggests that mechanisms of aggregation are not fast-acting. Future studies should assess the delay in aggregation differences based on TBI exposure in an attempt to identify long lasting secondary injuries that could contribute to aggregation of tau.

After confirming the levels of tau aggregation at day 10 and day 30, we investigated the effect of TBI on toxicity in flies with tau overexpression in astrocytes. Our hypothesis, as

mentioned above, proved to be true through both measures of toxicity that we performed, which were a MI_{10} and a lifespan analysis up to day 60. We found that the MI_{10} was a significantly higher in *astrocyte>tau* flies that were hit multiple times compared to flies of the same genotype who were hit once or not at all. This data matched well with the 60-day lifespan assay because we saw a significantly dose-dependent decrease in lifespan as TBI was more present in *astrocyte>tau* flies. This affirmed our hypothesis that multiple TBI events increases toxicity of tau expression in astrocytes. We hypothesized that this would occur because multiple reports have shown that more severe TBI, through the use of 2 different *Drosophila* TBI devices, is associated with shorter lifespans. One group showed that flies subjected to a severe hit had a significantly shorter lifespan than flies that experienced moderate or mild hit (Saikumar et al., 2021). While the frequency of TBI events was the same, the force of the hit increased, which resulted in higher toxicity. Another study that used the same TBI device as we did, the HIT device, demonstrated that more hits were associated with higher toxicity (Katzenberger et al., 2013). This was seen through a significantly shorter lifespan in flies that were hit 4 times versus flies hit once. These results complement our finding that multiple exposures to TBI enhance toxicity.

One caveat to this toxicity-related finding comes from the comparison of tau expression to both controls in all TBI paradigms that we investigated. In the MI_{10}, there was no significant difference when comparing *astrocyte>tau* flies to either protein overexpression control group or the negative control group. Consequently, in the lifespan analysis, we found that both controls had significantly shorter lifespans than the experimental group in all hit conditions. In past research, *W-* flies have shown to have lifespan decrease when exposed to TBI (Katzenberger et al., 2013). So, their lifespan decrease is expected and confirmed by our study. When comparing this group to *astrocyte>tau*, it does seem that tau has some protective quality due to their lifespan being significantly longer regardless of TBI exposure. Astrocytic tau overexpression may actually be beneficial because *astrocyte>tau* flies that were not hit had the longest lifespan of any group. This result is intriguing because Colodner and Feany, 2010 showed that expressing tau in all *Drosophila* glial subtypes significantly decreased lifespan compared to the *W-* control. This discrepancy may indicate that robust toxicity of tau may require additional glial cell types to

overexpress the protein. Since astrocytic tau overexpression is only in one of many glial subtypes, it can be assumed that it is not enough to elicit a toxic response. A potential mechanism that may induce this protective profile of astrocytic tau is activation of glial AP1, which is a transcription factor known to be involved in neurodegeneration (Byrns et al., 2021). Byrns *et al*., 2021 showed that appropriate activation of AP1 is protective in the presence of TBI. While this group also demonstrated that the interaction of pan-glial tau expression and glial AP1 is toxic in the context of TBI, potentially the subtype-only tau expression was able to modulate this relationship and generate a protective mechanism.

In addition to the *W-* control, it is important to mention the lifespan of all hit conditions with the *lacZ* genotype was not significant in pairwise comparisons. This demonstrates that astrocytic lacZ expression does not influence lethality in the context of TBI. Therefore, the overall decrease in lifespan in *astrocyte>lacZ* flies should be investigated further.

While assessing aggregates, no hit flies also had an unexpected result. In both aggregate assessments at day 10, the no hit flies have similar levels of aggregates among the groups. This illustrates that aggregation is not influenced by TBI at day 10. One reason that may be influencing this is a potential increase in kinase inhibitors that is present in rodent models and post-mortem human studies of TBI. GSK3β is a kinase that is highly associated with phosphorylation of tau in neurodegenerative diseases. Two inhibitor pathways of this kinase have shown to be up regulated in the presence of TBI in mice, rats, and human studies (Shim & Stutzmann, 2016). Subsequently, this upregulation also limited the activity of GSK3β, which defines the pathways' ability to potentially limit tau hyperphosphorylation that can be a precursor for aggregates in the CNS. From these past results, possible mechanisms for the presence of aggregates in the no hit *astrocyte>tau* flies could be due to the lack of inhibition of kinases linked to neurodegeneration.

Presence of tau aggregation does not predict toxicity

From assessing two different kinds of tau aggregates at day 10, we found that there were no significant differences in aggregate formation between conditions or TBI paradigms. However, we did find that there was a significantly higher mortality index at day 10 in *astrocyte>tau* flies that were hit 4 times compared to those that were hit once or not hit at all. This indicates that increased toxicity is independent of changes in tau aggregate formation and suggested that tau toxicity is not always indicated by the presence of increased aggregates.

This conclusion regarding toxicity independent of tau aggregation has been found by many other groups as well. Colodner and Feany, 2010 found that reducing tau expression in *Drosophila* lead to no difference in aggregates, but it does significantly lower levels of toxicity measured through the detection of apoptotic cells present. This is also shown in a tauopathy mouse model where PHF presence is consistent among mice with constant and interrupted tau expression but behavioral symptoms indicative of neurodegeneration are significantly less present in mice with interrupted expression (Santacruz et al., 2005). In unison with our data, these results suggest that toxicity may be independent of aggregation formation or presence.

The mechanisms to explain the lack of toxicity of the interaction between TBI and astrocytic tau aggregates may be linked to investigations of what structure of tau is pathological. Highly aggregated forms of this protein are associated with neurodegeneration, but they may not be the only version of tau structure to elicit pathology. Therefore, this result encourages the exploration of tau oligomers (Figure 22). As explained earlier, tau aggregates called NFTs are made up of numerous hyperphosphorylated tau proteins that have tangled into one protein aggregate. These NFTs have shown in some cases to drive tau toxicity, but that is not indicated in our study. To form into an NFT, tau monomers can dimerize/oligomerize to PHFs, which may mature into NFTs (Figure 22). Because our study shows that large aggregates are not significantly influenced by TBI, it is critical to investigate how hyperphosphorylated oligomers of tau can incite pathology and lethal toxicity since we saw a higher mortality index at day 10.

In future studies, groups should assess the role of soluble oligomers in tauopathy models exposed to TBI. However, this study must focus on specific oligomers that can be referenced by other research groups. Tau oligomers are hard to study due to their diverse nature. There are many levels of tau oligomers due to the difference in number of tau monomers present within the

oligomer, so comparing studies of these tangles is difficult (Cowan & Mudher, 2013). However, there is still evidence to describe that soluble oligomers of tau have the ability to incite pathology. One example of this is that the *Drosophila* model of tauopathy in neurons does not form NFTs (Wittmann et al., 2001). Although there are no NFTs in this model, pathologies related to neurodegeneration are still present in these flies (Wittmann et al., 2001). This was seen from degenerating neurons, decreased lifespan, and presence of vacuoles. Because there is evidence of neurodegeneration but no NFTs, it is likely that soluble tau oligomers play a role in toxicity. In addition to this result in *Drosophila*, human studies have also shown neurodegeneration occurring in the absence of advanced aggregates (Bird et al., 1999). In this study, three families with the same mutation on the *MAPT* gene was present and they all had similar levels of cognitive decline. However, the presence of NFTs were quite variable amongst the samples evaluated for tau aggregates. One family had a lot of NFTs, one had less, and another family had none. Additionally, the authors of this paper discuss another study by Huetink *et al*. 1997, which reports on a Dutch family that had the same mutation and an absence of NFTs (Heutink et al., 1997). This suggests tau aggregates are not required for neurodegeneration common in tauopathies. This study demonstrates this through the fact that mutations on tau's gene that known to incite the formation of NFTs do not always result in aggregates. As these two studies show that aggregated forms of tau are not necessary for neurodegeneration and dementia to occur, our results indicate that tau oligomers or other neurodegenerative mechanism related to tau presence may be influenced by chronic TBI at day 10.

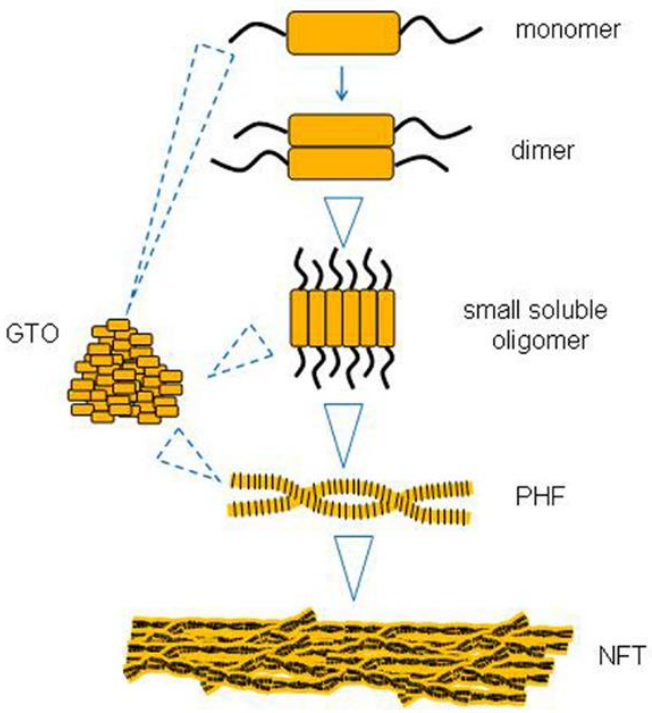

Figure 22. Monomers of tau can form into NFTs. Tau proteins are shown to start as monomers post-translation to perform its function of axonal stability. Pathologically, these monomers then dimerize and oligomerize with many tau proteins and form into a single structure. Paired helical filaments (PHF) are descendants from the oligomerized form of tau. Many PHFs can tangle together to form neurofibrillary tangles (NFTs). The development of granular tau oligomers (GTOs) is still unknown but is thought to either manifest from clusters of monomers or small soluble oligomers. GTOs are also hypothesized to form into PHF, which progress into NFTs. Adapted from Cowan and Mudher, 2013.

Limitations of the HIT paradigm of TBI

In this study, we used the HIT device to induce TBI. This device was developed by Katzenberger *et al.* 2013 to develop a model of TBI in *Drosophila*. This model works by attaching a vial of flies to a spring and releasing the spring at a 90-degree angle onto a rubber board. Before this device was developed, there was no way of modeling TBI with fruit flies. This gap in scientific research was important to fill because *Drosophila* model neurodegeneration well and are simple to handle due their size. Not only did this paradigm create a way to model TBI, but it is also easily recreated in most lab settings due to the fact that it is simple to assemble, and the materials are accessible. Since its discovery, the HIT device has been followed by the development of many other TBI mechanisms for *Drosophila* (Aggarwal et al., 2022).

To validate this model, Katzenberger *et al.* 2013 explored its ability to elicit a detrimental affect similar to that of TBI seen in other organisms. They found that flies who were exposed to TBI using the HIT device induced pathologies associated with neurodegeneration and these pathologies were more robust in flies exposed to TBI compared to a no hit control fly (Katzenberger et al., 2013). While there is evidence to demonstrate that this device can induce neurodegeneration, there are concerns with its reproducibility in inducing TBI. Because each fly is not in a fixed place within the vial, the individual performing TBI cannot control where flies hit against the edges of the plastic. This also means that TBI may not even occur. Flies may bump into each other or are jolted by the hit instead of having a physical interaction with the vial. Additionally, when the number of flies differ for each vial, the injuries may be more or less intense based on how full it is. Overall, these issues can lead to variability in the injury that occurs from fly to fly. The flies that receive the most severe injury may die promptly after exposure to TBI, and the ones that may have been exposed to a less intense hit are used for further analysis. This may influence our data, as flies that died immediately upon TBI were not examined for aggregate formation or included in the lifespan analysis.

Because there are variables that are not controllable while using the HIT device, each injury will be unique and potentially elicit a different outcome of pathology or toxicity. One argument that supports use for this device is the fact that head injuries in humans do not occur in a uniform matter either, as, there are many sources for TBI that vary on impact level and location of the hit on the head (Ginsburg & Huff, 2023; Yattoo & Tabish, 2008). Therefore, human TBI

cannot be described strictly by a list of requirements. So, the non-uniform nature of TBI in humans is somewhat recapitulated in *Drosophila* when the HIT device is utilized. Using a device that can elicit diverse levels of TBI severity allows for a broader understanding of how injuries can incite neurodegeneration. As this model was validated by consistent results of decreased lifespan and increased vacuoles (Katzenberger et al., 2013), it seems that the random nature of this device does not prevent it from being an appropriate for modeling TBI.

In conjunction with the lack of control around the level of injury each fly receives, there is also a critique about the specificity of the injury. While injuries that involve the whole body can also impact the head, some believe that the study of TBI should be specific to the head (Saikumar et al., 2020). This approach comes from the interest in knowing how infliction to the head/brain alone can induce neurodegeneration and its associated processes. In fact, some groups have developed TBI paradigms that allow for a hit directly and only to the head of the fly (Saikumar et al., 2020). This tool is important for uncovering what injuries the brain can withstand, but the translation for this research among TBI in humans is less clear. In many cases of human TBI, the head is not the sole site impacted by the injury (Yattoo & Tabish, 2008). This is supported by the fact that closed head trauma is the most common form of TBI, which occurs mostly in situations where the whole body is impacted by injury (Ginsburg & Huff, 2023). Along with these two ways of inducing TBI, there are many other models that work to recreate specific kinds of injuries common to humans and the level/location of impact that can have differing consequences for the person who is injured. This synergy gives scientists the tools to explore every avenue of TBI using *Drosophila* as the model organism, and it would be interesting to see if our results would be altered when using a different TBI paradigm.

Astrocyte-GAL4, UAS-hisRFP system's effect on protein overexpression

When creating our model for this project, we used *astrocyte-GAL4, UAS-hisRFP* flies, as there were limitations within the stocks present in our lab. Ideally, we would have used *astrocyte-GAL4* flies alone, but that stock was sick and unusable. In this light, we decided to use flies that were also overexpressing histone-RFP, as this would have potentially allowed us to perform cell count analyses using immunofluorescent stains. However, we found that the ethanol incubations quenched the fluorescence, so we did not pursue these cell count analyses. Therefore, we continued to use the *astrocyte-GAL4, UAS-hisRFP* recombinant flies, as they seemed to be a suitable replacement.

Notably however, hisRFP overexpresses histones within the nuclei of tau-expressing astrocytes. Histones are proteins that can condense chromatin to become heterochromatin or expose chromatin to become euchromatin, which is a known mechanism of gene regulation. This consequently influences whether specific DNA sequences of genes are transcribed into mRNA (Bannister & Kouzarides, 2011) (Figure 23). The histone protein that is illuminated using RFP is the His2A, which is a core histone protein. The structure of histones are made up of a round, coiled C-terminal domain and a tail in the N-terminal domain (Lee et al., 2020). The C-terminal can be phosphorylated but most of the post-translational modifications occur on the histone tail (Figure 23). RFP is a protein made of 4 monomers, and its structure mirrors a barrel (Marshall, 2000; Wall et al., 2000). Fluorescent protein tags are added by inserting their primary sequence into the sequence of the protein that is being tagged. The interaction between these two proteins in our fly model is unknown.

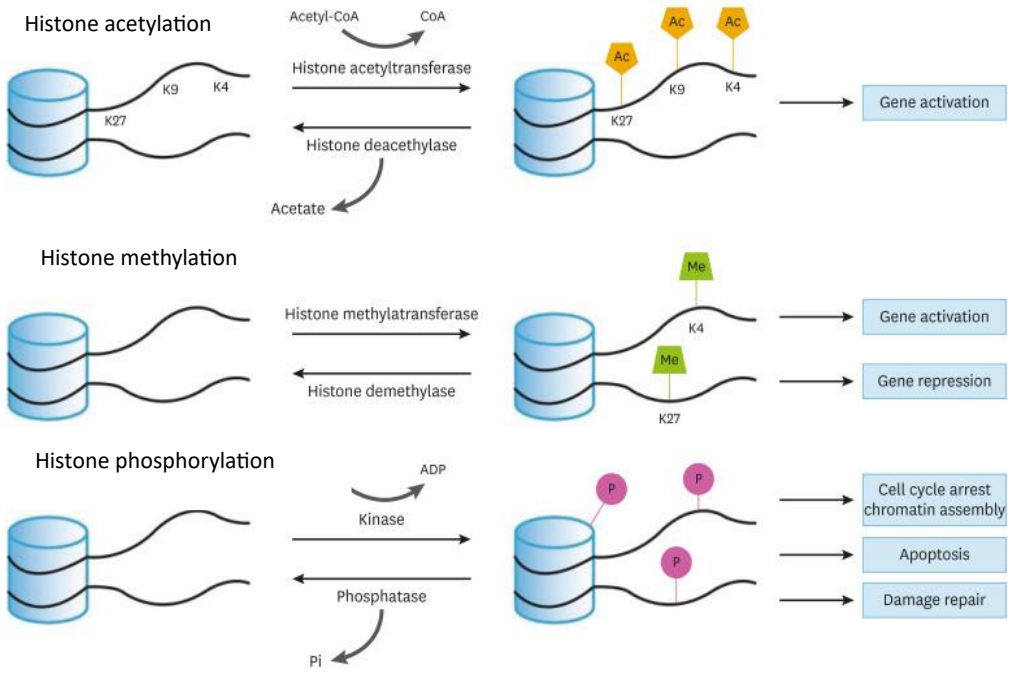

Figure 23. Post-translational modifications of histone proteins affect their function. Histones can be modified by functional groups, which can influence the activity of the histones. The addition of acetyl groups (Ac) on histones, through acetyltransferases, opens the histone tails and allows for increased gene expression/activation. Methyltransferases add methyl (Me) groups onto histone tails and can either activate or repress gene expression depending on location. Phosphorylation (P) from kinases can ignite apoptosis, damage repair, or chromatin condensation. Adapted from Lee *et al.,* 2020.

Our lab is interested in understanding the effect of tau expression in all fruit fly glial subtypes. Similar to the *astrocyte>hisRFP* stock used in this study, our lab has created stocks of flies that express hisRFP in all glial subtypes for other cell count projects. One of these glial cell subtype in particular is perineurial glial (PG). These cells are known to support the structure of the *Drosophila* CNS and make up the outer layer of the brain (Freeman, 2015). Tangentially,

previous experiments in the lab have shown that expressing tau in PG cells, using *PG-GAL4* and *UAS-tau* is lethal; flies are able to develop into pupae but do not eclose from their pupal casing. This suggests that PG-specific tau overexpression must interact in some way to prevent final development in the CNS and, therefore, causes death. Interestingly, however, when *PG-GAL4, UAS-hisRFP* flies are crossed with *UAS-tau* flies, tau is no longer lethal, and the flies develop normally and eclose from their pupal casing. This finding highlights a potential suppressive effect of histone overexpression on tau toxicity, which would potentially explain the neuroprotective effect of astrocytic tau from TBI that was indicated by the lifespan analysis up to day 60. If the hisRFP expression decreased tau toxicity in astrocytes like it did in the PG cells, then it is actually the hisRFP modulation of tau expression in astrocytes that is neuroprotective in the context of TBI and not astrocytic tau expression itself.

Additional experiments will need to be pursued in the lab to understand how histone overexpression may be suppressing tau toxicity, but two hypotheses to explain this finding are as follows. First, having proteins attached to the histones may limit their ability to expose DNA and prevent transcription and translation to occur at the rate of flies that don't overexpress hisRFP. Methylation on particular residues of the histone tails has shown to limit gene expression (Figure 23). We don't know how RFP is affecting the histone protein, but, if RFP is in a similar position and has the same effect as the methyl group, these flies may have reduced tau expression. Koson *et al.* 2008 compared two rat models of tauopathy with known differences in tau expression and found that rats with higher tau expression had a shorter life span (Koson et al., 2008). As mentioned above, mouse and *Drosophila* models have shown that reducing tau expression within the organisms' lifespan has decreased toxicity through means of less memory deficits and cell death (Colodner & Feany, 2010; Santacruz et al., 2005). These findings support this rationale as a hypothesis to explain lowered toxicity in hisRFP-expressing flies. If this is true, then one could use both the cell specific GAL4 alone and the *cell specific-GAL4, UAS-hisRFP* for other analyses comparing tau expression.

Another hypothesis that could explain this difference in lethality could come from known histone modifications that are able to modify tau toxicity within *Drosophila*. Potentially, the presence of RFP within the sequence of histone 2A is interacting with other aspects of cell

viability in a similar way to how functional groups added to histones via post-translational modifications can change the function of the protein. For example, phosphorylation on a specific site of histone tails alters its function and causes the histone to arrest promotion of the cell cycle (Figure 23) (Lee et al., 2020). In *Drosophila* models of tauopathy, markers of histone methylation decreased compared to the control without a change in the presence of methyltransferases (Frost et al., 2014). This indicates that tau expression can deter normal function of methyltransferases without degrading them. Potentially, histoneRFP presence prevents these mechanisms of disruption to proper histone function mechanisms by tau overexpression. Therefore, the toxic mechanism cannot take place, and overall toxicity of tau is reduced in flies with *UAS-hisRFP.* This theory should be explored through experimentation that can recognize and measure differences in post-translational modifications on histones in the presence of PG-specific tau expression and relevant controls.

No matter the reason, it appears that histoneRFP has the capacity to alter tau toxicity. This could influence the interpretation of our data. Because crossing *astrocyte-GAL4* flies with *UAS-Tau* flies is not lethal, we do not know if this change in toxicity is specific to PG tau expression. However, it is likely that similar mechanisms decreasing toxicity are present in both models. Therefore, to confirm the differences in toxicity levels of astrocytic tau expression, this study should be repeated with only *astrocyte-GAL4* (only) flies to understand how overexpression of tau, only, in astrocytes truly interacts with TBI and, when compared to our data, understand what levels of toxicity are suppressed when expressing both tau and histoneRFP in astrocytes using the GAL4/UAS system. Therefore, both *astrocyte-GAL4, UAS-hisRFP* or *astrocyte-GAL4* constructs allow for valid analyses of tau presence in *Drosophila melanogaster*.

Conclusions and future directions

Overall, our study found that the interaction between astrocytic tau and TBI affect both aggregation and lifespan. When TBI occurs earlier in life, astrocytic tau pathology increases, particularly in females. We found that aggregates that were marked with either S422 or AT100 antibodies aren't affected by TBI at day 10 when exposed to a single hit on day 9 or multiple hits on days 3, 5, 7, 9. We did, however, find that flies hit day 9 had a higher presence of AT100+ puncta compared to flies hit on day 29 or days 23, 25, 27, and 29. This was most apparent in females who were hit day 9 and had significantly more aggregates than females of both late TBI exposure and males who were hit on day 9 as well. Additionally, toxicity was measured by a MI_{10} and a life span analysis up to day 60. We found that elevated toxicity was present at day 10 in *astrocyte>tau* flies who were hit multiple times in the analysis from MI_{10}. We also studied how lifespan was affected by TBI and astrocytic tau expression. Through this, we found a dose dependent decrease in lifespan in *astrocyte>tau* flies in the context of TBI. These results demonstrate that TBI exacerbates tau pathology in both facets of aggregation and toxicity.

Our study also illuminates that tau aggregation is not linked to toxicity because our MI_{10} indicated *astrocyte>tau* toxicity was related to the number of hits while the number of aggregates did not change based on TBI exposure. Additional studies exploring other assessments of neurodegeneration through histological analyses would be beneficial. This assay would highlight the timing of when the brain loses mass and what types of TBI exposures are most vulnerable for these effects. Future studies should also examine how hits that are delivered later in life, like between days 23-29 and day 29, influences toxicity. Performing this study would help scientists differentiate between the effects of early and late TBI exposures on lifespan.

In conclusion, we found that astrocytic tau interacts with TBI. These discoveries can help inform future analyses of astrocytic tau or other glial tau pathologies that affect diverse populations. Our finding regarding the dose-dependent toxicity of TBI is also important to inform vulnerable individuals of what effects multiple TBI events can have in comparison to single exposures. All in all, our study addressed the unknown relationship between TBI and astrocytic tau pathology in *Drosophila melanogaster*. As more gaps continued to be filled by

analyses like this one, it is crucial that TBI and glial tau pathology continued to be studied in depth.

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