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The Effect of Glial Tau Expression and Traumatic Brain Injury on Heat Shock Protein Expression

by

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ABSTRACT

Recent studies examining the brains of footballers and boxers have shown that successive, low-impact traumatic injuries to the head lead to Chronic Traumatic Encephalopathy (CTE), a tauopathy characterized by the aggregation of misfolded Tau both in neuronal and glial cells. Glial cells play a crucial role in overall brain health, but the effect of glial Tau aggregation in CTE, as in other tauopathies, remains unclear. In this project, we used a *Drosophila* model of human glial tauopathy to investigate the effect of both glial Tau expression and Traumatic Brain Injury (TBI) on the expression of two Heat Shock Proteins (Hsps). Hsps are a class of molecular chaperones that prevent the aggregation of misfolded proteins, and the formation of Tau tangles suggests a deficit in this chaperone system. We have found that Hsp23 and Hsp70 levels attenuate with aging. Moreover, the presence of glial Tau in 3-day-old flies similarly results in a decline in Hsp levels. TBI also leads to a reduction in Hsp70 levels in young flies that do not express glial Tau, while Hsp levels in older flies were resistant to TBI-induced changes. Our results indicate that the Hsp chaperone system is indeed affected by TBI and compromised in tauopathies; the Hsp chaperone system represents a pathological pathway that may be targeted in an attempt to combat tauopathies.

INTRODUCTION

Chapter 1: General Overview

About 110 years ago, Alois Alzheimer first identified the hallmarks of Alzheimer's disease – extracellular amyloid plaques, composed of the peptide beta amyloid and intracellular neurofibrillary tangles, composed of the misfolded Tau protein. A century later, researchers have discovered many other diseases characterized by Tau pathology and have named this class of diseases "tauopathies." Tauopathies are often characterized by memory deficits, disorientation, and mood and behavior alterations. Chronic Traumatic Encephalopathy (CTE) is one such tauopathy that results from repetitive bouts of Traumatic Brain Injury (TBI) and affects athletes involved in contact sports such as football, wrestling, and boxing. Initially, these characteristic Tau tangles were thought to exist only in neurons of tauopathy-affected persons but studies have demonstrated that they also occur in the glial cells of the brain (Chin and Goldman, 1996). The precise mechanisms of Tau-induced neurotoxicity, particularly toxicity related to glial tangles, remain unresolved.

Protein aggregation is characteristic of many diseases in the body, and most cells possess machinery to combat this aggregation. Heat Shock Proteins (Hsps) are a class of molecular chaperones that interact with cellular proteins to ensure proper folding and prevent aggregation of misfolded proteins (Feder and Hoffman, 1999). Hsps are often upregulated in response to stressors such as oxidative stress and heat shock, as these stressors induce protein misfolding and consequently, protein aggregation (Dickey et al., 2006). Under normal conditions, Hsps have been shown to bind to misfolded Tau to

prevent aggregation (Dou et al., 2003; Dickey et al., 2006) and the presence of Tau tangles, therefore, suggests a deficit in the Hsp chaperone system.

Many studies have posited that overexpressing Hsps in cells slows the onset and suppresses the symptoms of tauopathies (Kim et al., 2015; Eroglu et al., 2014); however overexpressing of Hsps has also been associated with the proliferation of cancerous cells (Jindal et al., 2016) and may therefore not be the most effective treatment for tauopathies. On the other hand, little is known about how the presence of Tau, particularly in glial cells, and the incidence of TBI, influence endogenous Hsps. To develop better treatments for tauopathies, it is necessary to characterize the endogenous Hsp response to glial Tau expression and to TBI. This will elucidate mechanisms underlying CTE and related tauopathies, allowing for the development of therapies that target specific pathways involved in the Hsp chaperone system.

Chapter 2: Tau And Tauopathies

Tau is the most abundant microtubule-associated protein (MAP) found mainly in the neurons but also in the glial cells of the brain. Its first known function was to regulate microtubule assembly in brain cells (Weingarten et al., 1975) and in recent years, it has also been shown to interact with the cell membrane, ribosomes, and DNA (Bukar Maina et al., 2016). In its misfolded state, Tau forms the neuronal and glial aggregates characteristic of a group of neurodegenerative diseases known as tauopathies. Tauopathies include the more common Alzheimer's Disease (AD), as well as Progressive Supranuclear Palsy (PSP), and Frontotemporal Dementia and Parkinsonism (FTDP). Some cases of these diseases involve mutations in the *mapt* gene that codes for Tau (Spillantini, 1998; Stanford et al., 2000; Pérez, 2000), but most tauopathies are sporadic and are not thought to be caused by genetic mutations. Some other tauopathies are associated with environmental causes. Chronic Traumatic Encephalopathy (CTE), for instance, is caused by external hits to the brain. In all tauopathies, tau inclusions are found to varying degrees in neuronal and glial cells. The presence of insoluble tau inclusions and neurodegeneration in these diseases suggests that there are common mechanisms underlying tau aggregation across tauopathies.

2.1 Tau Structure

Tau is an intrinsically disordered protein; it does not have a fixed three-dimensional structure, and as a result, easily self-aggregates into β-sheet structures, which are the core of the protein tangles in tauopathies (Kolarova et al., 2012). There are six isoforms of Tau in the human body. An isoform may have either three or four

repeat-regions in its microtubule-binding domain and have between zero to two inserts in its projection domain (Figure 1; Buee et al., 2000).

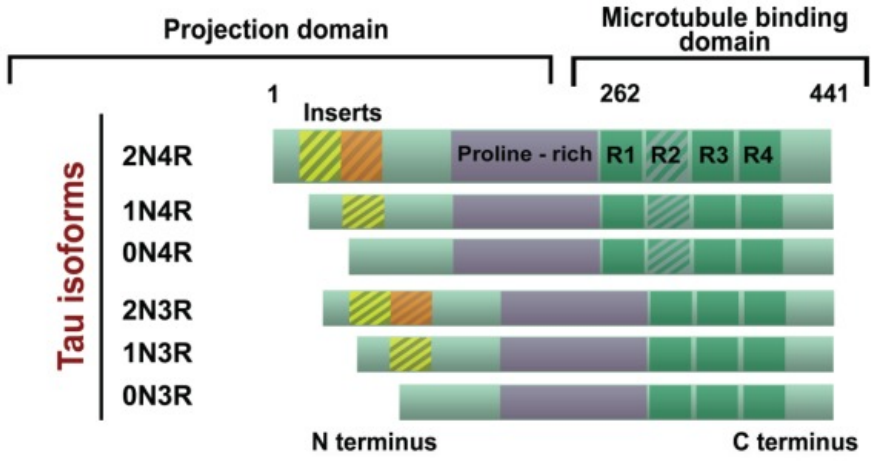


Figure 1. The six isoforms of Tau (Adapted from Simic et al., 2016)

Tau self-aggregation has been attributed to intermolecular interactions between the amino acids in its microtubule-binding domain (Peterson et al., 2008). This study demonstrated that self-aggregation of Tau led to the formation of initial Tau oligomers, which researchers such as Ward et al. (2012) have described as the key toxic species of Tau. In this study, we used 0N4R Tau, which is less prone to self-aggregation than 3R Tau (Barghorn and Mandelkow, 2002), but more prone to aggregation due to phosphorylation in the proline-rich region (Alonso et al., 2004). This Tau isoform was chosen both for its prevalence in neurofibrillary tangles and for its susceptibility to Tau phosphorylation, which has been found to play a key role in the progression of tauopathies.

2.2 Tau Phosphorylation

Phosphorylation appears to be a critical post-translational modification that controls Tau's normal function as well as its pathological nature. For its normal functioning, the coordinated phosphorylation and dephosphorylation of Tau regulates its binding to axonal microtubules in order to control axonal transport (Figure 2). Kinases such as GSK-3β and phosphatases such as PP2A have been found to modulate Tau's association with microtubules (Kolarova et al., 2012).

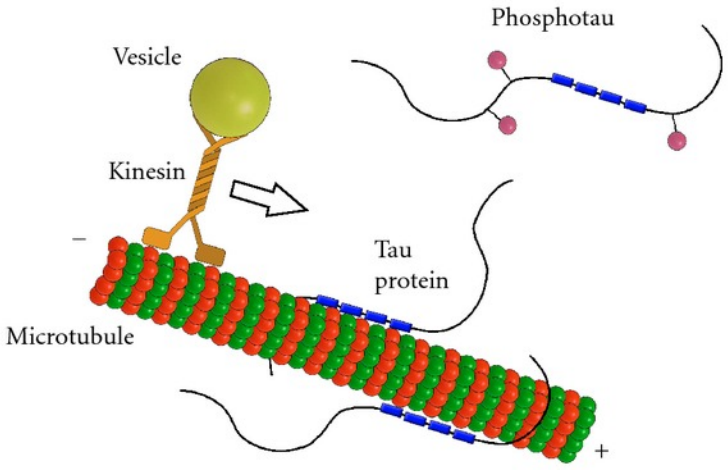


Figure 2. Normal Tau function. Tau stabilizes microtubules with its tubulin binding domains (blue boxes). Phosphorylation (pink balls) causes detachment of Tau from microtubules, regulating axonal transport (Adapted from Kolarova et. al, 2012).

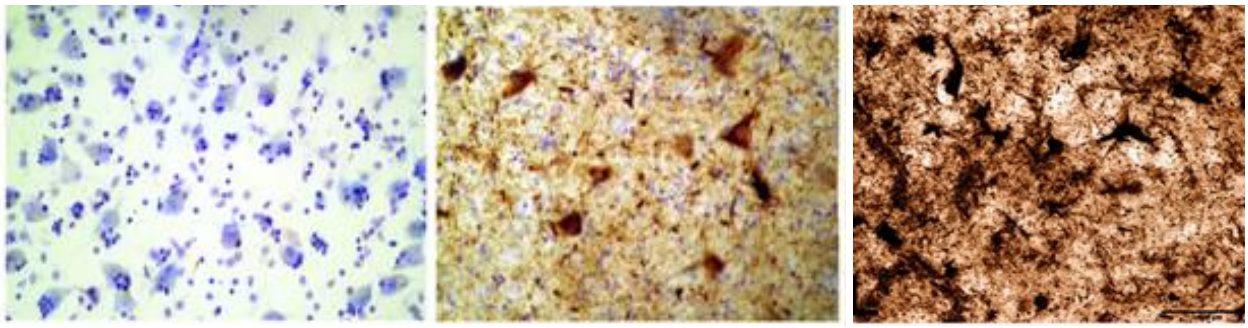
Studies have shown that Tau phosphorylation in the proline-rich region also causes dramatic changes to Tau structure – the protein loses its ability to bind to microtubules, and it also becomes more susceptible to aggregation (Fischer et al., 2009; Mukrasch et al., 2009). In tauopathies, Tau is abnormally hyperphosphorylated. Tau found in the tangles of the AD brain, for instance, has about four fold more

phosphorylation than normally functioning Tau (Iqbal et al., 2009), which is potentially caused by the upregulation of kinases and/or downregulation of phosphatases (Kolarova et al., 2012). Tau hyperphosporylation has been proposed to contribute to neurodegenerative disease pathology in two main ways. The first follows a toxic loss of function hypothesis, in which hyperphosphorylated Tau loses its ability to bind to microtubules. This leads to impaired microtubule function that in turn inhibits the transport of essential molecules along the axon (Cleveland et al., 1977). The second hypothesis attributes a toxic gain of function to tau phosphorylation. Phosphorylation distorts normal Tau structure, increasing the tau-tau interactions that lead to oligomer and aggregate formation and consequently, the activation of toxic pathways (Grundke-Iqbal et al., 1986).

2.3 Tau Pathology in Glial Cells

Originally considered as non-functional "glue" for neurons, the molecular functions of glial cells were neglected for years, and most attention was paid to neuronal cell function. However, glial cells make up about 50% of the brain (Azevedo et al., 2009), and over the years, the subgroups of glial cells (astrocytes, microglia and oligodendrocytes) have been found to play crucial roles in neuronal communication, neuronal support and neuronal protection from insults such as brain injury. In response to injury, for instance, glial cells undergo a process known as reactive gliosis, in which the glial cells are activated and secrete molecules to prevent further neurodegeneration (Ridet et al., 1997). The functions of glial cells under pathological conditions, including Tau pathology, remain unresolved.

In tauopathies such as CBD and PSP, more Tau aggregates have been found in glial cells than in neuronal cells (Binder et al., 1985; Ikeda et al., 1998). Tauopathies such as CTE are also characterized by a combination of glial and neuronal tangles. The presence of Tau tangles in glial cells remains a mystery, considering that the Tau protein itself is largely localized to neurons. Some researchers have suggested that in tauopathies, Tau tangles are phagocytosed by glial cells (Bolós, 2016); others have demonstrated that dormant genes are aberrantly transcribed in the presence of neuronal Tau pathology (Frost et al., 2014) and this may explain why Tau also accumulates in glial cells in neurodegenerative diseases.



Healthy Brain Tissue Neuronal Tau Aggregation Glial Tau Aggregation

Figure 3. Tau aggregation in the neuronal and glial cells of CTE brains (Adapted from McKee et al., 2009; McKee et al., 2015).

Glial Tau pathology has been shown to have functional consequences. Forman et al. (2005) have demonstrated Tau tangles in glial cells lead to neuronal degeneration even in the absence of neuronal Tau tangles. Similarly, Akiyama et al. (2006) have shown that neuronal degeneration begins after Tau has accumulated in glial cells. These studies suggest that glial cells might play early pathogenic roles in tauopathies, and

highlight the importance of elucidating mechanisms of glial Tau pathology in order to get closer to developing better treatments for tauopathies.

Chapter 3: The Drosophila Model of Tauopathy

Despite the contrast between the size of a fruit fly, *Drosophila melanogaster*, and a human, the fruit fly genome contains homologs of approximately 70% of human genes. The first ever detection of Heat Shock Proteins (Hsps), molecules crucial to human health, was made in the fruit fly (Ritossa, 1962), highlighting the potency of fruit fly genome studies.

The fruit fly brain is just as complex as the human brain, containing specialized cells that share anatomical and molecular similarities with humans. For instance, the fly brain comprises functionally diverse neurons that possess all of the structural features, neurotransmitters, and ion channels found in human neurons (Littleton et al., 2000). Like the mammalian brain, the fly brain also contains well-defined glial subtypes (astrocytes and ensheathing glia) that function to protect the fly brain using mechanisms similar to those in the human brain (Logan and Freeman, 2007).

Since early tauopathy research was limited to observation of post-mortem brains at advanced disease stages, cognitive decline and neuronal cell death could only be linked to the final stages of Tau aggregation. However, the development of animal models of tauopathies has facilitated the identification of much earlier pathogenic events. For instance, the formation of toxic Tau oligomers that appear upstream of tangle formation was demonstrated in a mouse model of neuronal tauopathy (Ojo et al., 2016). Transgenic mice that express human Tau also exhibit similar pathological features as humans do in the diseased state, such as the formation of Tau tangles (Andorfer et al., 2005; Berger et al., 2007). These mice also demonstrate cognitive decline

when tasked with behavioral experiments, and the similarities of these symptoms to human symptoms make these animal models useful in the investigation of tauopathies.

Several *Drosophila* models have been used successfully to model human tauopathies such as Alzheimer's Disease, where they have identified key pathological processes in the progression of the disease. For instance, Frost et al. (2014) used a fly model of neuronal tauopathy to demonstrate that Tau led to heterochromatin relaxation, which in turn led to the aberrant expression of certain genes. Fortini and Bonini (2000) have also used a fly model of neuronal tauopathy to identify apoptotic neuronal cell death as an important pathological feature of tauopathies.

In this study, we used the fruit fly model of glial tauopathy originally described by Colodner and Feany (2010). This system capitalizes on the yeast GAL4/UAS system to express 0N4R human Tau specifically in the glial cells of the fruit fly. Colodner and Feany demonstrated that flies expressing Tau in their glial cells exhibited characteristics similar to those observed in human tauopathies: glial tangle formation accompanied by a shortened lifespan and age-dependent cell death in neurons and glia.

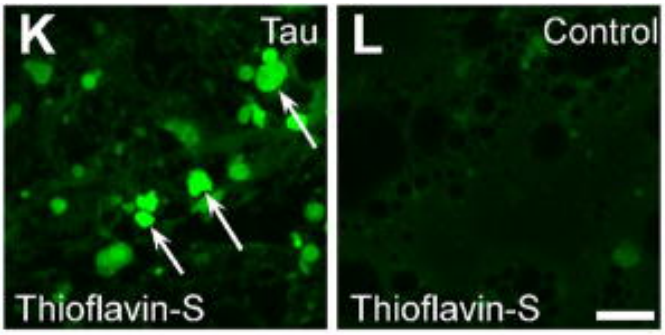


Figure 4. The *Drosophila* model of glial tauopathy produces glial tangles similar to human glial tangles. Arrows point to the glial tangles (Adapted from Colodner and Feany, 2010).

The similarities between human and fruit fly glial Tau pathology, as well as the utility in using the fruit fly model to identify upstream pathogenic events, makes this system a reliable one for elucidating the mechanisms underlying tauopathies, as well as the role that glial Tau accumulation may be playing in the disease process.

Chapter 4: Traumatic Brain Injury and Chronic Traumatic Encephalopathy

Traumatic Brain Injury (TBI) is a leading cause of morbidity and mortality in children and particularly, in adults around the world. A single mechanical blow to the head can result in a concussion, with the sufferer experiencing temporary headaches and vision impairments.

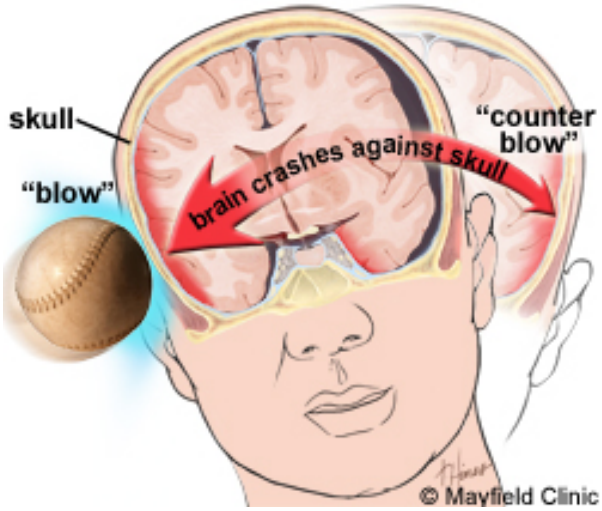


Figure 5. Schematic of Traumatic Brain Injury. During impact, the brain bounces back and forth against the skull causing bruising, bleeding, and shearing (Adapted from www.mayfieldclinic.com)

Repetitive TBI usually has more damaging long-term effects. Postmortem brain studies have shown that athletes involved in contact sports, where they receive multiple sub-lethal blows to the head, can develop a neurodegenerative disease called Chronic Traumatic Encephalopathy (CTE). The symptoms of CTE are often insidious: the affected person first displays deficits in attention, concentration and memory, as well as disorientation and occasional dizziness. As the disease progresses, their judgment declines and they develop dementia. In severe cases, the sufferer may experience slowed muscular movements, impaired speech and vertigo. The severity of CTE

depends on the length of time spent playing a sport and the number of traumatic brain injuries experienced. Currently, CTE can only be diagnosed postmortem (McKee et al., 2009).

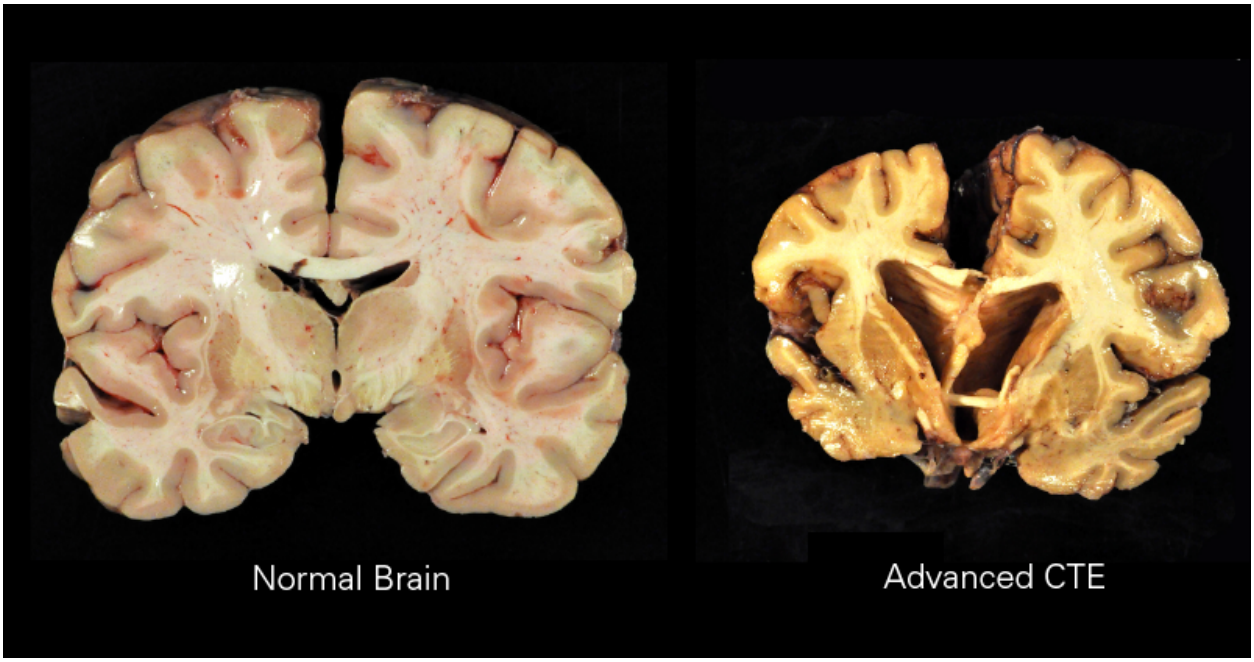


Figure 6. Neuropathological features of CTE. CTE brains have reduced brain weight, coupled with atrophy of the frontal and temporal cortices and medial temporal lobe. The lateral ventricles and the third ventricle are often enlarged, and in rare instances, the fourth ventricle is dilated (Adapted from www.commons.wikimedia.org)

Living humans with acute TBI and those who have died from CTE exhibit neurofibrillary Tau tangles, neuropil neurites, and glial Tau tangles in multiple brain regions including the hippocampus, the medial temporal lobe, and the frontal cortex. This pathology is also accompanied by neuronal and glial degeneration (McKee et al., 2009). Interesting to note, is that the human brain can limit the spread of TBI-related damage by creating a glial scar that seals off the injured region and prevents further damage. For instance, reactive astrocytes in this glial scar produce nutrients to and

support the viability of the surviving cells (Ridet et al., 1997). This highlights how important glial cells are in the process of recovery from TBI.

Studies suggest that TBI causes mechanical damage to the blood-brain barrier and induces the release of local neurotoxins, explaining why the Tau tangles in CTE often occur in regions surrounding the blood vessels (Geddes et al., 1999; Buee et al., 1994). Astrocytes play a prominent role in maintaining the blood-brain barrier, and the accumulation of tau in astrocytes surrounding blood vessels is also a prominent feature of CTE (McKee et al., 2009). However, the mechanism by which Tau aggregation itself occurs in acute TBI and CTE, in both neuronal and glial cells, like in many other tauopathies, remains to be determined.

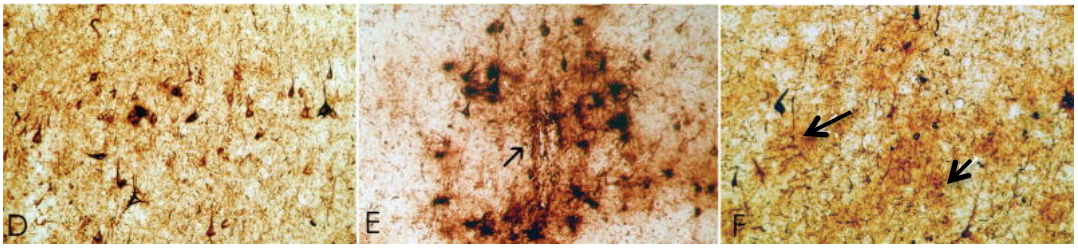


Figure 7. Tau pathology in postmortem CTE brains. There are dense tau-immunoreactive neurofibrillary tangles (NFTs) and neuropil neurites throughout the cortex (D), astrocytic tangles around small blood vessels (shown by the arrows in E) and plaque-like clusters of tau-immunoreactive astrocytic processes distributed throughout the cortical layers (shown by the arrows in F) (Adapted from McKee et al., 2009).

Findings from animal models of TBI and CTE parallel those in humans. In a mouse model expressing human Tau in neurons, repetitive mild TBI led to increased levels of hyperphosphorylated Tau and Tau oligomers and injured animals exhibited memory impairment and behavioral changes (Ojo et al., 2016). Glial changes were also observed in this mouse model of TBI. Following brain insult, glial cells (particularly astrocytes and microglia) underwent morphological transformation into an activated

state to combat the effects of TBI (Ojo et al., 2016). These pathological changes to glial cells are known to promote recovery and/or underlie the pathobiology of certain deficits.

Researchers have also successfully developed fly models of TBI. Katzenberger et al. (2013) created a closed head fly TBI model that used a mechanical device to subject flies to brain injury. Similar to humans with TBI, flies that had received injuries to the head demonstrated temporary incapacitation, ataxia, immune response activation, neurodegeneration, and death. Barekat et al. (2016) developed a similar closed head TBI device and used a fruit fly model of neuronal tauopathy to show that repetitive, mild TBI led to increased Tau phosphorylation in neurons, as well as the activation of inflammatory and autophagy responses.

All these animal studies have revealed some repercussions of TBI in the absence and presence of neuronal Tau; however, the effects of TBI in the presence of glial Tau remain to be outlined.

Chapter 5: Heat Shock Proteins

Heat Shock Proteins (Hsps) were first identified in *Drosophila* in 1962, after flies exposed to elevated temperatures displayed swellings known as "puffs" on their chromosomes. These puffs indicated that genes had been transcriptionally activated in response to excessive heat to combat the heat-induced protein misfolding. Since their discovery, Hsps have been found to occur in all cells of all plant and animal species studied, an indication that these genes are highly conserved across species. While Hsps were originally named for their induction by heat, they are now known to respond to a range of environmental and metabolic stressors including viral infection, fever, hypoxia, radiation, ischemia, heavy metal ions, ethanol, nicotine and surgical stress. Consequently, researchers such as Whitley et al. (1999) prefer to refer to Hsps as "stress proteins." While some Hsps are induced by stress, others are expressed constitutively in unstressed cells, in which case they are known as Hscs, short for Heat Shock Cognates (Morimoto et al., 1997).

Hsps are classified into families based on their molecular weights. They include the Hsp110, Hsp100, Hsp90, Hsp70, Hsp60, and the Hsp40 families, as well as the small HSP family. In eukaryotes, many of these groups are comprised of multiple members that may differ by how inducible they are, by their intracellular localization, and by their function. The functions of several Hsps have been outlined in *Drosophila* (Mehlen et al., 1996; Michaud et al., 2002; Chang et al., 2002).

5.1 Hsp Mechanism of Action

Proteins adopt non-native conformations either due to protein-denaturing stress or because they have not been fully synthesized, folded, assembled, or localized to their appropriate cellular compartments. Hsps act as molecular chaperones, interacting with misfolded proteins to reduce the likelihood that these proteins will interact improperly with one another. Hsps recognize and bind to misfolded proteins usually by association with and/or hydrolysis of nucleotides (Feder and Hoffman, 1999). Protein denaturation exposes hydrophobic amino acid residues, which are also the binding sites for Hsps.

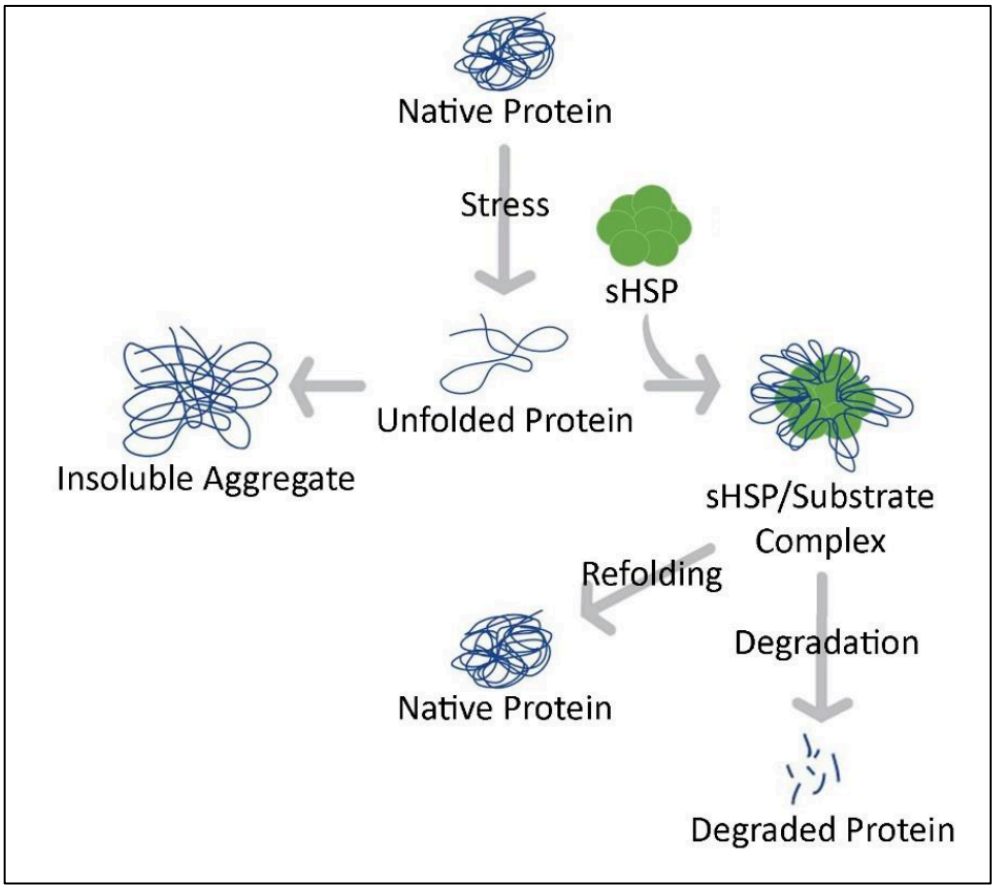


Figure 8. Hsp mode of action (Adapted from Kahlson, 2015).

Hsp expression is mediated by HSF1, a transcription factor that binds to promoters upstream of the Hsp genes to activate their transcription. After their production has been stimulated, some Hsps such as Hsp70 deactivate HSF1 through a feedback inhibition loop to prevent further Hsp transcription (Krakowiak et al., 2018).

5.2 Hsp70

Localized to the cell nucleus and cytosol, Hsp70 is the most characterized heat shock protein. It binds transiently to the hydrophobic regions of nascent proteins and prevents them from adopting non-functional conformations. Hsp70 functions together with co-chaperones such as the Hsp40s and CHIP (carboxy terminus of Hsc70 interacting protein), a molecule that contains E3 ubiquitin ligase activity and facilitates protein degradation (McDonough and Patterson, 2003). Hsp70 relies on the hydrolysis of ATP to ADP to adopt different conformations that ensure efficient chaperone activity (Sharma and Masison, 2009).

Several studies in *Drosophila* models of neurodegenerative disease have reported that overexpressing Hsp70 can prevent neuronal loss and reduce the symptoms of neurodegenerative diseases (Auluck et al., 2002; Kim et al., 2015) and interference with endogenous Hsp70 activity has been found to exacerbate the pathological phenotype in some neurodegenerative diseases (Auluck et al., 2002). This demonstrates the therapeutic potential of Hsp70 in neurodegenerative diseases.

5.3 Small Hsps

The small Hsps (sHsps) range in size from 10 to 40kDa and four of these sHsps have been extensively studied in *Drosophila*. Following exposure to heat shock, all four

sHsps are upregulated (Marin et al., 1996; Michaud et al., 1997). To carry out their functions as molecular chaperones, sHsps assemble into large oligomeric structures. They do not require ATP hydrolysis, but they maintain proteins in a folding-competent state until they are refolded by downstream ATP-dependent chaperone systems (Haslbeck et al. 2005). The four sHsps have developmental and tissue-specific expression patterns (Table 1).

Table 1. Small Hsps of *Drosophila* are expressed in different cell-types throughout development. (Adapted from Morrow and Tanguay, 2003)

		Developmental stages		
sHsp	**mRNA/protein**	**Embryonic**	**Larval**	**Adult**
Hsp22	mRNA	Metameric ectodermal patches	Absent	Head and thorax
Hsp23	Protein	CNS	Present	Gonads, fat body, CNS
Hsp26	Protein	CNS, male gonads	Male gonads, CNS, ventral ganglion	CNS, gonads
Hsp27	Protein	Ubiquitous	CNS, gonads	CNS, gonads

CNS = Central Nervous System

There are also differences in subcellular localization of these chaperone proteins. Hsp22 is expressed mainly in the mitochondrial matrix (Morrow et al., 2000), Hsp23 and Hsp26 are localized to the cytosol, and Hsp27 is localized to the nucleus (Beaulieu et al., 1989; Marin and Tanguay, 1996). These differences imply that each sHsp has different intracellular protein targets or regionally specialized effects. Some sHsps have also been found to be more efficient chaperones than others. For instance, Hsp22 and Hsp27 prevent heat-induced protein aggregation better than Hsp23 and Hsp26 (Morrow et al., 2006), which may also affect cellular processes at different places within the cell.

5.4 Hsps and Tau

Some Hsps bind directly to Tau, independent of its phosphorylation status, to promote Tau's interaction with microtubules and limit Tau aggregation (Matts et al, 2011) while others preferentially bind to hyperphosphorylated Tau to enhance its degradation and dephosphorylation (Shimura et al, 2004). Dou et al. (2003) demonstrated, using a model of Alzheimer's disease, that Hsp70 binds directly to Tau to promote microtubule binding, decreasing tau phosphorylation and aggregation. Patterson et al. (2011) have also shown that Hsp70 preferentially binds to oligomeric Tau over fibrillary Tau, another possible indication that oligomeric Tau plays a key role in Tau toxicity. Few studies have investigated sHsp-Tau interactions but Hsp27 has been shown to preferentially bind to hyperphosphorylated Tau to enhance its degradation and dephosphorylation (Shimura et al, 2004).

5.5 HSPs and aging

Protein-folding diseases are more common in older persons than in younger people. This is thought to be because aging comes with increased oxidative stress, as well as an increased probability for biosynthetic errors during protein formation (Sohal et al., 2002). At the same time, Hsp levels attenuate with aging in most organisms, diminishing the protein quality control mechanisms that prevent protein aggregation in the cell (Tower, 2009; Morrow and Tanguay, 2003).

Small Hsps in particular, have been implicated as supporters of healthy aging. For instance, the overexpression of Hsp26 in *Drosophila* cells led to a 15% increase in lifespan while overexpression of Hsp70 had no effect on lifespan (Morrow and

Tanguay, 2003). This indicates once more that different Hsps may target different cellular processes that influence general cellular health.

5.6 Hsps and TBI

A number of animal models of TBI have been developed and some of these have been used to examine Hsp expression in relation to TBI. Brown et al. (1989) mimicked TBI-related brain damage by making surgical lacerations in the cerebral cortex of anesthetized rats and determined that Hsp70 mRNA was activated in both glial and neuronal cells in the cerebral cortices of the TBI rats. This induction of Hsp70 was transient, with elevated mRNA levels being observed 2 hours after injury and a reduction to basal levels in 24 hours. Another study conducted by Hayes et al. (1995) used the fluid-percussion TBI model to investigate the impact of TBI on Hsp70. In this model, brain injury is inflicted through a craniotomy, where a brief fluid pressure pulse is applied onto the intact dura. They also found that Hsp72 mRNA was induced after injury and returned to control levels after 24 hours. With regard to Hsp protein levels, DeGracia et al. (2007) have also used surgical incisions in a rat model of TBI to demonstrate that Hsp70 protein levels are steadily increased from 4 to 48 hours following traumatic brain injury. In this experiment as well, rats were anesthetized prior to TBI and Hsp protein induction was localized to the region where the incision was made.

It is expected that the upregulation of Hsp mRNA will lead to an increased expression of the Hsp proteins that could potentially rescue deficiencies resulting from brain injury. Indeed, studies have been conducted to demonstrate the therapeutic potential of Hsps. In a study conducted on genetically modified mice overexpressing

Hsp70, Eroglu et al. (2014) observed that injured mice had an improvement in memory deficits compared to those that did not overexpress Hsp70 (Eroglu et al, 2014). Kim et al (2015) have shown, similarly, that pharmacologically inducing Hsp70 expression, by intraperitonial injection of 17-AAG, protects mice from repercussions of brain injury such as reduced alertness levels. Despite their advantages in improving the symptoms of diseases, it has been suggested that overexpression of Hsps could possibly also play a role in the progression of carcinogenesis (Jindal et al., 2016), highlighting the need to better characterize the factors that control Hsp expression in disease states.

Though animal TBI models have played a significant role in investigating endogenous HSP expression in response to TBI, they often use approaches that are not similar to physiologically experienced TBI. For instance, athletes do not experience TBI while under anesthesia but many of the TBI models used by researchers involve anesthetizing the animals used in experimentation. Anesthetizing rats for instance, has been shown to alter protein levels in the rat brain, an effect that lasts up to 28 days (Kalenka et al., 2007). The effect of anesthetics on protein expression might thus confound the interpretation of results obtained from TBI studies. It is important therefore to investigate the impact that more physiologically experienced TBI, without anesthesia, can have on endogenous protein expression, particularly when examining the effect of TBI on the dynamic Hsp system.

Chapter 6: Aim of Study

As evidenced by Forman et al. (2005), Akiyama et al. (2006) and Colodner and Feany (2010), glial Tau aggregations represent a significant pathological event within the context of tauopathy progression. The goal of this study is to first describe whether the presence of glial Tau pathology (or glial tau expression) impacts endogenous Hsp expression in an age-dependent fashion. Secondly, we aim to characterize the response of two heat shock proteins, Hsp23 and Hsp70, to our *Drosophila* model of TBI in flies of different ages that either express or do not express glial Tau. The ultimate objective of this project is to elucidate molecular mechanisms underlying CTE by examining how Hsp expression is affected by TBI impact and/or the presence of Tau in glial cells. These studies may thus help in the development of new and targeted therapeutic measures for prevention and treatment of CTE and related tauopathies.

MATERIALS AND METHODS

1. *Drosophila* Stocks and Genetics

1.1 The GAL4/UAS System

To model glial tauopathy in *Drosophila*, we utilized the GAL4/UAS (upstream activating sequence) system for the ectopic expression of a human Tau specifically in glial cells. To develop this system, the gene that encodes the yeast transcriptional activator, GAL4, is inserted into the *Drosophila* genome. The GAL4 gene is usually placed downstream of a cell- or tissue-specific promoter. Flies with this GAL4 gene in their genome are then crossed to flies containing a UAS-human transgene construct to yield progeny expressing both cell-specific GAL4 and the UAS-human transgene construct. In cells where GAL4 is produced, the protein binds to the UAS region upstream of the human transgene, initiating the transcription of the human protein (Figure 9).

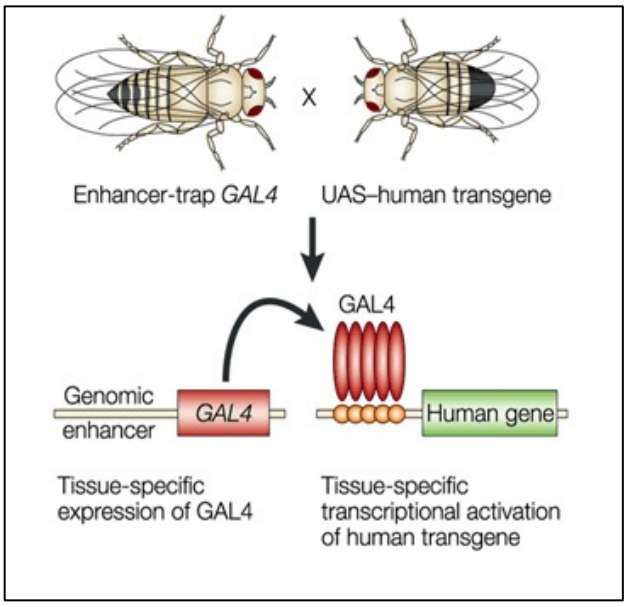


Figure 9. The GAL4/UAS System. (Adapted from Muqit and Feany, 2002)

In the fly lines used in this experiment, GAL4 expression was targeted to glial cells using the *repo-GAL4* gene construct. Repo is a glia-specific promoter that directs the transcription of our transgene, human Tau (0N4R) to glial cells only. Since overexpression of glial Tau in developing fruit flies is lethal, our fly stocks also expressed the temperature sensitive gene, *tub-GAL80^{ts}* to allow for temperature-regulated expression of glial Tau. GAL80 is a transcriptional repressor of the GAL4 protein. At 17°C, GAL80 fully suppresses GAL4, preventing the transcription of human Tau. At 25°C, GAL4 is only partially suppressed, allowing non-lethal levels of Tau to be produced in the glial cells of the flies. At 28°C, GAL4 is free from inhibition by GAL80, and will fully induce glial Tau overexpression. In most experiments using this system, flies are crossed at 17°C in order to prevent progeny mortality. The flies are then transferred to a 28°C incubator to allow for full transcriptional activation of glial Tau at the desired ages. In this project however, all flies were crossed at 25°C. Hsps are often activated at temperatures above 28°C and we aimed to ensure that any effect on Hsps resulted either from glial Tau expression or from TBI and not from the heat shock experienced at elevated temperatures.

1.2 *Drosophila* Crosses and Aging

The experimental fly stocks were of the genotype *repo-GAL4, tub-GAL80^{ts}, UAS-tau^{wt}/ TM3, Sb*. Control flies were genetically similar to the experimental flies, lacking only the *UAS-tau^{wt}* gene. They had the genotype *repo-GAL4, tub-GAL80^{ts}/ TM3, sb*. All fly stocks contained the fruit fly third chromosome balancer, *TM3*. Balancer chromosomes have modifications that prevent crossing over during meiosis. This prevents recombination and ensures that the inserted gene constructs (*repo-GAL4, tub-*

GAL80^{ts}, *UAS-tau^{wt}*) remain intact as they are being passed on from one generation to the next. The third chromosome balancer is also expressed phenotypically as Stubble bristles (*Sb*), allowing for differentiation between the progeny carrying the balancer and the progeny expressing the desired GAL4, GAL80 and human Tau genes. Virgin females from the fly stocks were crossed to *w-* males at 25°C to obtain experimental progeny. *w-* is a common fruit fly strain that lacks the red-eye phenotype found in wild type fruit flies, and the *repo-GAL4, tub-GAL80^{ts}, UAS-tau^{wt} / TM3, Sb* fly line was developed on a *w-* background. Virgin females from the Tau-expressing and control stocks were therefore crossed to *w-* males to remove the third chromosome balancer. Approximately 50% of the resulting progeny expressed white eyes with Stubble bristles, with the remaining 50% expressing desired phenotype – red eyes and normal bristles. All flies were aged to 3 days, 10 days, and 30 days at 25°C, and flies being aged were transferred to a new vial with fresh food every other day.

2. Traumatic Brain Injury (TBI)

2.1 TBI HIT Protocol

We inflicted acute closed head TBI on flies using the high impact trauma (HIT) device developed by Katzenberger et al. (2013). This device is comprised of a metal spring attached to a vial at one end and a polyurethane pad at the other end (Figure 10). A maximum of 20 unanesthetized flies were placed in a standard plastic vial and were confined to the bottom quarter of the vial using a plunger that is connected to the spring (Figure 10A). The spring is lifted to 90° and released, allowing the vial to contact the polyurethane pad very rapidly (Figure 10B). This delivers a mechanical force to the flies

as they bounce back and forth against the vial wall with different regions of their head and bodies striking the vial. Each fly hits the vial wall with a different force, and so degrees of primary injury vary among the flies. Closed head TBI mimics the randomness of brain injury that occurs in falls, sports collisions, and automobile crashes and the phenotypic effects on flies subjected to HIT device-induced TBI are similar to those in humans.

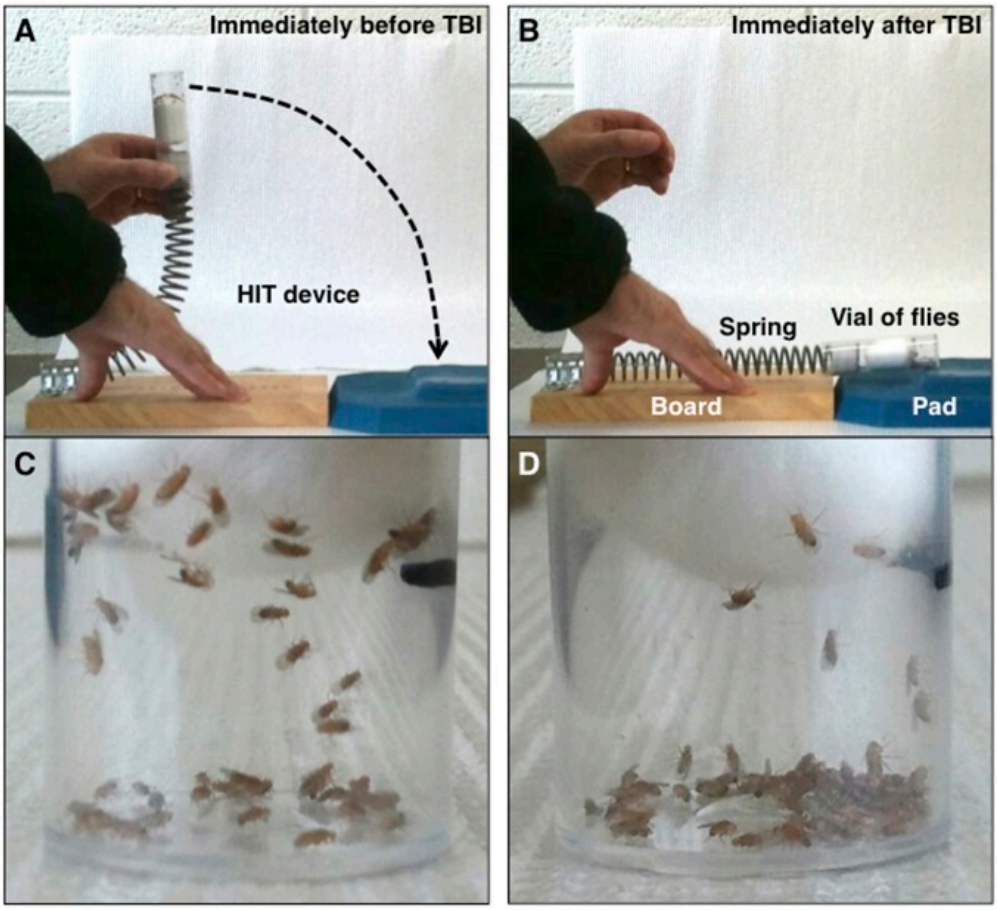


Figure 10. The HIT device used to inflict TBI on fly heads. (A) HIT device bent to 90° before a strike, (B) the HIT device immediately after a strike, (C) Flies in a vial before a strike, and (D) after a strike. (Adapted from Katzenberger et al., 2013)

In our experiments, each acute TBI event consisted of 4 successive hits with 5-minute recovery intervals between each hit. We used different hit protocols to examine

the impact of different hit paradigms on Hsp expression. In one experiment, 10 day and 30 day flies were hit on days 9 and 29 respectively, and were frozen at -80°C, 24 hours after they had recovered from the hit. This experiment was done to determine how Hsp protein levels changed after 24 hours of injury at different stages in the *Drosophila* lifespan. Another cohort of flies was hit on one of the following days during their lifespan – day 3, day 7, or day 9 – and frozen on day 10. This hit protocol was used to determine if HSP levels were influenced over a longer recovery time. A final cohort of flies was hit successively on days 3, 5, 7, and 9 and frozen at -80°C on day 10 to investigate the impact of successive brain injuries on Hsp expression.

2.2 Quantifying Mortality after TBI

With the knowledge that TBI in humans can be lethal, we investigated mortality levels in fruit flies using a measure known as MI_{24}. The MI_{24} measures fruit fly Mortality Index after 24 hours. For this analysis, we calculated the number of flies that died 24 hours after receiving acute brain injury as a percentage of the surviving flies. For flies that were hit successively over a span of one week, this calculation was done after every hit.

$$\text{Mortality Index} = \frac{\text{Number of dead flies 24 hours after TBI}}{\text{Number of surviving flies 24 hours after TBI}}$$

3. Western Blotting

3.1 Western Blot Procedure

Western blotting was used to determine changes in Hsp protein levels following TBI, as well as the changes caused by glial Tau expression. One head from a female fly of a specified age and condition was homogenized per microcentrifuge tube in 2X Laemli Buffer (Bio-Rad) to release brain proteins into solution. The resulting solution was boiled for 10 minutes in order to denature the proteins. For each experimental condition, 5-6 biological replicates were pipetted into separate wells in a 26-well Polyacrilamide gel (Bio-Rad). The sample material was then subjected to Polyacrilamide gel electrophoresis (PAGE) for an hour at 120V to separate the proteins in the sample by size (Figure 11). Separated proteins were then transferred onto a Polyvinylidene fluoride (PVDF) membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The membrane containing the transferred proteins was then washed in blocking solution (1% powdered milk, 0.1% Tween® in TBS) for 20 – 30 minutes to improve antibody specificity and signal-to-noise ratio. The membrane was treated overnight with the primary Hsp antibodies (all used at 1:1000) at 4°C. Primary antibodies were washed off after treatment and mouse and rabbit Anti-HRP secondary antibodies (used at 1:10,000) were applied to the membrane depending on the host species of the primary antibody. The secondary antibody was also washed off after 2 hours and ECL substrate (Bio-Rad) was run over the blot to bind to the secondary antibodies and allow for chemiluminescence imaging using a Fujifilm Image Reader LAS-3000.

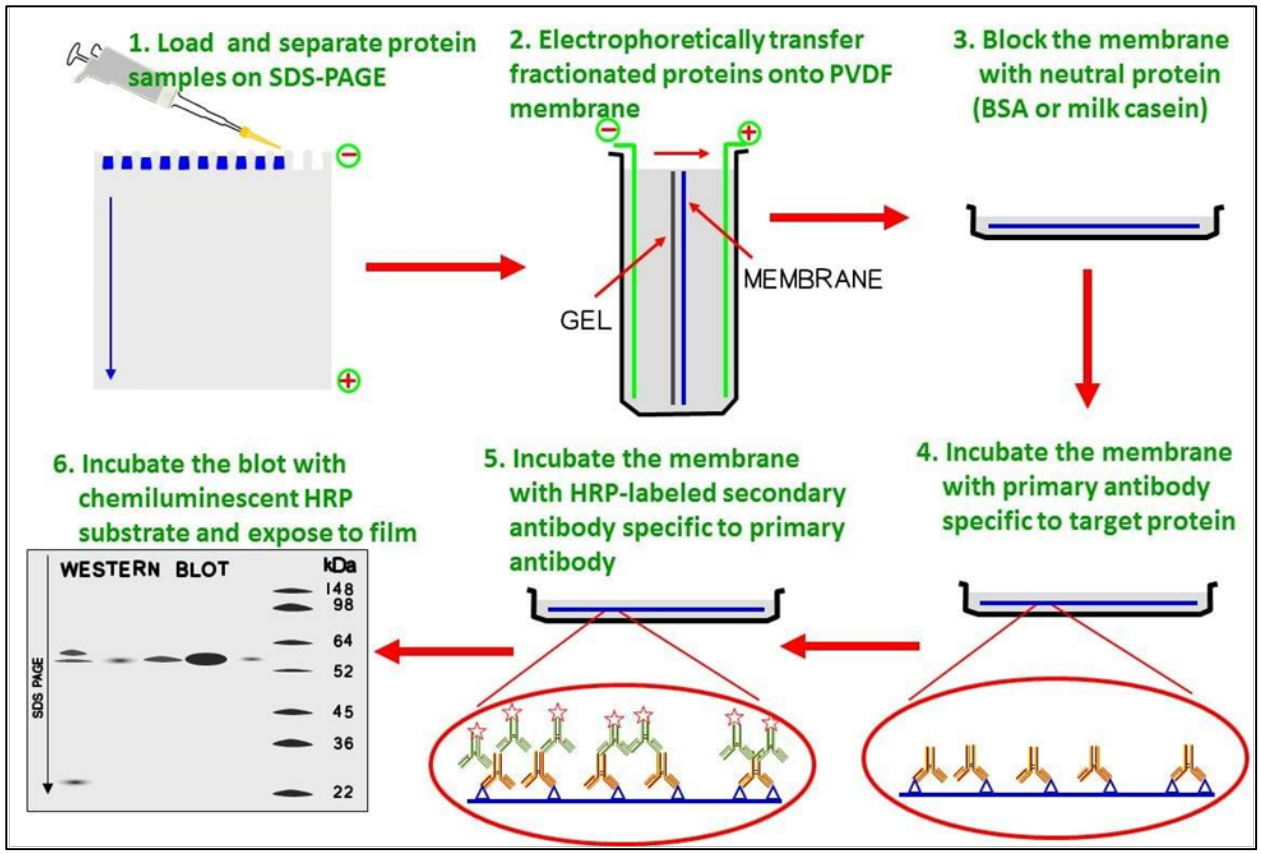


Figure 11. Western blotting procedure. (Adapted from www.microbeonline.com)

In order to draw accurate conclusions from Western blots, we had to ensure that any observed differences were due to altered protein expression levels and not gel loading or protein transfer errors. To do this, we used actin as a loading control (used at 1:1000) to indicate the total amount of protein present per fly head. In our analysis, Hsp levels are normalized with this actin loading control in the quantification process described below.

3.2 Western Blot Quantification

To quantitatively examine the changes in Hsp levels, we performed a densitometric analysis of the Western blots using the ImageJ software. In this analysis, the density of each protein band is measured and the software produces a numeric value corresponding to the darkness of the band. The Hsp band density is then calculated relative to the density of its corresponding actin loading control, to adjust for protein loading errors. Relative Hsp density is calculated as follows:

$$\text{Relative Hsp density} = \frac{\text{Raw Hsp band density}}{\text{Corresponding Actin band density}}$$

This relative density was then normalized to the average relative density of control samples, to allow for comparison between control and experimental samples. The formula used was:

$$\text{Normalized Hsp density} = \frac{\text{Relative Hsp band density}}{\text{Average Hsp band density of control samples}}$$

The averages of normalized Hsp band densities were calculated and used in plotting graphs. Average Hsp levels for control samples were always 1, since normalization was done relative to the control. The Welch's unpaired t-test was used to determine whether or not there was a significant difference between control and experimental Hsp levels. An ANOVA was also used to analyze the differences among different experimental means. All Western blot images and quantitative comparisons of respective proteins are from samples that were run on the same gel.

RESULTS

Hsp expression decreases with age

To determine if basal levels of Hsp expression change as the fly ages, we performed Western blot analysis to compare levels of Hsps in the heads of 3-day and 30-day-old flies. Densitometric analysis revealed a marked decline in Hsp70 and Hsp23 levels in both control and glial Tau-expressing flies (Figure 12), confirming the age-related decline in Hsp protein expression reported by Morrow et al. (2003).

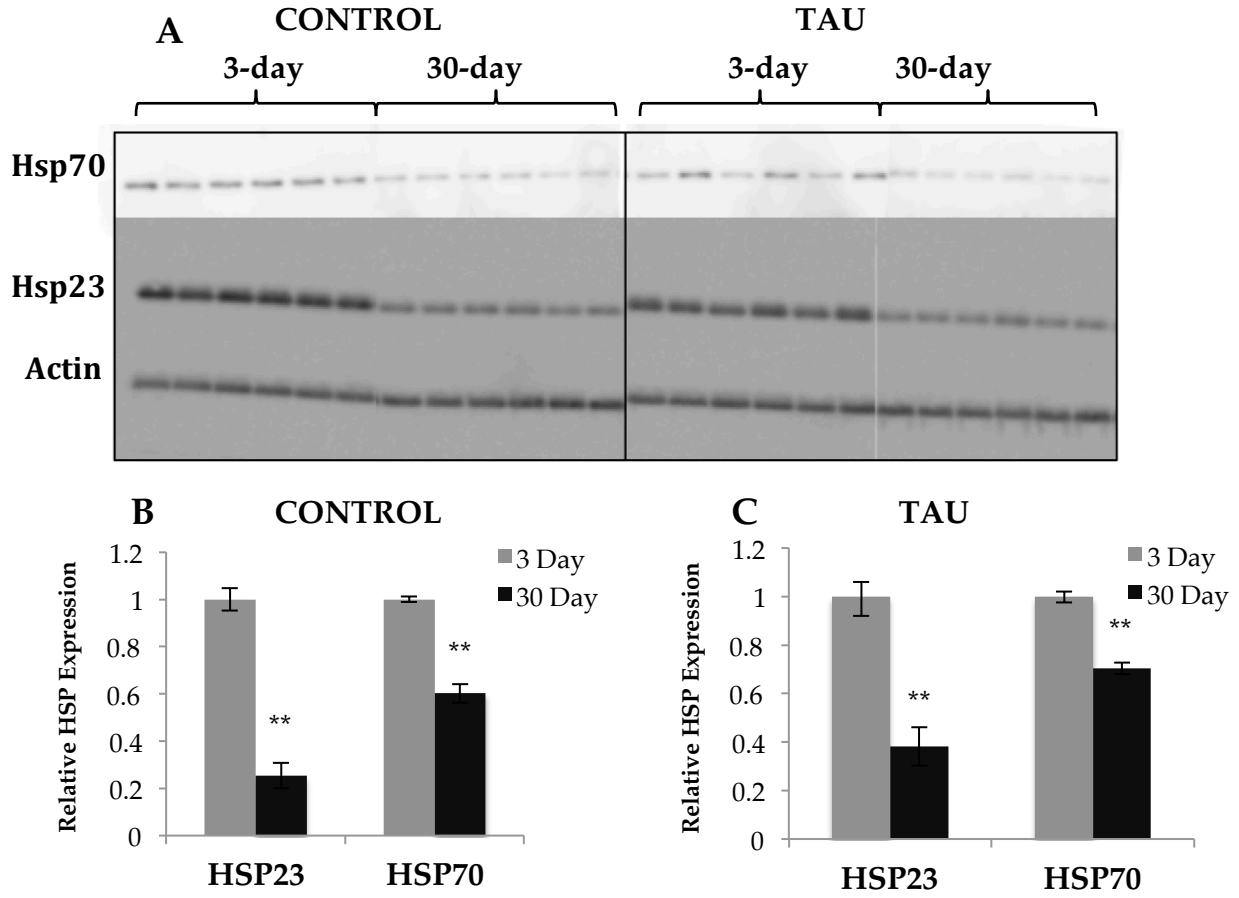


Figure 12. Hsp expression decreases with age. (A) Western blot analysis of Hsp23 and Hsp70 levels in 3-day and 30-day old control and glial Tau-expressing flies. Actin was used as a loading control. Densitometric analysis of Western blots comparing relative Hsp levels in 3 and 30-day-old (B) control flies and (C) glial Tau-expressing flies. n=6, ** $p<0.01$, t-test. Error bars represent ± SEM.

Glial Tau expression suppresses Hsp23 protein levels in younger flies

To investigate the impact of glial Tau expression on Hsp23 and Hsp70 levels, we compared Hsp protein levels in Tau and control flies at 3 and 30 days of age. Western blot results revealed an age-specific suppression of Hsp23 in 3-day-old glial Tau flies, compared to 3-day-old control flies. There was no difference in Hsp23 levels in 30-day-old control and glial Tau flies, and Hsp70 levels remained unchanged between 3 day and 30-day control and glial Tau flies (Figure 13).

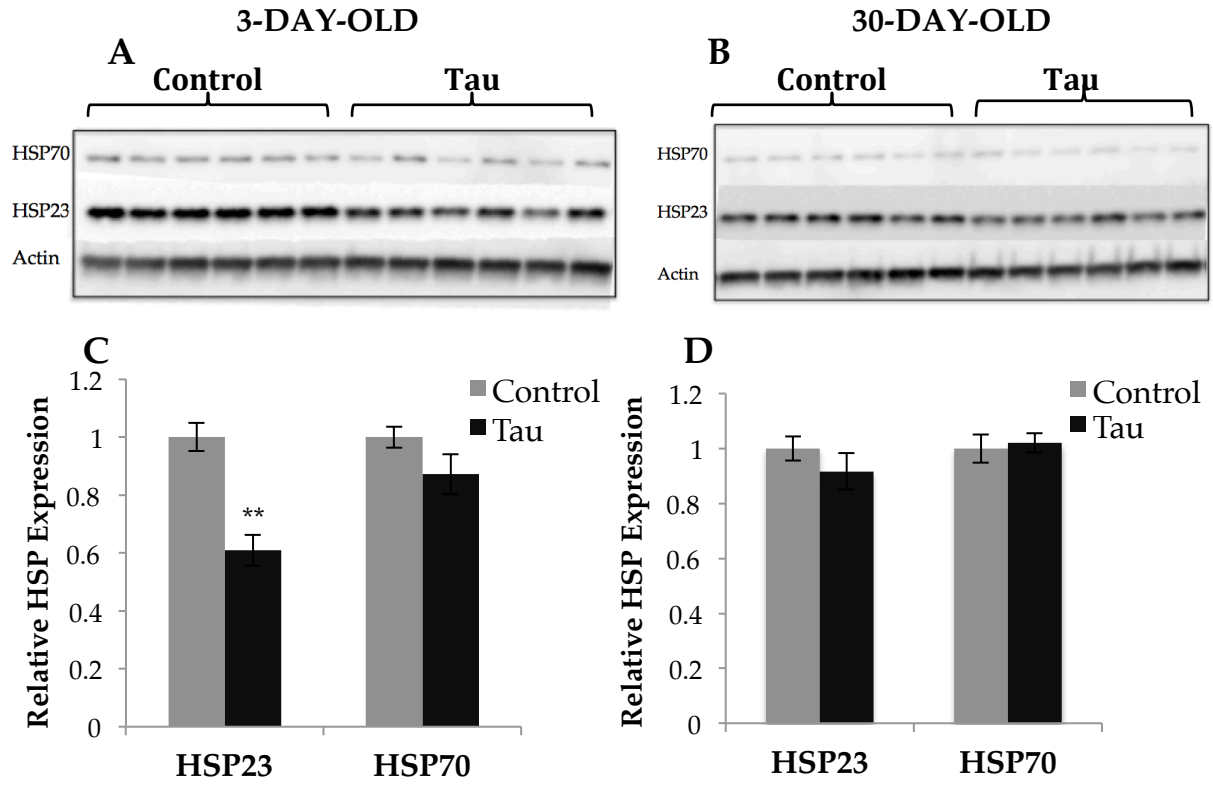


Figure 13. Glial Tau expression inhibits Hsp23 expression in 3-day-old flies. Western blots showing Hsp levels in control and glial Tau-expressing (A) 3-day-old flies and (B) 30-day-old flies. Densitometric analysis showing relative Hsp levels in (C) 3-day-old flies and (D) 30-day-old flies. Actin was used as a protein loading control. n=6, $^{**}p<0.01$, t-test. Error bars represent ± SEM.

Age-dependent effect of TBI on Hsp23 and Hsp70 expression 24 hours after injury

We proceeded to examine the effect that TBI had on Hsp levels 24 hours after TBI infliction. This time frame was selected because it provides a reasonable amount of time for translation of induced mRNA into protein. Also, previous research that surgically inflicted TBI in a mouse model demonstrated that Hsp levels remained elevated between 4 and 48 hours after TBI (DeGracia et al., 2007). To determine if there were age-related differences in the response of Hsp expression to TBI, 10 and 30-day-old control and glial Tau-expressing flies were examined.

In 10-day-old flies without glial Tau, Hsp23 levels remained unchanged, while Hsp70 expression was significantly reduced 24 hours after TBI. Hsp23 and Hsp70 levels remained unchanged in glial Tau-expressing flies, indicating that glial Tau flies were less prone to Hsp70 expression alteration in response to TBI (Figure 14).

In 30-day-old flies, there was no significant difference in Hsp23 or Hsp70 levels between flies that were subjected to TBI and those that were not for both control and glial Tau flies (Figure 15).

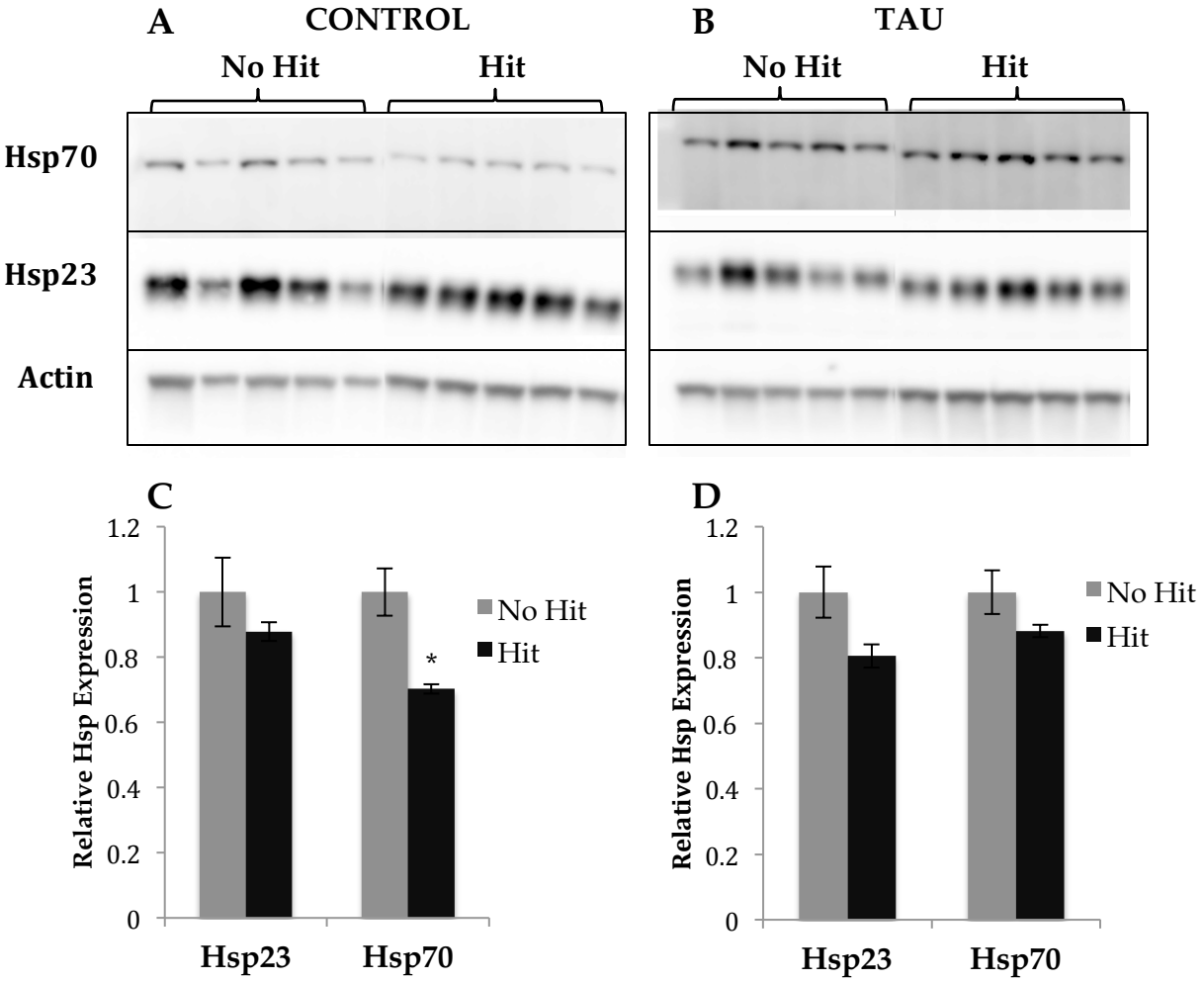


Figure 14. TBI reduces Hsp70 levels, but does not affect Hsp23 levels in 10-day-old control flies, while Hsp 23 and 70 levels remain unchanged in Tau flies post-TBI. Western blots showing Hsp levels in hit and no hit flies for (A) Control flies and (B) Tau flies. Densitometric analysis showing relative Hsp levels in (C) Control flies and (D) Tau flies. Actin was used as a protein loading control. n=5, *p<0.05, t-test. Error bars represent ± SEM.

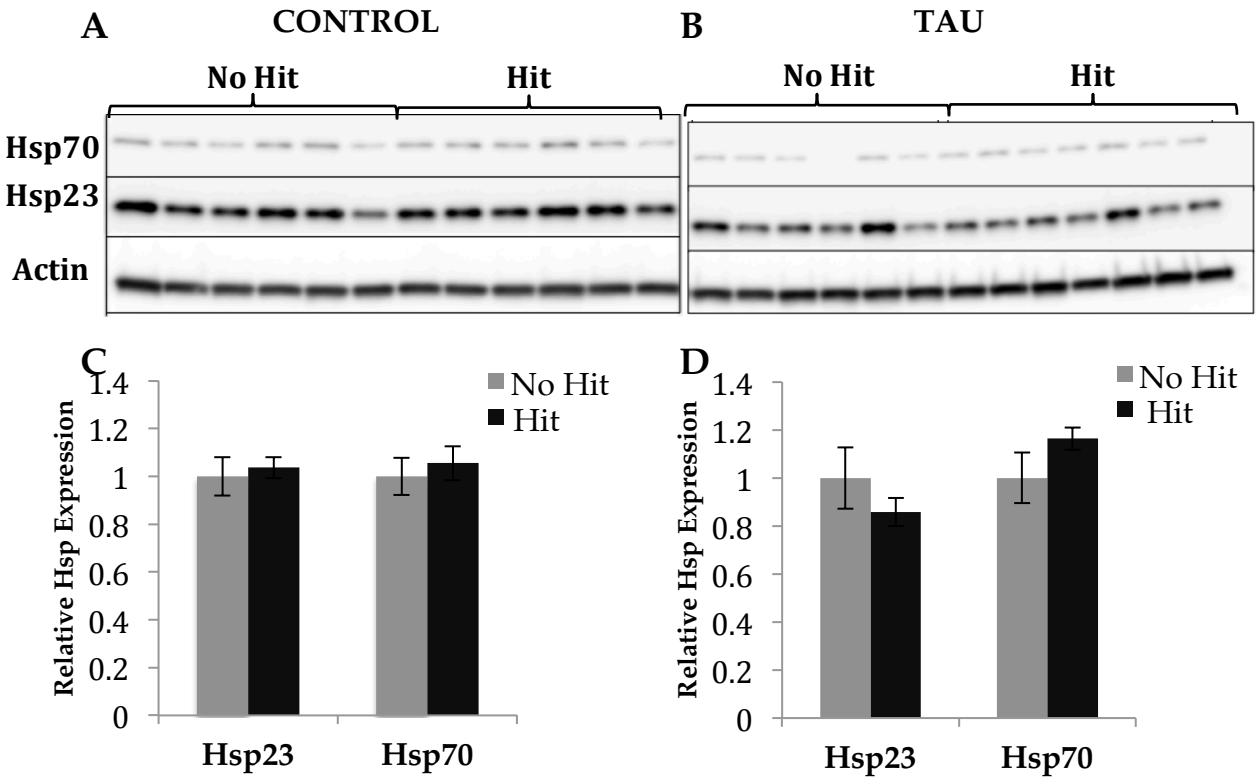


Figure 15. Hsp23 and Hsp70 levels are unaffected both in 30-day-old control and Tau flies 24 hours after TBI. Western blots showing Hsp levels in hit and no hit flies for (A) Control flies and (B) Tau flies. Densitometric analysis showing relative Hsp levels in (C) Control flies and (D) Tau flies. Actin was used as a protein loading control. n=6 or 7. Error bars represent ± SEM.

Hsp70 levels reduce 24 hours post-TBI and return to basal levels after 3 or 7 days

In a separate TBI experiment, we examined if HSP levels were affected by the length of recovery time after injury. Hsp levels were analyzed for control and glial Tau-expressing flies that had sustained a TBI on day 3, day 7 or day 9 and were allowed to recover until day 10, resulting in a recovery time of 7, 3, or 1 day.

In control flies, Hsp70 levels were significantly reduced in flies hit on day 9 and allowed to recover for 24 hours. Hsp70 levels, however, were restored back to normal after recovery times of 3 and 7 days post-TBI (Figure 16). Hsp23 levels remained

unchanged between no hit flies and flies that experienced TBI with 1, 3, and 7 days of recovery (Figure 16). In Tau flies, neither Hsp23 nor Hsp70 levels change with any of the recovery paradigms (Figure 17).

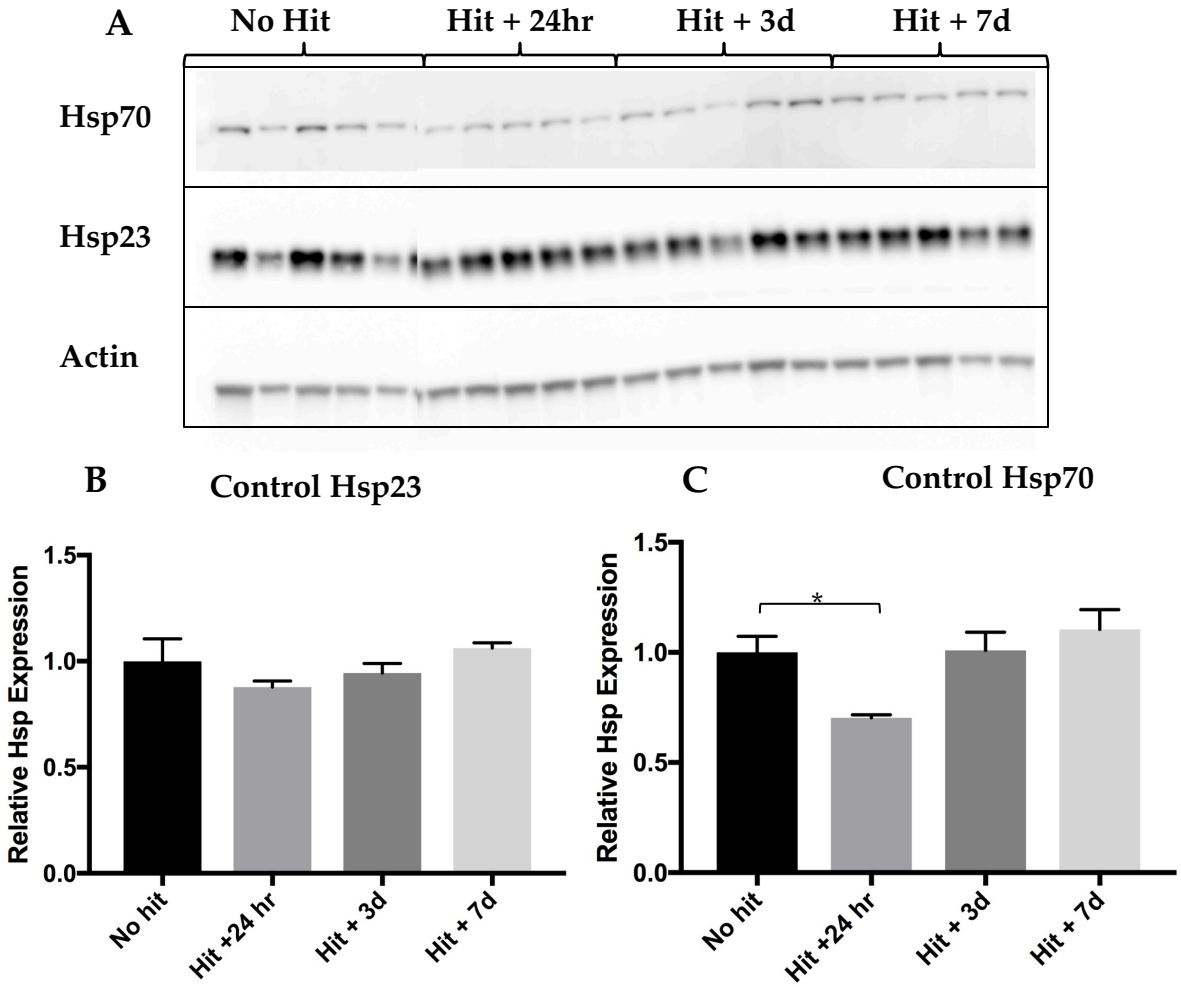


Figure 16. Hsp70 levels decrease after 24 hours post-TBI but are restored to normal levels in control flies 3 and 7 days post-TBI. (A) Western blot showing Hsp levels in 10-day control flies that were allowed to recover for 1 day, 3 days or 7 days. Densitometric analysis was used to show how (B) relative Hsp23 levels and (C) relative Hsp70 levels changed for the different hit paradigms. Actin was used as a protein loading control. n=5. Error bars represent $\pm$ SEM. Note: Bands were shifted to be consistent with graphical representation; however, all bands are from the same membrane with the same imaging exposure.

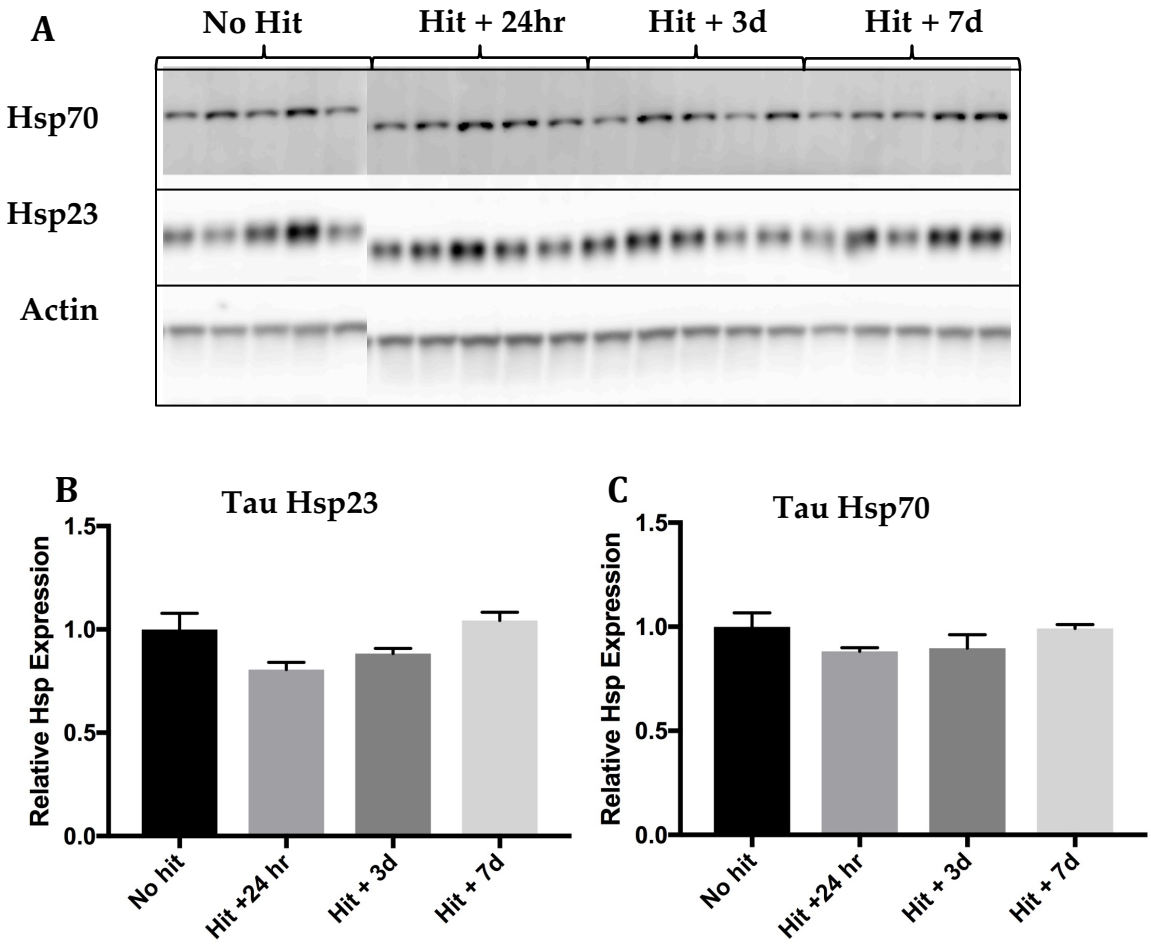


Figure 17. Hsp23 and Hsp70 levels are unaffected by different recovery times in Tau flies. (A) Western blot showing Hsp levels in 10-day Tau flies that were allowed to recover for 1 day, 3 days or 7 days. Densitometric analysis was used to show (B) relative Hsp23 levels and (C) relative Hsp70 levels for Tau flies in the different hit paradigms. Actin was used as a protein loading control. Error bars represent ± SEM. Note: Bands were shifted to be consistent with graphical representation; however, all bands are from the same membrane with the same imaging exposure.

A mortality analysis run on the flies in the different hit paradigms revealed that acute TBI was not lethal to 10-day old Tau and control flies. There were no deaths recorded 24 hours after TBI in flies hit on day 3, 7 or day 9.

Repetitive TBI leads to decreased Hsp70 expression and increased mortality

Western blot analysis was again used to determine if Hsp levels were affected by a repetitive TBI protocol. Multi-hit flies received TBI on day 3, day 5, day 7 and day 9 and were frozen at day 10, 24 hours after the last hit. Multiple hits led to a decline in Hsp levels specifically for control flies (Figure 18).

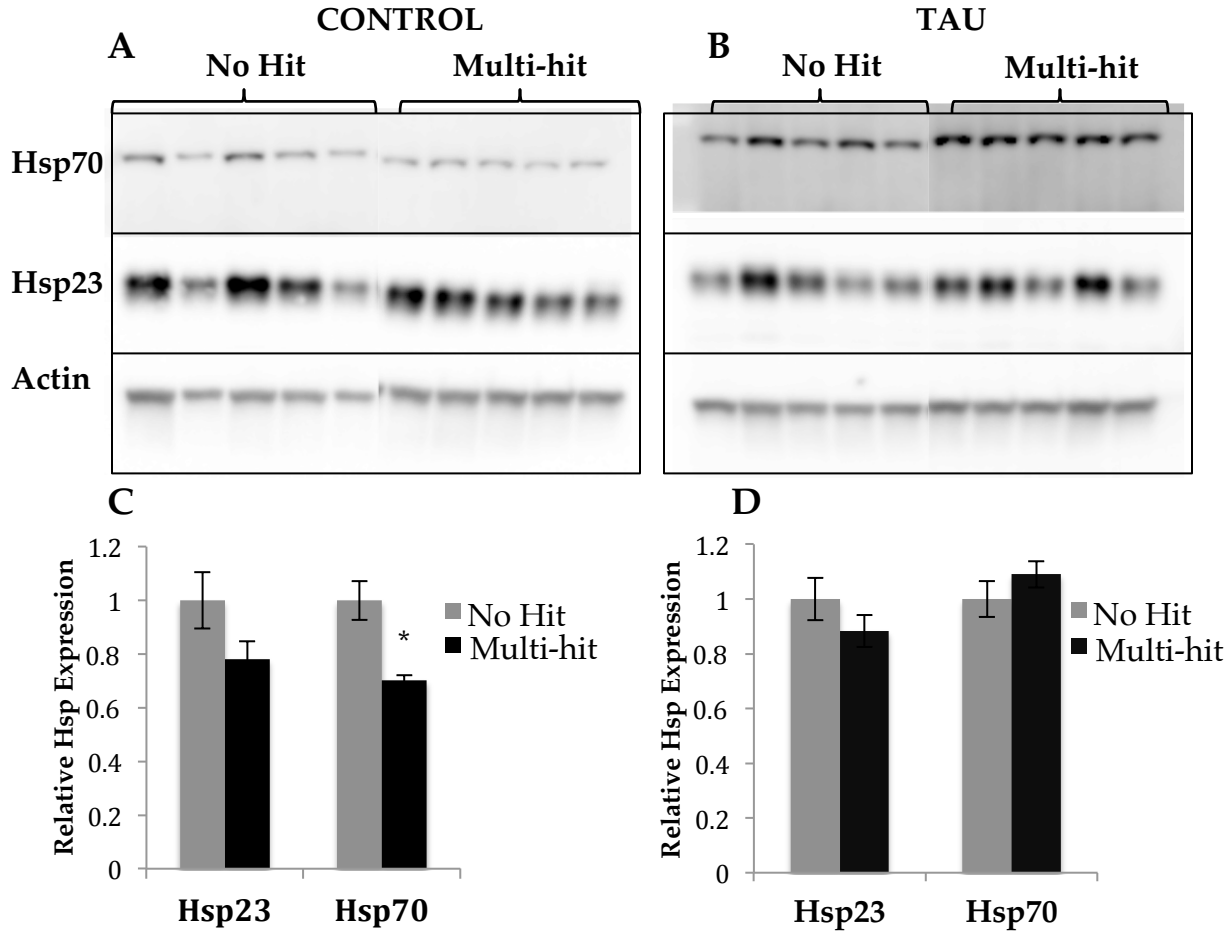


Figure 18. Repetitive TBI leads to a decline in Hsp70 levels in control flies. Western blot showing Hsp levels in 10-day flies that were hit on days 3, 5, 7 and 9 in (A) control flies and (B) Tau flies. Densitometric analysis was used to show (C) relative Hsp23 and Hsp70 levels in (C) control flies and (D) Tau flies. Actin was used as a protein loading control. n=5, *p<0.05, t-test. Error bars represent ± SEM.

Qualitative observation of the Western blots shows an increase in Hsp levels for multi-hit Tau flies but quantitative analysis reveals that this induction is not significantly different from basal Hsp levels in no hit flies (Figure 18). The actin loading control levels for Tau multi-hit flies is also significantly greater ($p<0.05$, t-test) compared to non-TBI flies. Thus, actin levels appear to increase significantly with repetitive TBI as well. This overall reduces relative Hsp70 levels, as they are calculated relative to total actin levels.

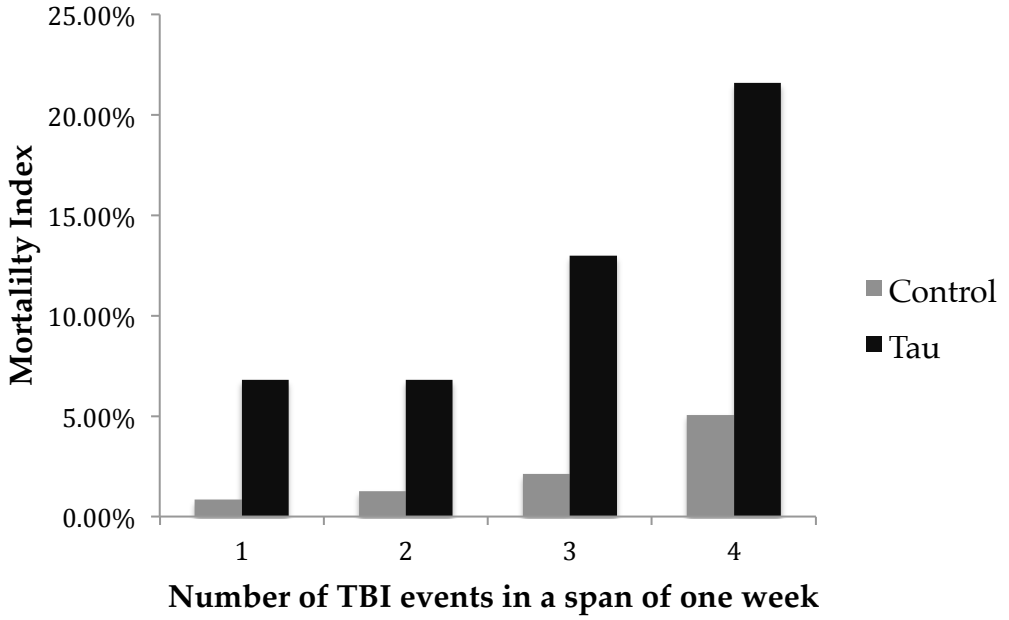


Figure 19. Repetitive TBI leads to increased mortality. Flies were hit on day 3, day 5, day 7 and day 9. The percentage of dead flies was recorded 24 hours after each hit for control and glial Tau flies. n = 60-110.

We also explored the effect of multiple acute TBI events on mortality in Tau and control flies by recording percentage mortality 24 hours after each hit. Mortality increased with each successive hit (Figure 19) and while this increase was observed in both Tau and control flies, it was more pronounced in Tau flies. Tau flies that had

received 4 successive hits had a mortality rate of about 22% whereas control flies that had experienced 4 successive hits had a mortality rate of 5%.

DISCUSSION

Summary of results

We have found that Hsp23 and Hsp70 levels attenuate with age both in glial Tau-expressing flies and control flies, and that glial Tau expression selectively reduces Hsp23 protein levels in young flies. Acute traumatic brain injury led to decreased Hsp70 levels in 10-day-old control flies 24 hours after injury, while Hsp23 levels were not changed. However, TBI did not affect Hsp70 or Hsp 23 protein levels 24 hours after injury in 30-day-old control flies. Hsp23 and Hsp70 levels were also unaffected by TBI in glial Tau flies. The reduction in Hsp70 levels in control flies 24 hours post-TBI returned to basal levels with 3 and 7 days of recovery. Repetitive TBI also led to Hsp70 repression 24 hours after the last TBI event; however, this did not happen in glial Tau-expressing flies. Overall, TBI affected only Hsp70 levels and not Hsp23 levels. In all flies expressing glial Tau, Hsp23 and Hsp70 levels remain unchanged following TBI.

Age-induced suppression of Hsp23 and Hsp70 proteins

The free-radical theory of aging suggests that an organism's lifespan is dependent on its resistance to reactive oxygen species (free radicals), which are the natural by-products of oxygen metabolism. Free radicals attack proteins, lipids and DNA, and lead to oxidative damages such as reduced DNA stability, protein misfolding, and subsequent protein aggregation. In older populations, increased protein aggregation is observed particularly in tissues with postmitotic cells such as brain tissue (Sohal, 2002). Hsps, which are part of the cell-defense mechanisms that

combat protein aggregation, appear to be implicated in the aging process. Our experiments have shown that both Hsp23 and Hsp70 protein levels decrease in older flies compared to younger flies (Figure 12), indicating that older flies have weakened cell-defense mechanisms to combat increased protein aggregation. In a similar aging experiment, Morrow et al. (2003) demonstrated that while thoracic *Drosophila* Hsp23 mRNA levels increased with aging in response to increased oxidative stress, this mRNA induction was not reflected at the protein level in whole fruit fly extracts. Rather, Hsp23 protein levels, as shown by Western blots of whole fruit flies, were reduced at day 30 compared to day 1 of the fruit fly's lifespan. The fact that Hsp23 mRNA is induced in the thorax, but the protein level is not increased in the whole fruit fly may indicate that while the fly is transcribing genes in response to oxidative damage at older ages, translation from mRNA to protein is apparently inhibited and could ultimately result in increased protein aggregation.

Previous experiments have also explored Hsp70 induction with fruit fly aging. Wheeler et al. (1995) demonstrated that Hsp70 protein levels increased with aging in a tissue-specific manner, as the increase was restricted to *Drosophila* flight and leg muscles. Our aging experiments were localized to the *Drosophila* brain, which likely follows a different expression pattern. Western blotting revealed that there was an age-related decrease in Hsp70 levels in the *Drosophila* brain (Figure 12A & C). It is likely that age-related expression of the Hsp70 protein varies in the different regions of the body and that there are cell-type specific effects of aging on Hsp70. Morrow et al. (2003), conducting a similar aging experiment on whole fly extracts, also found that Hsp70 protein levels were reduced in 30 day, 60 day, and 90 day flies, compared to 1 day flies.

This age-dependent suppression of Hsp23 and Hsp70 in 30-day-old flies was observed both in our control and glial Tau-expressing flies, indicating that regardless of Tau expression, aging results in a decline in Hsp protein production in the fly brain. The accumulation of damaged proteins is often prevalent at older ages and the physical symptoms of neurodegenerative diseases such as Alzheimer's Disease and Chronic Traumatic Encephalopathy are also often observed in older populations. The attenuation of Hsp23 and Hsp70 expression with age represents a deficit in the chaperone system meant to combat protein accumulation.

Selective inhibition of Hsp23 by early glial Tau expression: Another case for oligomeric Tau as the key toxic species?

Multiple experimental models that have overexpressed Tau in neuronal and glial cells have demonstrated that the expression of Tau in neuronal and glial cells is toxic, as it leads to apoptotic cell death, and subsequent mortality. The quest to fully uncover mechanisms by which Tau confers its neurotoxicity is still underway. Frost et al. (2014) have pointed to aberrant gene expression due to Tau-induced heterochromatin relaxation as a potential mechanism for neuronal Tau toxicity. More recent studies have suggested that Tau induces neurotoxicity by impairing protein synthesis in cells. In the diseased state, when it adopts disordered conformations, Tau binds to ribosomal proteins that participate in RNA translation, inhibiting the translation process (Meier et al., 2015). In our experiments, we have identified a suppression of the Hsp23 protein expression in young flies expressing glial Tau (Figure 12), which may be indicative of impaired Hsp23 mRNA translation due to aberrant tau-ribosome interactions in glial cells.

Interestingly, the suppression of Hsp23 protein that we see is age-specific, a result that may be explained by the suggested toxicity of oligomeric Tau. While insoluble Tau aggregates were originally thought to be the key toxic species in tauopathies, emerging research has demonstrated that the soluble, oligomeric Tau that precedes tangle formation is likely the central toxic species in tauopathies. In mouse and fly models overexpressing varying isoforms of human Tau, neurodegeneration still occurs in the absence of Tau aggregations (Wittman et al., 2001; Andorfer et al., 2005; Berger et al., 2007). Additionally, suppression of Tau expression in animal models rescued memory deficits without altering existing neurofibrillary tangles (Santacruz et al., 2005; Sydow et al., 2011). Taken together, these studies suggest that Tau aggregations might not be as toxic as Tau oligomers, and that oligomeric Tau species that later aggregate to form the insoluble tangles, might play a more important role in Tau toxicity. Similarly, Colodner and Feany (2010) found that glial Tau aggregates are largely absent in young flies, but abundant in 30-day-old glial Tau-expressing flies. At 3 days, therefore, we expect that misfolded glial Tau has not yet aggregated, and that pathological Tau may be present in the more toxic oligomeric form. Abisambra et al. (2013) have demonstrated that this soluble, oligomeric Tau associates with proteins that participate in endoplasmic reticulum (ER)-associated degradation (ERAD), impairing the protein quality control mechanisms that take place in the ER, leading to increased toxicity. At 3 days, oligomeric Tau may confer toxicity in this way, ultimately suppressing Hsp23 protein expression. The decline in Hsp23 levels in response to Tau toxicity signifies further inhibition of protein control mechanisms, which would, in turn, enhance toxicity as protein aggregation becomes more prevalent.

Recent studies have also demonstrated that Tau expression leads to a loss of HSF1, the transcriptional activator of all heat shock proteins. A study conducted by Kim et al. (2017) has revealed that HSF1 suppression is an early event that precedes Tau aggregation in Alzheimer's disease. In their experiment, young rats expressing wild type human Tau had about 30% less HSF1 than controls. Tau-induced HSF1 loss at younger ages suggests that the chaperone system is compromised early on in the fly's lifespan. With lower HSF1 levels, the transcription of Hsps is inhibited, ultimately leading to reduced levels of protein being translated even at that younger age. This sets the stage for the protein accumulation that is seen at later ages, and highlights the importance of determining HSF1 levels in young and old flies.

Our results also showed that at 3 days, only Hsp23 and not Hsp70 expression is affected by glial Tau expression. It may be the case that the factors that contribute to Hsp70 transcription, translation, and/or degradation are more resistant to degradation by Tau toxicity, though more susceptible to other perturbations such as TBI.

In 30-day-old flies, glial Tau-expression has no effect on either Hsp23 or Hsp70 levels (Figure 12) despite the accumulation of glial fibrillary Tau tangles. This could be another indicator that insoluble Tau tangles, which are present at 30 days, are in fact, less toxic to cells than soluble Tau oligomers.

TBI and Hsp70 suppression in 10-day control flies

After a 24-hour recovery period, 10-day-old control flies that were subjected to one bout of acute TBI on day 9 showed a decrease in Hsp70 levels (Figure 14). A similar reduction in Hsp70 levels was observed for flies that were subject to repetitive TBI (Figure 18); however, this reduction cannot fully be distinguished from the reduction

seen in flies that sustained injury only on day 9. After a 3 or 7-day recovery period, Hsp70 levels returned to basal levels. It is therefore highly probable that in repetitive TBI flies, it was only the final hit on day 9 that contributed to decreased Hsp70 levels by day 10. For a better understanding of how repetitive TBI affects Hsp levels, it might be necessary to wait 2-7 days after the last bout of TBI on day 9 before analyzing Hsp levels. All the same, the decrease in Hsp levels 24 hours following TBI is again symbolic of a decline, rather than an upregulation in the chaperone system that would be expected to combat stress-induced protein misfolding.

In mouse TBI models involving surgical lacerations on the cerebral cortex or injury inflicted with the fluid-percussion model, there was an upregulation of Hsp70 mRNA between 2 and 24 hours after injury, and others have observed an increase in Hsp72 protein levels that lasts 4 to 48 hours following injury (Brown et al., 1989; Hayes et al., 1995; DeGracia et al., 2007). If TBI increases mRNA production, then a reduction in Hsp levels points to a defect in the translation processes. TBI is known to induce misfolding of the Tau protein both in animal models and in humans (Ojo et al., 2016; Barekat et al., 2016; Yang et al, 2017). This misfolded Tau, as discussed earlier, may be interacting with translation-inducing ribosomal proteins in a way that prevents translation of Hsp70 mRNA. Nonetheless, we have not yet characterized the response of Hsp mRNA levels to our closed head TBI model and so cannot confirm an increase in mRNA levels that does not translate into increased protein levels following TBI. Future experiments should look into mRNA expression following TBI using our model.

Although few studies have investigated Hsps in relation to TBI, several have been conducted on the relationship between TBI and the ubiquitin proteasome system

(UPS), a large protein complex that, like Hsps, degrades damaged proteins in the cell. TBI has been found in multiple studies to lead to a decline in the activity of the ubiquitin proteasome system (Mizukami et al., 2002; Yao et al., 2007; Yao et al., 2008). Nonetheless, other researchers have demonstrated that chemical inhibition of the ubiquitin proteasome system leads to a marked increase in Hsp levels in order to counter the toxicity induced by reduced proteasome function (Lee and Goldberg, 1998; Awasthi and Wanger, 2005). In our TBI model, however, we observe a decrease in Hsp70 levels after 24 hours. Perhaps chemical inhibition of the ubiquitin proteasome system and proteasome inhibition by TBI have differing resulting effects on the Hsp system. Given that both proteasome activity and Hsp chaperone activity are decreased following TBI, it is evident that TBI leads to very toxic consequences for the cell as protein aggregation is further enhanced. The identification of these two pathways in TBI-related pathogenesis is key in the development of treatments for brain injury.

Older flies are unresponsive to TBI

In all 30-day-old flies, Hsp levels remain unchanged following TBI. This is an intriguing result, considering that protein aggregation is prevalent at this age, particularly in the presence of glial Tau. Although Tau oligomers are proving to be the key toxic species, the toxicity of insoluble Tau aggregates has not been completely ruled out. For instance, it still stands that more severe symptoms of tauopathies correspond with increased Tau aggregation in neuronal cells (Arriagada, 1992), and studies have shown that filamentous Tau confers toxicity by impairing fast axonal transport (LaPointe et al., 2009; Patterson et al., 2011). Patterson et al. (2011) have also

demonstrated that a direct interaction between Hsp70 and aggregated Tau abolishes the effects of insoluble Tau fibrils on fast axonal transport. These data indicate that Tau aggregates indeed confer some level of toxicity to cells. With the knowledge that Tau expression from an early age inhibits HSF1 production (Kim et al., 2017), it is possible that at older ages, Hsp genes are unresponsive to stress as a result of this early inhibition of HSF1 that continues to impair Hsp protein synthesis. Nonetheless, TBI has no effect on Hsp levels in 30-day control flies either. Therefore it is evident older flies generally do not respond to TBI as a stressor. Some researchers have demonstrated that the ability of some Hsps to be induced by heat-induced stress diminishes with aging (Morrow and Tanguay, 2003); it has been suggested that this happens because during aging, chronic expression and accumulation of Hsps might become toxic by disrupting of signaling pathways (Tower, 2009). This may explain why Hsp levels remain unchanged in 30-day-old flies.

Tau inhibits changes to Hsp expression after TBI

In all flies expressing Tau, TBI has no effect on Hsp expression (Figure 14B & D, Figure 15B & D, Figure 17, Figure 18B & D). I have suggested two hypotheses for this result. The first is that the presence of Tau in 10-day-old flies counters the suppression of Hsp levels in response to TBI that was observed in controls. Filipick et al. (2015) have demonstrated that in transgenic mice expressing neuronal tau, Hsp27 levels are increased 3-fold compared to control mice. This increase was observed almost exclusively in a specific subtype of glial cell, reactive astrocytes, rather than in the tau-expressing neurons themselves, once more highlighting the key role that astrocytes play in maintaining overall brain health. The presence of Tau in astrocytes and other glial

cells of our fly model may similarly induce Hsp23 and Hsp70 proteins in the fly's glial cells, leading to an induction of Hsp levels that reverses the suppression observed in control flies 24 hours post-TBI. This allows Tau flies to maintain basal levels of Hsp after TBI.

The second hypothesis involves Tau-actin interactions, which also presents itself as a limitation in our experiment. Beyond its microtubule-stabilizing properties, Tau is also involved in regulating the actin cytoskeleton (Griffiths and Pollards, 1982). Fulga et al. (2007) have demonstrated that human Tau enhances neurodegeneration in *Drosophila* by interacting with and stabilizing the actin cytoskeleton. In flies expressing wild type or pathological Tau in neurons, actin-rich rod formation accompanied neurodegeneration in the fly brain, and overall actin levels were increased in Tau flies compared to control flies. Elie et al. (2015) have similarly shown that in mice expressing pathogenic Tau, actin levels are increased compared to actin levels in control mice, leading to toxicity via the restriction of synaptic vesicle mobility. In our Western blot on 10-day Tau flies, the density of Hsp70 protein for multi-hit flies is visibly higher than that of unhit flies, though this apparent Hsp70 induction is not statistically significant (Figure 18). An issue here is that actin levels themselves increase in Tau flies subjected to repetitive TBI ($p<0.05$, t-test); this increase in actin levels is not observed for the control blot (Figure 17), indicating that it is specific to Tau flies. The increase in actin band density with Tau expression and with repetitive hits will affect relative Hsp levels, as these levels are calculated compared to actin levels. With repetitive hits, pathological Tau increases and accumulates in brain cells, and there is now evidence that increased pathological Tau levels correspond with increased actin levels (Fulga et al., 2007). These

increased actin levels likely make significant alterations in Hsp expression in injured Tau flies appear insignificant after quantitative analysis. GAPDH is another well-used loading control that is constitutively and ubiquitously expressed in cells. Use of GAPDH antibodies in Western blotting may provide a more accurate analysis of Hsp levels in Tau-expressing flies, as GAPDH has not been shown to vary with Tau levels.

Glial Tau suppresses normal glial function and/or inhibits the protective glial response

A key feature of TBI and CTE brains is the disruption of the blood-brain barrier (BBB) caused by the mechanical force inflicted on the brain. This BBB disturbance is thought to lead to the release of neurotoxins into the brain to enhance degeneration (Geddes et al., 1994). Astrocytes, the more extensively studied subset of glial cells, play a prominent role in the formation and maintenance of the blood-brain barrier (Cabezas et al., 2014). In our study, we have identified that flies expressing Tau in their glial cells (which include astrocytes) have higher mortality rates following repetitive TBI compared to control flies (Figure 19). Our results indicate that glial Tau might be playing a role in altering the normal astrocytic function that would maintain the BBB. Bartels et al. (2009) have demonstrated, in the absence of TBI, that the expression of human Tau specifically in astrocytes of aged mice leads to a disruption of the blood-brain barrier using reduced P-glycoprotein function as an indicator of reduced BBB integrity. Additionally, in a mouse model of neuronal tauopathy, it was found that suppressing Tau expression reduced damage to the blood-brain barrier, indicating once more that Tau plays a role in blood-brain barrier depletion (Blair et al., 2015). It is therefore evident that even in the absence of TBI, the blood-brain barrier is not

effectively maintained as a result of Tau expression. TBI might thus further enhance blood-brain barrier dysfunction, leading to increased neurotoxicity and subsequently, increased mortality among Tau flies.

A second hypothesis is that the presence of Tau in glial cells inhibits not only normal glial function, but also the protective glial response that is activated after TBI. In an attempt to combat the neurodegenerative damage caused by TBI, glial cells are activated in a process known as reactive gliosis. Reactive astrocytes, for instance, provide nutrients to and support the viability of the surviving cells in order to prevent further degeneration (Ridet et al., 1997). In an *in vivo* experiment conducted by Myer et al. (2006), mice that received an ablation of reactive astrocytes following moderate TBI showed a pronounced loss of neuronal tissue (about 60% more compared to controls). This experiment demonstrated that reactive gliosis plays a vital role in preventing the degeneration that follows brain injury. It is therefore likely that the presence of glial Tau in flies inhibits this protective glial response, leading to increased neurodegeneration that makes the glial-Tau expressing flies more susceptible to death by TBI. In fact, there is some evidence for Tau's inhibition of the protective glial response. One of the features of reactive gliosis is the activation of the STAT signaling cascade, a process which releases cytokines and growth factors essential for homeostasis in cells (Sriram et al., 2004). Colodner and Feany (2010) have found that glial Tau expression the fly brain reduces STAT activity. This provides evidence for a Tau-induced obstruction in the protective glial response to brain injury, explaining the increased mortality observed in Tau flies compared to control flies following TBI.

Limitations

There are currently no experiments in the literature that have used closed-head TBI to investigate the impact of TBI on endogenous Hsp expression. Those that have conducted similar experiments involve surgical lacerations or impacts to the dura under anesthetic conditions. In our TBI model, each fly receives a random impact with a different intensity in a different location on its brain and/or body. There are 10-20 flies present in a vial when the HIT apparatus is released from a height onto a platform. Upon hitting the platform each fly strikes the inside of the vial with a different amount of force, with different regions of its body being impacted. As a result, some flies may sustain more acute injuries to the brain than others, making subsequent changes in the chaperone machinery variable among flies. In our analyses that usually consider 5-7 biological replicates, the variability in Hsp levels between flies could be attributed to differential severity of injury from fly to fly. A new TBI model that has recently been developed by Sun and Chen (2017) delivers impacts of adjustable strength directly to one fly head. A specific CO_2 pressure is used to drive an impactor against the fly head. The localization of injury to the head, as well as the ability to regulate force of impact, may help to reduce the variability in Hsp levels resulting from our random TBI model. However, Sun and Chen's TBI model involves anesthetizing the flies and our lab is currently working to use a version of this model that eliminates the anesthesia portion of the TBI protocol.

As has been outlined earlier, there appears to be an interaction between Tau and actin that increases actin levels in Tau expressing flies following TBI (Fulga et al., 2007). Overall accuracy in this experiment could be improved by using other loading controls

such as GAPDH, which will be less likely to vary with levels of Tau, in addition to using a more precise TBI-inflicting apparatus.

Future directions

Since Hsp mRNA levels have not been described following TBI with our model, it will be important to outline this mRNA response and compare this with the reduction in Hsp protein levels that we have observed. It may also be helpful to measure levels of Hsp activity rather than just the amount of Hsp protein present in the fly head. It may be the case that Hsps do not need to be upregulated in order to increase misfolded protein turnover.

From previous TBI studies, we learn that Hsp activation is often localized to the particular brain regions that have experienced injury. As such, changes in Hsp levels may not be effectively visualized in the whole brain. Immunostaining of brain tissue with Hsp protein antibodies may provide a better means of identifying brain areas in which Hsp levels are activated following injury. This may also reveal the specific brain areas that were impacted by the TBI event. Additionally, co-localization of Hsps and glial cells with immunostaining techniques will also confirm if Hsp suppression in the presence of Tau or Hsp suppression following TBI is a result of glial cell death.

Conclusion

We have identified a decrease in Hsp23 and Hsp70 protein expression in response to aging, and we have found that glial Tau expression also diminishes Hsp23 levels in younger flies. Additionally, we have characterized the effect of TBI on Hsp23 and Hsp70 expression using our closed head TBI model. While Hsp23 levels remain unchanged, Hsp70 expression is suppressed in younger control flies following TBI, and Hsp levels in aged flies are unaffected by TBI. Thus, individual Hsp levels are differentially affected by Tau expression and TBI.

In summary, both glial Tau expression and TBI attenuate Hsp levels in younger flies. Our results suggest that targeting transcriptional and translational regulation of Hsp expression, in addition to targeting Tau pathology in glial cells, represents a viable therapeutic strategy in the treatment of tauopathies and TBI.

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