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_____	_____
Joseph A. Behnke	Date

***Drosophila melanogaster* as a model to investigate lifelong brain deficits and underlying mechanisms following repetitive mild head trauma**

By

Joseph A. Behnke
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Neuroscience

_____
James Q. Zheng, Ph.D.
Advisor

_____
Victor Faundez, M.D., Ph.D.
Committee Member

_____
Zixu Mao, M.D., Ph.D.
Committee Member

_____
Kenneth H. Moberg, Ph.D.
Committee Member

_____
David Rye, M.D., Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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Joseph A. Behnke
B.A., Rutgers University, 2014

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An abstract of
A dissertation submitted to the Faculty of the
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Abstract

***Drosophila melanogaster* as a model to investigate lifelong brain deficits and underlying mechanisms following repetitive mild head trauma**

By Joseph A. Behnke

Mild head trauma, including concussion, can lead to chronic brain dysfunction and degeneration but the underlying mechanisms remain poorly understood. Here, we report the utilization of a novel head impact system to investigate the long-term effects of mild head trauma on brain structure and function, as well as the underlying mechanisms in *Drosophila melanogaster*. We find that *Drosophila* subjected to repetitive head impacts develop long-term deficits, including impaired startle-induced climbing, progressive brain degeneration, and shortened lifespan, all of which are substantially exacerbated in female flies. Interestingly, head impacts elicit an elevation in neuronal activity and its acute suppression abrogates the detrimental effects in female flies. Together, our findings validate *Drosophila* as a suitable model system for investigating the long-term effects of head trauma, suggest an increased vulnerability in brain injury in female flies, and indicate that early altered neuronal excitability may be a key mechanism linking mild brain trauma to chronic degeneration.

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This work could not have been made possible without the continued support of my close colleagues, friends, and family.

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I owe a debt of gratitude to my undergraduate mentors, Drs. Janet Alder and Smita Thakker-Varia, who took me under their wing during my time as an undergraduate at Rutgers. It was here that my fascination for neuroscience research grew, which has since led me down a path of trying to understand brain function in health and disease. It was readily apparent that Janet and Smita made their students an important priority of theirs, and I can only hope to have an opportunity to pay this forward.

My family has remained a pivotal part of everything that I do. My parents, Henry (III) and Marie, instilled values of hard work and determination that have pushed me to challenge myself every day. My older brothers, Henry (IV) and David, and the rest of my close family have all been actively involved in my personal development and have helped shape the person I am today.

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Chapter 1 An Introduction to Traumatic Brain Injury

1.1 Introduction and Epidemiology

Traumatic brain injury (TBI) is, "an alteration in brain function, or other evidence of brain pathology, caused by an external force" [1], that oftentimes consists of a heterogeneous set of physical signs, and cognitive and emotional symptoms [2-4]. The Centers for Disease Control and Prevention (CDC) estimates an annual TBI incidence of 2.8 million individuals in the US, including 50,000 TBI-related fatalities[5]. Over 5 million Americans are currently living with long-term TBI-related disability[5]. Closed-head injuries, most commonly a result of falls and automobile accidents, are the most prevalent type of TBI, of which mild TBI (mTBI), also known as *concussion*, account for ~75% of total TBI incidence[6]. This is likely an underestimate as mTBI is likely to go undiagnosed, or even unrecognized in cases where medical care is not pursued. mTBI is particularly elusive, given that it may present with subtle or even no obvious indications, yet 11-38% of affected individuals experience symptoms past 6-months after injury[7], indicating that mTBI may have long-lasting neurological effects[8]. A history of repetitive mTBI, especially in high risk individuals such as military personnel[9, 10] and contact athletes is associated with the insidious progressive neurodegenerative disease known as chronic traumatic encephalopathy (CTE), whose onset occurs years after injury[2, 3, 11-17]. A prior history of head trauma is also associated with the development of age-related neurodegenerative diseases[15, 18-22], including Alzheimer's (AD)[9, 23], Parkinson's disease (PD)[13], and Amyotrophic Lateral Sclerosis (ALS)[19], which collectively represent a leading cause of long-term morbidity and mortality worldwide[18]. Despite the critical concern related to repetitive head

trauma exposure, its underlying mechanisms involved in neurodegeneration and long-term complications remain poorly understood[21, 24, 25]. *Therefore, there is a great need to understand the immediate cellular mechanisms underlying mechanical brain trauma and how they lead to long-term brain dysfunction.*

1.2 Traumatic Brain Injury Classification

Human TBI severity has long been clinically classified using the Glasgow Coma Scale (GCS)[26]; a 3-15 point scale equally based on i. motor response, ii. best verbal response, and iii. eye opening. The aggregate score of these responses is then used to calculate overall injury severity using a three-tier classification system: i. ***mild (13-15)***, *ii.* ***moderate (9-12)***, and *iii.* ***severe*** **(3-8)**[27]. Another severity classification is made based on duration of loss of consciousness (LOC) and post-traumatic amnesia (PTA) sustained following trauma: i. ***mild (<30m LOC*** &/or ***<24h PTA)***, *ii.* ***moderate (30m-1d LOC*** &/or ***1-7d PTA)***, and *iii.* ***severe*** **(>1d LOC** &/or **>7d PTA)**[15]. This severity classification system is of particular importance for measuring acute function following injury, especially as it pertains to the need for potential life-threatening measures related to trauma exposure, such as hemodynamic control and initiating urgent protocols to resuscitate and maintain vital function.

Additional classification measures related to TBI involve pathoanatomic type (i.e. focal vs diffuse) and physical mechanism (i.e. contact [“impact loading”] vs noncontact [“inertial loading”])[28]. Typically, focal injuries are sustained as a result of contact “impact loading”, such as a blunt object striking the head. Injuries that involve impact

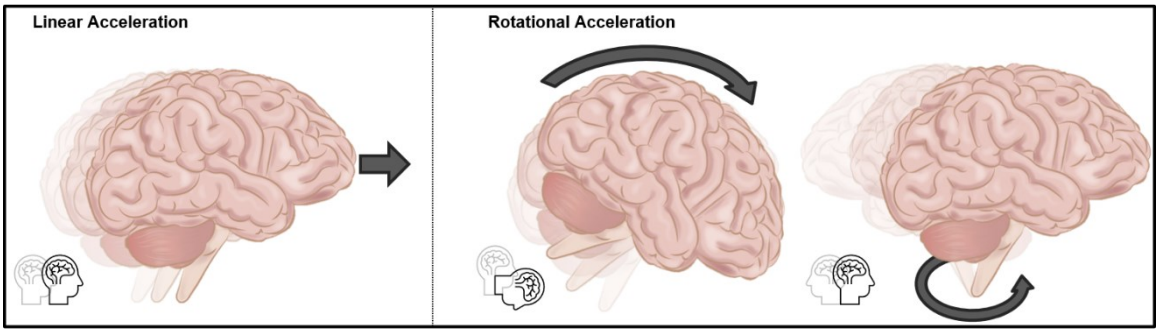

Figure 1-1 **Brain movement during head trauma**

loading are generally considered more severe and carries a greater risk for skull fracture, contusion, and hemorrhaging[29]. Conversely, inertial loading, such as that seen during sudden acceleration/deceleration movements of the head/body involve linear and rotational forces (**Figure 1-1**) that are more likely to result in diffuse brain injuries, such as those commonly associated with concussion[29]. Although focal injuries, such as those sustained via blunt force trauma, gunfire/explosive wounds, extreme falls and serious automobile accidents are generally considered more severe than diffuse injuries, they represent a small percentage of overall TBIs sustained (~20%). TBIs that involve penetrating wounds are referred to as open-head injuries, whereas injuries that preserves the skull are considered closed-head injuries.

The much more innocuous type of injuries such as concussive or sub-concussive impacts to the head represent a vast majority of head injuries. Although their seemingly unsuspecting danger associated with individual incidence remains low, the long-term cumulative effect of subsequent injury exposure is unknown. The relationship between successive injuries is particularly important when assigning long-term risk to head injury exposure as this may affect vulnerability to future injury or even neurodegeneration or long-term symptoms that develop years after exposure. Further improvement of injury severity classifications will be important for risk stratification, especially as it pertains to

treating and advising head injury patients about their vulnerability to related comorbidities and further injury exposure.

1.3 A Historical Perspective on Mild Traumatic Brain Injury

> *"... our conception of concussion of the brain must be modified. It is no longer possible to say that 'concussion is an essentially transient state which does not comprise any evidence of structural cerebral injury.' Not only is there actual cerebral injury in cases of concussion but in a few instances complete resolution does not occur, and there is a strong likelihood that secondary degenerative changes develop. We feel, therefore, that the post-concussion neuroses should properly be called cases of traumatic encephalitis"*
>
> **Michael Osnato, MD & Vincet Giliberti, MD**
> *Read before the 52nd Meeting of the American Neurological Association (Atlanta City, NJ 1926)

The concern surrounding mild head trauma and its long-term implications is not a new concern. In 1926, two Italian-born New York City neurologists, Drs. Michael Osnato and Vincent Giliberti, read a report during the Fifty-Second Meeting of the American Neurological Association in Atlantic City, NJ challenging the idea of *concussion* as being a, *" 'transient state which does not comprise any evidence of structural cerebral injury' "* [30, 31], which had been a generally accepted theory at the time. Osnato and Giliberti believed that post-concussion neurosis served as an inadequate description for concussion and its sequelae as they believed an organic physical cause was to blame. This conceptual framework led to the condition known as "traumatic encephalitis", a precursor to the present-day neuropathological condition known as chronic traumatic encephalopathy (CTE), an insidious neurodegenerative disorder seen in individuals with a past history of repetitive head trauma that appears years following injury exposure[15]. The work by Osanato and Giliberti also brought clarification to the use of "concussion" to refer to cases of mild head trauma that elicited temporary clinical manifestations, like loss of consciousness, but in the absence of gross contusions, skull fractures, swelling or bleeding which confound the interpretations brain-specific injury response[31].

Two years later, in Martland's 1928 seminal piece *Punch Drunk*, he referred to a phenomenon by the same name found in amateur and professional boxers[11] describing a condition that left boxers in a state resembling a drunken stupor. Martland described a rather important realization he had made while studying the punch drunk condition, saying, "*I have found that the opinion of shrewd laymen, many of whom are making a living by observing the physical fitness, actions and characteristics of the professional fighter, is perhaps more substantial than the opinion of medical experts*", which speaks to early history of the long-term effects related to repetitive head trauma being communicated by those directly involved, and not yet realized by the medical community. Martland's *Punch Drunk* concluded with a *New York Daily News* excerpt of commentary from Gene Tunney, a former world heavyweight professional boxer with 65 wins/1 loss and no recorded "knock outs" sustained during his career, who described his thought process for retiring from boxing, saying that he, *" 'wanted to leave the game that threatened [his] sanity before [he] met with an accident in a real fight with six ounce gloves that would permanently hurt [his] brain' "* [11]. This commentary is a profound example of concern expressed by a well-known professional athlete who, at the time of his retirement was still at the pinnacle of his sport of boxing. While the association between head trauma sustained during boxing and its long-term effects drew further validation during the decades that followed, it remained understudied and underappreciated in other contact sports involving exposure to concussive and sub-concussive head impacts. In fact it wasn't until the beginning of the following century, in 2005, when Omalu et al. published the first peer-reviewed case study identifying CTE in a former professional American football player[32]. Subsequently, this high-profile

report connecting the long-term risks associated with head trauma sustained by a former NFL player gave rise to an increase in publications related to head trauma, including a greater emphasis on repetitive and mild head trauma (**Figure 1-2**). The last 15 years of preclinical and clinical TBI research has put forth an incredible effort to better understand the fundamental pathophysiology surrounding mild head trauma, especially as it pertains to long-term dysfunction seen years following injury exposure.

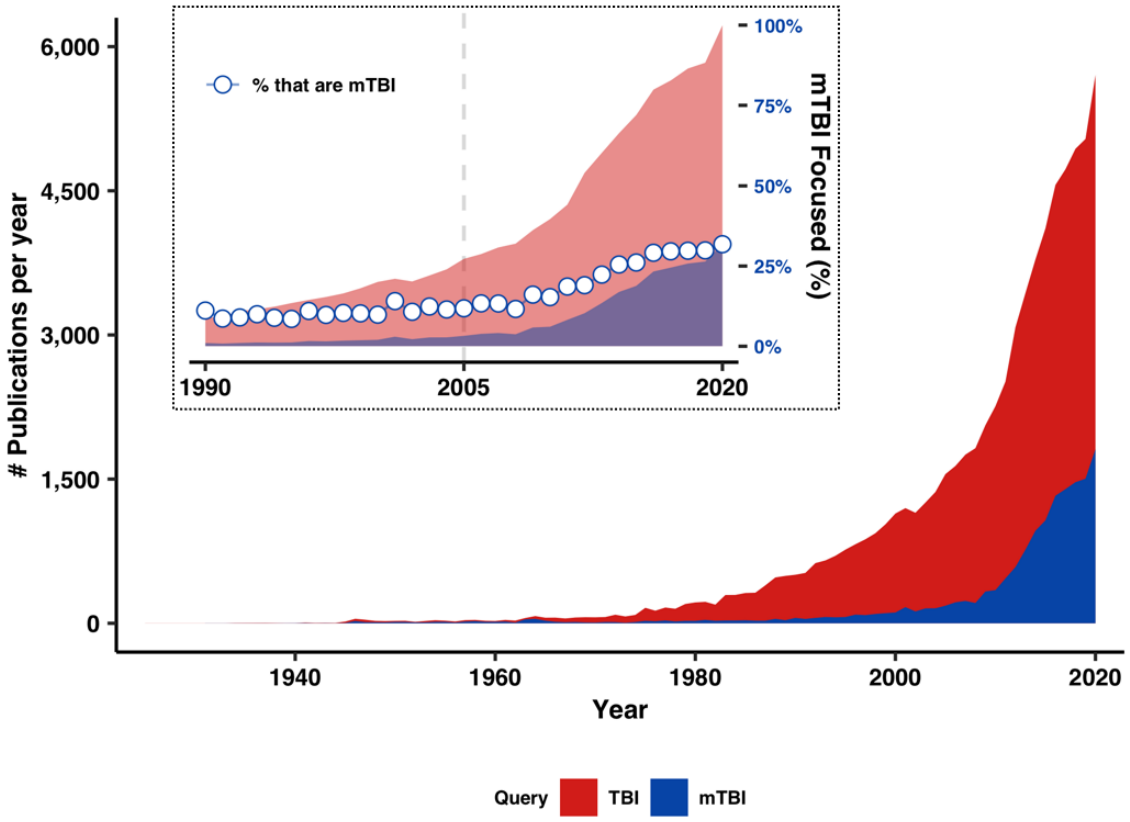

Figure 1-2 **PubMed search query of TBI publications (1925-2020)**

There has been a substantial increase in the number of TBI publications, *(insert)* with a growing emphasis on mild/repetitive traumatic brain injury relative to 2005, when the first report connecting American football to CTE was published [32]. PubMed search query for **mild/repetitive TBI** included: "dementia pugilistica"[Title/Abstract] OR "concussion"[Title/Abstract] OR "punch drunk"[Title/Abstract] OR "mild head trauma"[Title/Abstract] OR "mild head injury"[Title/Abstract] OR "mild traumatic brain injury"[Title/Abstract] OR "repetitive traumatic brain injury" [Title/Abstract] OR "mild tbi"[Title/Abstract]. The **TBI** search query additionally included:"head injury"[Title/Abstract] OR "traumatic brain injury" [Title/Abstract] OR "head trauma" [Title/Abstract].

Table 1 **Terminology of Head Trauma-Related Neurological Disease**

	Traumatic Encephalitis (1926) [31]	**Punch drunk** (1928) [11]	**Dementia Pugilistica** (1937) [33]	**Chronic Traumatic Encephalopathy** (1940) [34]
Signs & Symptoms	headache Memory impairments Sleep disturbances dizziness photophobia "Emotional symptoms"	Clumsiness Ataxia Confusion	Gait abnormalities Jealousy/paranoia Physical tremors Cognitive Impairment	Cognitive Impairment Impulsive behavior Depression/apathy Emotional instability
Pathology	Diffuse perivascular hemorrhagic infiltrations "moth-eaten"/vacuolated appearance état criblé	punctate concussion hemorrhages Septal defects	AD-like pathology (1954) [35] NFT Formation (1973) [36] Phospho-tau accumulation(1988) [37]	
Investigators	Osnato & Giliberti	Martland	Millspaugh	Bowman and Blau

1.4 Pathophysiology of Traumatic Brain Injury

Traumatic brain injury can be broken down into two injury components that are sustained in tandem: the primary injury, which is the initial immediate mechanical impact/insult sustained by the brain that results from linear and/or rotational head movement, followed by the secondary injury, which involves the subsequent myriad of downstream pathophysiological processes[38]. The primary injury can involve direct contact, such as a blow or collision to the head, where transference of momentum from one mass, such as an object or helmet to the receiving skull and subsequent brain. This can also include penetrating injuries, such as a gunshot wound, which result in open-head injuries. Alternatively, the primary injury can involve indirect contact, including whiplash and other acceleration/deceleration-mediated injuries. During this process, sudden movement of the head involves transference of momentum and energy from the

skull to brain matter. At the whole-brain level, the brain can be impacted by the skull (coup) and momentum can carry the brain to be subsequently impacted at the opposite end of the skull (contrecoup)[39]. Various commissural fibers that are perpendicular to the insult are most vulnerable to the injury, such as the corpus callosum[40].

Blast injury, which is most often sustained by military personnel, involves a shock-blast wave as the primary injury component, where overpressure compresses nearby airwaves and delivers a diffuse impact to the skull and subsequently to the brain[41]. Blast injuries can also involve shrapnel and other projectiles that are liable to make direct contact with the affected individual[41].

Following the primary injury exposure, the secondary injury response ensues immediately, and can carry on for days, weeks, months and even potentially longer depending on the nature of the injury. Secondary injury consists of excitotoxicity, free radical production, mitochondrial dysfunction, and inflammatory mediators [38, 42]. Axons are thought to be the most vulnerable portion of the neuron to mechanical trauma, given their large surface area to volume ratio[43] and high degree of anisotropic organization of cytoskeletal elements[44], of which is principally composed of microtubules[45]. Shear stress and strain forces elicited by mechanical head trauma can cause rapid stretching of axons, followed by an unregulated influx of cations (Na^+ & Ca^{2+}) through sodium and calcium channels along the axolemma, resulting in indiscriminate depolarization and release of excitatory neurotransmitters[24, 46-51]. Persistent neuronal hyperexcitability can overburden Na^+/K^+ pumps needed to restore proper ionic homeostasis, and can last into the chronic phase of injury[52]. Elevated

activity can also overwhelm mitochondrial buffering of calcium and eventually result in metabolic dysfunction[53].

In addition to ionic disturbances, structural abnormalities are seen immediately following injury, including the breaking of microtubules directly in response to physical trauma[54] (strain of 100% at a rate of 44 s-1). Concurrently, elevated calcium can activate calcium-sensitive proteases, such as calpain, which cleave cytoskeletal components, such as microtubules within the axon[55-57]. Microtubule degradation is evident in other parts of the neuron as well. Hippocampal microtubule-associated protein 2 (MAP2), which is localized mainly to the soma and dendrite is also reduced following injury[58]. Inhibiting calpain activation shortly after injury mitigates behavioral deficits and cytoskeletal breakdown in preclinical animal models of injury[57, 59]. Subsequent processes include axonal transport deficits[60], swelling[61], retraction[44], cellular inflammation[62] and eventual cell death[62]. The culmination of these processes are thought to give rise to the insidious neurodegenerative condition, chronic traumatic encephalopathy (CTE), found in post-mortem brains from individuals with a past history of repetitive trauma[17].

CTE pathology is characterized by the presence of hyperphosphorylated tau accumulated within neurons and glia surrounding small blood vessels (perivascular) at sulci depths in a pattern distinct from Alzheimer's disease[15, 17]. Other supportive pathological features include abnormal TDP-43 accumulation[15, 17, 63], and may include beta-amyloid (Aβ) deposition[17]. The subsequent accumulation of toxic protein aggregates is thought to result in chronic neurodegeneration seen following injury exposure[19, 22, 64]. In addition to parenchymal brain loss, neurodegeneration is also seen in other nerve

fibers, including the progressive response that is evident in retired veterans who experience a decrease in longitudinal retinal nerve fiber layer (RNFL) thickness[64]. The mechanisms that connect discrete injury exposures to long-term neurodegeneration remain known. The use of models that recapitulate aspects of human trauma while enabling hypothesis-driven studies remain an invaluable resource to further dissect underlying mechanisms of pathology.

1.5 Mild TBI Signs and Symptoms

In addition to the potential loss of consciousness and amnesia, mild head trauma can result in a heterogeneous set of cognitive, emotional/psychiatric and physical signs and symptoms, as well as sleep disturbances (Table 1). Importantly, the onset of these symptoms may not appear immediately after injury and may arise days to weeks

Table 2 **Mild TBI Signs & Symptoms [65-67]**

Cognitive	***Neuropsychiatric***	***Physical***	***Sleep-Related***
Difficulty concentrating/remembering Slowed reaction times	Irritability Sadness Nervousness Anxiety	Headache Blurry vision Dizziness (vertigo/imbalance) Lethargy Nausea Photophobia Hyperacusis	Sleeping more or less than usual Difficulty falling asleep

following the traumatic insult. Additionally, while many individuals achieve a full recovery within 7-10 days following injury, 18-38% of individuals develop post-concussion syndrome and may experience symptoms past 6-months after injury[7, 68-71], indicating that mTBI may have long-lasting neurological effects. Exposure to mild head trauma has been shown to be a risk factor for the development of psychiatric

disorders, including major depression (MDD) and/or post-traumatic stress disorder (PTSD) [72], seen in both civilian[72] and military populations[73] subjected to mild TBI.

Careful attention and consideration for the presence of signs and symptoms is especially critical for documenting the recovery process as they may be the only indication of neurological injury/perturbation given that standard neuroimaging and other biomarker diagnostics remain largely absent from standard mild TBI care. A greater understanding in fundamental disease processes related to head trauma exposure will provide insight into the development of potential diagnostic/prognostic tools to aid in the identification and recovery from head injury.

1.6 TBI Risk Factors

The discovery of risk factors related to both TBI incidence and recovery is an active area of ongoing research, however this is complicated by both how injuries are sustained and whether they go noticed or not. That being said, several important risk factors have been identified, including age, prior exposure, sex and genetic factors, which are discussed below.

Age

Age remains to be one of the strongest outcome predictors for complications following head trauma, including following mild trauma [74-76]. The cause of injury varies by age, with motor vehicle accidents being the greatest source of injury in young adults and falls being the greatest contributor to injury in elderly populations[77]. Older age is associated with an increased incidence of head trauma, as well as greater morbidity and mortality following injury[78], including a slower overall recovery process.

Functional outcome 6-months following injury reveals a slower recovery in affected older individuals (aged 60+) who initially present with the same degree of mild injury severity compared to younger adults[79]. An additional study looking at mildly-injured 65+ individuals revealed that the level of one's education positively affected the chances for reaching a full recovery 6-months following injury[80]. Together, these findings lend support to the concept of cognitive reserve, which decreases with age and is associated with the onset of age-related neurodegenerative disorders such as AD[81], as a potential protective factor against the effects of mild head trauma. This is further supported by the finding that cognitive reserve (estimated premorbid IQ) is associated with improvements in multiple tested cognitive domains (memory, verbal fluency, and executive function) across all injury severities of head trauma 1-year following injury in an injured adult cohort ranging from 19-79 years of age[82]. It is difficult to disentangle the added effect of comorbidities and increasing frailty associated with aging, but future preclinical work can precisely dissect these potential mechanisms that will then inform further clinical investigation.

Prior Exposure

A prior history of concussion is a risk factor for sustaining future concussion, which has been studied most within the context of contact sports, primarily American football. As is the case for older age, a prior history of head trauma increases both the incidence of future head trauma, as well as hastens the length of recovery from concussion[83]. Importantly, the vulnerability to injury re-exposure appears to be greatest shortly following the initial injury, as a majority of affected athletes who experience multiple concussions within a given football season did so 7-10 days following their

initial concussion[83]. Not only does prior exposure to injury lower the threshold for further injury, and lengthen the subsequent recovery time, but it also results in the exacerbation of early deficits seen following injury exposure, seen as a difficulty in integrating multi-sensory information[84]. Collectively, this highlights the cumulative effect of repetitive injuries, and reinforces the concept behind looking at mild forms of repetitive trauma not as isolated discrete events but as multiple related processes.

Sex Differences

The use of both sexes within preclinical TBI research has garnered greater attention recently, which followed a period of time that incorporated limited female representation in studies in prior years[85], and has become a greater point of emphasis since the NIH mandated that sex be considered a biological variable in 2016[85, 86]. A growing body of evidence demonstrates that there exists a sex-dependent effect related to incidence and recovery from neurotrauma[87-91]. This should come as little surprise given that there are sex differences in anatomy[89, 92], sex gonadal hormones[93], and immunological responses[85, 94, 95], which presumably affect vulnerability to injury. That being said, there is an interesting disparity between clinical and preclinical sex-dependent findings, where female sex is associated with worse outcomes in human studies and better outcomes in preclinical studies[96]. Part of this disparity in findings is attributed to the use of different animal models and varying severity of injuries [96].

In a large literature review, human studies involving mild injury showed that the female sex was associated with worse outcome measures[96]. Human data from the TRACK-TBI study demonstrated that the female sex was associated with decreased six-month functional outcome measured using the Glasgow Outcome Scale-Extended

(GOSE)[88] following mild TBI. A recent cross-sectional human study revealed that females were more likely to report a less favorable health-related quality of life (HRQoL) during the chronic stage of mild TBI (10 years post-injury)[87]. Additional evidence demonstrated that young female athletes may take longer to become symptom free following sports-related concussion[90].

Interestingly, preclinical data showing that the female sex is associated with better outcomes in severe injury models served as the foundation for testing the neuroprotective effects of progesterone in the ProTECT (Progesterone for Traumatic Brain Injury, Experimental Clinical Treatment) human clinical trials for severe neurotrauma[97]. Despite preclinical evidence demonstrating neuroprotection, exogenous progesterone failed to meet the clinical end point (a 10% improvement in the Extended Glasgow Outcome Scale 6-months post-injury) [97]. The paradoxical finding that exogenous progesterone exhibits neuroprotective properties in preclinical models, yet females experience a worse prognosis may be attributed to the cyclical nature of progesterone, and forms the basis of what is known as the "withdrawal hypothesis" [98, 99]. The "withdrawal hypothesis" posits that the relative abundance of progesterone dictates injury vulnerability. In the case of females who sustain a mild TBI during the luteal phase of menstruation (when progesterone is high) report a lower quality of life (EuroQoL/EQ5D) and worse self-reported outcome measures (Rivermead Post Concussion Questionnaire) 1-month following injury compared to injured females on birth control, who exhibit elevated levels of progestins[99]. A similar trend is seen across age-groups within females: pre-menarche and post-menopausal women report lower 3-month post-concussive symptoms compared to women of child-bearing years[100], indicating that

injury vulnerability may be related to the disruption or natural cycling of female gonadal hormones. Taken together, this demonstrates sex differences related to recovery from TBI and substantiates the importance of using both sexes in both clinical and preclinical studies.

Genetic Factors

Genetic predisposition to traumatic brain injury and its sequelae has become an important avenue of study in recent years. To date, several genetic variants have shown significance towards increased susceptibility to mild TBI, including the APOE promoter -219G/T polymorphism and the brain derived neurotrophic factor (BDNF) Met/Met variant, the latter of which is associated with greater recurrence of concussion[101]. Additionally, the development of post-traumatic stress disorder and worse functional outcome is associated with the Catechol-O-methyltransferase (COMT) Val158Met polymorphism following mild traumatic brain injury[102]. Further work is needed to understand the fundamental pathophysiology for each of these respective predispositions.

To date, there are no FDA-approved drugs explicitly developed for treating TBI. This dearth of therapeutics leaves the treatment of moderate and severe cases of trauma with limited options to promote functional recovery aside from supportive and life-saving hemodynamic strategies. Furthermore, with increasing evidence that mild head trauma, especially repetitive exposures, has long-lasting and cumulative effects there is even greater need to develop neuroprotective strategies for even the mildest of head traumas. With limited therapeutics at our disposal for the treatment of head trauma, the identification of risk factors associated with worse outcomes following mild head trauma

is an important component for mitigating risk in vulnerable populations and should remain a priority for future research.

Chapter 2 Preclinical Modeling of Traumatic Brain Injury

While humans remain the quintessential model organism for studying human disease, there oftentimes are limitations and constraints that necessitate the use of simpler experimental model organisms. Animal models allow for carefully controlled, hypothesis-driven research that is important for identifying novel disease pathways, genetic and environmental risk factors, biomarker discovery and preliminary testing of therapeutic interventions prior to human clinical trials[25].

In the case of head trauma, small mammalian (rodent) models have served as the primary go-to *in vivo* model system, given their highly conserved genetics and biological processes. Large mammalian injury models, including porcine, cats, dogs and non-human primates have also been developed during this time, albeit their utilization is far less than that of small mammalian models[103]. One major neuroanatomical advantage present within large mammalian subjects that is absent in smaller rodents is the gyrencephalic nature of their brains, which better recapitulates the folding patterns found within the cortex of the human brain[103]. Rodents have lissencephalic brains, which may not represent the adequate substrate for modeling tissue deformation following mechanical injury[103]. Interestingly, the pathognomonic distribution of hyperphosphorylated tau associated with CTE is found at the depths of sulci, where the gyrencephalic brain experiences the greatest degree of mechanical stress[103]. That being said, smaller model organisms provide a considerably significant cost advantage with greater standardization of outcome measurements (behavioral and pathological)[104]. Not one model can fully recapitulate human trauma, especially given that human trauma is in itself highly heterogeneous, but precise aspects of trauma can neatly be dissected using model

organisms, especially smaller model organisms with greater tractability, that would otherwise not be feasible within humans.

In addition to mammalian model systems, invertebrate models have found a foothold within the field, offering unique advantages over existing strategies[105]. An overview of commonly used mammalian and invertebrate head injury models, including *in vitro* cell-culture models, and their advantages and disadvantages are discussed below. The central focus of this thesis will be placed on repetitive exposure to mild injury.

2.1 *In Vitro* Trauma Models

Traumatic brain injury is a highly complex, heterogeneous process. The use of cell culture models to investigate the cellular injury response to mechanical trauma allows one to deconstruct the complex injury response into a much more simplified system. A number of cell culture-based injury models have been developed to better understand various types of mechanical trauma, including transection, compression, shear stress and strain[51, 68, 106-108]. Importantly, these models recapitulate *in vivo* injury models, thus validating their utility to study novel mechanisms of injury[106]. In doing so, their use has enabled investigators to study the immediate cellular and molecular injury response, which is much more difficult to do in larger model systems (*see chapter 5*). Importantly, cell culture injury models have been used for screening therapeutic targets and drug discovery that can then be used to assess relevancy and efficacy *in vivo*. For example, a high-throughput *in vitro* transection injury screen identified a novel compound, known as 1-ARA-35b, which has since shown to exhibit therapeutic efficacy in preclinical *in vivo* animal models[109]. Additional peptide screens initially conducted *in vitro* have also demonstrated efficacy when applied to *in vivo* preclinical models[110].

There are several important considerations to bear in mind with respect to in vitro injury models. Cell culture experiments enable investigation of isolated cell-specific responses to mechanical injury, however, *in vivo* trauma involves a complex organization of different extracellular environments and tissue made up of different cell types[106]. Therefore, it is important to consider that cells in isolated cultures may respond differently than *in vivo*. That being said, the use of cell culture injury models provides enormous tractability and potential to pursue highly-controlled experiments that can guide future *in vivo* studies.

2.2 Mammalian Models

Prior to the growing concern surrounding boxing that came to prominence in the 20th century, Koch and Filehne provided some of the earliest experimental evidence demonstrating the detrimental effects of repetitive mild head trauma in 1874. Their injury model delivered concussive blows to the head of dogs using a 250-500g percussion hammer[111-113]. Mortality was seen only after delivery of a high number of impacts. Interestingly, no overt tissue damage was detected upon post-mortem inspection, and because the injuries were individually considered small, Kock and Filehne concluded that the deficits seen were attributed to the functional injury response elicited by iterative injuries, rather than a gross structural lesion such as a contusion or hemorrhage[111, 114]. Subsequent experimental injury modeling carried into non-human primates during the latter half of the 20th century[115-117], which revealed the significance of rotational head acceleration towards the mild head injury response. Since then, a bulk of the basic TBI research has been derived from smaller mammalian models[118], providing a great wealth of fundamental pathophysiology of traumatic brain injury [25, 48, 119-124], across all

injury mechanisms and severities. Discussed below are the most commonly used small mammalian models for modeling mild head trauma.

Fluid Percussion Injury (FPI)[125-130]

The fluid percussion injury model delivers a fluid pulse into the epidural space of rodents following craniotomy. To do so, a weighted pendulum is released from a specified height, which then contacts a plunger at one end of a fluid cylinder which transduces the fluid pulse to a surgically implanted Luer-Lock on the opposite end connected to the exposed dura. A fluid pressure detector measures the imparted fluid pulse. A mild impact can be selectively tuned by setting the pressure range of the pulse to 0.9-1.5 atm[125], by adjusting the height at which the weighted pendulum is released. The LPI model produces focal cortical contusion at the site of impact and diffuse subcortical neuronal injury[104]. Injury severity using the FPI can vary depending on where the craniotomy is performed The FPI model does not result in skull fracture, which is typical of most mild forms of neurotrauma[104].

Controlled Cortical Impact (CCI)[48, 123, 124, 130]

Within the controlled cortical impact model, a pneumatic (compressed air) or electromagnetically driven piston penetrates the exposed dura resulting in cortical deformation. A mild focal impact can be delivered by tuning the depth (<1mm) and speed (<4m/s) of the piston.

Weight-Drop Models[130-132]

There are several variations of the weight-drop model using rats and mice, all of which involve dropping a free-falling weight onto the skull. Both models also deliver their respective injuries to anesthetized rodents. In Feeney's model, a craniotomy exposes the

dura, and the weight is dropped atop the dura to deliver a focal injury[133]. In contrast, Marmarou's model involves placement of a metal disk secured to the exposed skull, which delivers a diffuse injury when the weight is dropped onto the metal disk, thus distributing the impact force throughout the skull[131]. Marmarou's diffuse injury model can be adjusted to deliver a milder injury by using a lighter weight and or by dropping the weight from a shorter height[104, 133]. Mechanical ventilation is often required with the Marmarou model as respiratory depression is known to occur[104]. Weight-drop models are associated with high variability in injury severity[104].

CHIMERA[134]

The Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) model is a nonsurgical approach involving a pneumatically driven piston that delivers an impact to the intact skull of a restrained mouse. Mice are exposed to isoflurane anesthesia during the injury induction.

Boston University Model[135]

Tagge et al. (Boston University) recently developed a novel model of closed-head lateral impact injury that uses a pneumatic powered transfer device to rapidly induce traumatic head acceleration in unanesthetized, bodily restrained mice. The device is tunable, enabling the delivery of mild injuries in a repetitive fashion. Furthermore, it provides several key advantages over existing strategies, such as the absence of needed surgical manipulation and use of anesthesia required by the aforementioned models to deliver injuries.

Blast Model[136]

To model blast TBI, a shock-wave tube has been developed to deliver various amounts of overpressure to rodents. Delivery of an 80–145kPa pulse corresponds to a mild diffuse injury*[137]*. That being said, there is a high degree of variation in blast injury models across institutions, making it challenging to compare results without standardization[104].

Caveats with existing models

The FLP, CCI and weight-drop models all involve surgical manipulation, either incisions to the head to expose the skull, or even craniotomies to expose the dura. Additionally, these methods employ the use of anesthetics during the injury delivery, likely resulting in some degree of confounding effects on the study of traumatic brain injury[121]. This is especially important in the context of repetitive injuries which would require repetitive exposure to anesthesia. Furthermore, because subject animals are subdued and incapacitated during delivery of the injury, no post-injury neuroscoring or characterization of immediate behavioral signs can be assessed, thereby restricting behavioral examination to subacute phases and beyond.

Other considerations related to the use of small rodents lies in their neuroanatomical differences with humans. For example, aside from the considerable difference in sheer size, the human brain composition of white to gray matter (60:40) is strikingly different from that of rodents (10:90)[138]. Also, drastically different is the ratio of glia to neurons, which are 10:1 in humans and 1:1 in rodents. While these findings do not preclude rodent studies from contributing to our understanding of traumatic brain injury, they are important considerations to bear in mind.

2.3 Non-Mammalian (Invertebrate) Models

2.3.1 A Quick Primer on *Drosophila Melanogaster* Neurobiology

Drosophila have served as a powerfully tractable model organism to investigate fundamental neurobiological processes and mechanisms of neurodegeneration[139-146]. This is made possible due to conserved neurobiology that exists between both *Drosophila* and mammalian species, including Na^+/K^+-based action potentials and inhibitory and excitatory neurotransmitters with shared neurosecretory-released mechanisms[147]. Like that of the mammalian brain, the fly brain consists of an organized arrangement of discrete neuronal structures and circuitry, but exists within a much smaller, more easily dissectible brain size that enables the study of individual neurons and their corresponding functional roles[148]. The brain of *Drosophila melanogaster* is comprised of ~100,000 neurons [148, 149] and 10,000 glia [147, 149], which makes it several orders of magnitude smaller than the human brain (86 billion neurons[148], with an equal number of glia[150]).

Specifically, the fly brain consists of a central brain and two large optic lobes, each with an outer cortex that contains cell bodies and a synaptically-dense inner neuropil inner[151]. This is in contrast to the vertebrate brain[152], which features a synaptically-dense outer region within the superficial cortical layers. The outer cortex of the fly brain is surrounded by a perineurium that serves a functional equivalent to the blood-brain barrier within vertebrates[151]. The ventral nerve cord is the invertebrate equivalent of the mammalian spinal cord, which extends into the thorax where it relays motor-sensory information [153]. At the cellular level, *Drosophila* neurons share the same three subcellular compartments as mammalian neurons (axon, soma, dendrite), which make up a mix of unipolar and multipolar neurons[154]. However, unlike mammalian dendrites,

Drosophila dendrites do not possess clearly demarcated spines that occupy post-synaptic sites[154].

2.3.2 Benefits of *Drosophila Melanogaster*

The use of *Drosophila melanogaster* as a model organism has provided a plethora of genetic, molecular and cellular insight towards the contribution of understanding fundamental neurobiology. This is perhaps most recently appreciated in the study of the molecular and genetic mechanisms underlying circadian rhythm function for which the 2017 Nobel Prize in Physiology or Medicine recognized. Several key characteristics of *Drosophila melanogaster* make its use as an ideal model organism for the study of neurological diseases. 75% of disease-related genes in humans have corresponding fly orthologs[155]. They have a short reproductive and life cycle, which enables lifelong processes such as those related to neurodegeneration and aging in a much more considerable time frame[137, 141, 146, 155-158]. Adult fly exhibits complex behaviors and an organized central nervous system, all while sharing conserved neural mechanisms with that of humans[155]. Perhaps most impressive is the genetically tractable nature of fruit flies, which is unparalleled by any mammalian system. The ability to systematically probe protein function and control transgene expression in a cell-specific manner enables easy access to interrogate various signaling mechanisms, and opportunities for high-throughput screens[137, 157]. This approach has led to the study of cellular and molecular injury responses following axonal injury within the fly. Using a variety of genetic screens inherently accessible within *Drosophila* has led to the identification of several key genes and their respective proteins that are involved in axonal survival and degeneration[159-162]; this includes Wallenda (Wnd), a fly homologue of dual leucine kinase (a conserved

mitogen-activated protein kinase (MAPK) kinase kinase important in relying cell-autonomous axonal degeneration following axonal transection). Importantly, these discoveries have translated to both murine[163-165] and human cell-based[166] studies, which validates the utility of using *Drosophila* as a simple *in vivo* approach to investigate conserved neurobiological processes. Given the success of modeling axonal transection injury within the fly, it has garnered attention as a model system to study traumatic brain injury, which offers many of the same aforementioned benefits. The existing published fly traumatic brain injury models are described below.

Physiological Considerations for Modeling Human Trauma using *Drosophila*

There are several physiological caveats to consider within the present study given that small mammals are the traditional animal model type used to study neurotrauma. The mammalian brain is a highly vascularized structure that is suspended and cushioned in cerebralspinal fluid (CSF), and contained within a boney skull, whereas the *Drosophila* brain is surrounded by trachea and air sacs that distribute nutrients and oxygen similar to that of the mammalian circulatory system, and is enclosed within a chitinous exoskeleton. Although the *Drosophila* brain is not surrounded by CSF, the surrounding air sacs serve as a fluidic equivalent in being able to suspend the brain and serve as a buffer between the brain and outer enclosure. Also, similar to the aforementioned anatomical considerations for using rodents to model trauma, the ratio of glia to neurons in *Drosophila* is 1:10[147], which is far less than that of rodents and humans (1:1).

2.3.3 Existing *Drosophila* Head Injury Models

The first published fly head injury model was reported in 2013 by Katzenberger et al. [167, 168] . This design, known as the “high-impact trauma” (HIT) device featured a

metal spring secured to a board on one end, and the free end secured to a standard fly vial containing a large cohort (~60) of flies. To elicit trauma, the spring is loaded so that the free end is perpendicular (90°) with respect to the surface to which the secured end of the spring is attached to. The vial (attached to the spring) is then released, undergoing rotational acceleration, and rapidly makes contact with a polyurethane pad, after which the forward momentum of flies carries them until acceleration/deceleration-mediated contact is made. Injury severity can be modified within this model by adjusting the angle at which the spring is brought back. This first report provided early characterization of fruit flies subjected to mechanical trauma, including mortality/lifespan effects, climbing deficits, neurodegeneration and immune responses secondary to trauma exposure. It also took advantage of the high-throughput nature of *Drosophila* by performing a genetic screen to compare mortality outcomes in mutant lines for innate immunity [additional results]. The primary limitation of this first approach is that body orientation, and thus the degree of head trauma, is not controlled for. The type of injury sustained is rather heterogeneous, as the author's noted that, *"individual flies presumably contacted the wall with different region of their head and/or body and with different forces, so primary injuries will vary among flies in the same vial."* By nature of the vial constantly changing velocity while it undergoes rotational acceleration, flies experience a tumbling motion thus resulting in impacts sustained in a non-specific fashion (**Figure 2-1B**), thus limiting the conclusions drawn as being head/brain specific deficits. Since its initial publication, the model has additionally been described as a full-body trauma model[169]. That being said, it is worth noting the contributions that have been made to date using the HIT model. Loss of

highwire, a protein involved in Wallerian degeneration, which was first studied within the fly, is protective against cell death and degeneration following head injury exposure[170].

In 2016, Barekat et al. developed a different approach to deliver head trauma to individual fruit flies using a tissue homogenizer[171]. Each fly was first incapacitated using CO_2, placed in a 2mL screw cap tube, then subjected to either a single bout of "severe" TBI (1×, 4.35 m/s intensity) or repetitive (10x) "mild" TBI (2.1 m/s) of shaking. This approach demonstrated activation of innate immunity (acute and chronic[1week] post-injury), shortened lifespan, climbing deficits, mitophagy/autophagy, sleep disturbances as well as intestinal dysfunction, in response to injury, the last of which is likely attributed to non-specific trauma sustained throughout the body. This homogenizer approach, like that from Katzenberger et al., delivers trauma to the entire body, thus complicating the conclusions that can be drawn. Additionally, this model involves the delicate handling of individual flies delivered to one tube at a time, after they have been subdued using CO_2, yielding low-throughput potential and the exposure to repeated rounds of CO_2 which affects neurological function.

Additional head-specific methods have since been developed to provide a more precise injury delivery. The first head-specific method developed involved trapping the fly body in a 200 μL pipette tip, and delivering blunt trauma to the exposed head using a CO_2 powered impactor [172]. Preliminary characterization from this model showed acute mortality and shortened lifespan and locomotion that was correlated to the CO_2 flow rate[172]. Unfortunately, this model employs continuous supply of CO_2 throughout the injury process[172].

A more recently developed model delivers precise and accurate head impacts using a piezo-electric actuator that compresses the head of individually restrained flies[173, 174]. This model can be scaled up to deliver trauma to a small cohort (up to 5 flies at a time). However, CO_2 is required to position the flies within the injury rig. That being said, this approach still recapitulates several key aspects of trauma found in humans and other preclinical models of trauma including progressive neurodegeneration and glial-specific responses. This model also enables precise manipulation of injury severity, by specifically tuning the actuator. In addition to showing neurodegeneration seen in the HIT model, this report demonstrated the first evidence of glial reactivity, which consisted of an activated astrocytic phenotype and perturbed unsheathing glia, which potentially compromised blood brain barrier function. Both of these cellular responses have orthologous processes that have been documented in mammalian injury models, thus demonstrating a conserved cellular response seen in invertebrates. This model also demonstrated acute stress responses, including oxidative stress and lysosomal activity. While this group demonstrated feasibility of delivering milder injuries, most of their results were limited to severe forms of trauma. They also limited their study to using only male flies.

2.4 Our Novel Repetitive Mild Traumatic Brain Injury *Drosophila* Model

Here, we developed a tractable *Drosophila melanogaster* model that enables lifelong interrogation of specific processes and their downstream cellular and behavioral effects. Several fly models have been developed to study traumatic brain injury in *Drosophila* but the lack of controlled head-specific impacts may confound brain neuronal-specific responses [167, 171, 175]. Head-specific impact models have recently

been developed in which physical head impacts were delivered to a single restrained fly one at a time, thus limiting the throughput of the study on large populations of animals [172, 173]. Developing a reliable and precise method for inflicting repetitive mild head trauma to flies will allow for eventual high-throughput genetic, environmental, and drug screens similar to that of *in vitro* experimentation with the same benefits of working *in vivo*.

2.4.1 Results

To investigate the long-term consequences of repetitive mild head trauma, we developed a novel repetitive head injury model using *Drosophila* that accurately and precisely delivers headfirst acceleration-deceleration-mediated head impacts (**Figure 2-1A,Video S1**). Multiple flies (10-15 flies at a time) are contained within a customized plastic injury vial designed to fit within our injury rig consisting of a cradle that is connected to a pulley system with a specific counterweight. Each injury trial consists of pulling the cradle downward to the base of the injury rig, followed by lightly tamping the cradle three times for all the flies to fall to the bottom of the vial before releasing it for upward acceleration. When the cradle, which contains the injury vial with flies suddenly stops at the top, the momentum of the fruit flies continues to propel them upward, where they sustain headfirst impacts against the glass ceiling at the top of the vial (**Figure 2-1A**). The light tamping is employed for two purposes: to cause all the flies to fall to the bottom so they will travel the same distance before the head impact and to allow the flies to orientate in a head-upward posture as a part of their startle-induced negative geotactic climbing response. Indeed, our high-speed video recording revealed that most of the flies hit the top ceiling with their heads first, resulting in an impact angle of nearly 90 degrees

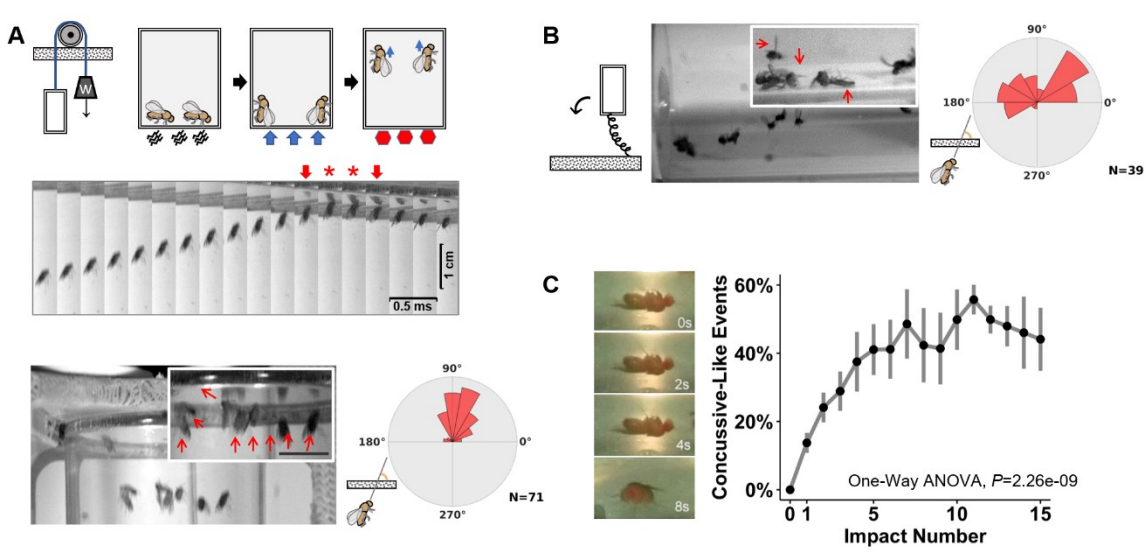

Figure 2-1 **Development of a novel repetitive head impact *Drosophila* model.**

(A) (Top) Diagram of our novel headfirst impact model that utilizes a counterweight pulley system to accelerate a vial of flies upward ultimately resulting in acceleration-deceleration headfirst impacts. To prime proper body orientation, the vial is lightly tamped down, resulting in the natural negative geotactic upwards orientation, after which the vial is released, causing the counterweight to accelerate the vial unidirectionally. The unidirectional movement upwards, together with the priming sequences encourages facilitates headfirst orientation at impact once the vial reaches its maximum height. (Middle) High-speed image sequence showing headfirst movement through impact with the surface of the injury vial (arrows indicate frame immediately before and after contact, which is denoted by red stars, which lasts ~1ms long). (Bottom) Angular histogram comparisons demonstrate more reliable and consistent headfirst impacts and improves upon **(B)** previously existing strategies which encourage rotational body movement that results in inconsistent head orientation at impact. **(C)** Immediately following headfirst impacts, flies sustain acute signs of neurological injury, such as temporary loss of consciousness that becomes more prevalent with increasing number of repetitive head impacts. Impact number increases concussive-like events (one-way ANOVA, $F(1,89)=44.26645$, $P=2.26e-09$). Data represented as mean ± SD, n=4-8 vials (10 flies per vial) of both male and female flies.

(anterior-posterior body axis perpendicular to the impact surface). This tailored approach improves upon an existing strategy that delivers traumatic injury to flies within a vial that is accelerated in a circular motion, which causes the flies to undergo a tumbling motion, thereby prompting subsequent nonspecific impacts sustained to systemic regions of the body other than the head (**Figure 2-1B**) and limiting the head-specific conclusions that can be drawn from this type of injury. With our current impact configuration, we determined using the high-speed video recording that each fly experienced an impact that involves ~1 ms of contact at a speed of ~5 meters/s with the glass ceiling (**Figure 2-1A**, arrows: immediately before the contact as indicated by the gap between the fly and its mirror; asterisks: contact). Immediately following headfirst impact, a subset of flies fell to the floor of the vial and exhibited a concussive-like behavioral response indicative of neurological injury, including temporary loss of consciousness and other uncoordinated behavioral responses (**Figure 2-1C**, **Video S2**). The frequency of this immediate behavioral response increases with the number of successive head impacts sustained. Incapacitated flies eventually regain consciousness within seconds, in line with mild human trauma that is associated with temporary loss of consciousness. Furthermore, flies subjected to impact injuries do not feature any gross head or body damage (**Figure 2-2A**), which is reminiscent of a closed-head injury. Interestingly, these impacts are individually sublethal in the acute phase of injury, and only elicit mortality when iteratively delivered repetitively after a high number of sustained impacts **(Figure 2-2B)**.

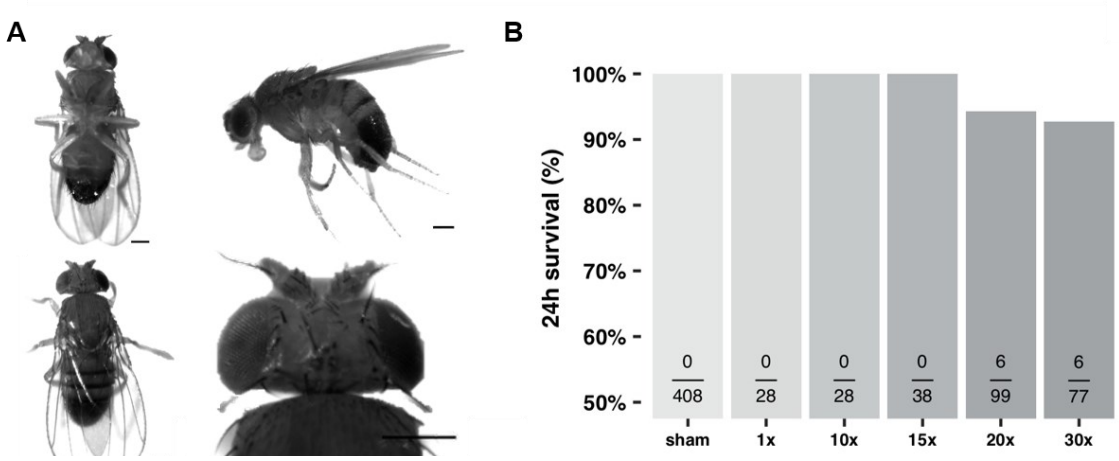

Figure 2-2 **Gross morphology and acute survival following repetitive head impacts**

(A) Representative whole-body micrograph of an injured *Oregon R* male fly showing no signs of gross morphological damage to the head or body following repetitive head impact exposure. Scale bar= 100 μm. **(B)** Bar plot of acute survival (24h) following varying number of iterative successive head impacts delivered 10s apart. Black text indicates (# dead/# at risk).

2.4.2 Discussion

The present study developed a novel acceleration-deceleration impact model that can deliver repetitive headfirst impacts to awake and unrestrained fruit flies. Our newly developed *Drosophila* head impact model possesses several unique features that distinguish it from other systems. First, the physical impacts are delivered mostly to the head of awake and unrestrained flies. The headfirst collision of flies to the impact ceiling is achieved in part through the upward body orientation at the start of acceleration due to the innate negative geotactic escape response during which flies orient headfirst against the force of gravity when startled. The aerodynamic body shape of the flies is also believed to contribute to the headfirst impact orientation as we observed mostly headfirst impacts with flies injured while immobilized and incapacitated (**Figure 5-1B**). Second, the headfirst impacts are delivered to a large cohort of flies (10-15 flies at a time), thus increasing the efficiency for investigating the effects on large populations that are essential for analyzing genetic variations and long-term age-dependent changes; the latter of which is made possible by the relatively short overall lifespan of fruit flies. Third, injury severity, or the impact force sustained by the fly head can be specifically modified by adjusting the hardness of the impact surface. For example, our current glass impact surface, which is a circular glass coverslip, can be replaced with a material surface of different hardness, such as plastic or Styrofoam, which can provide a specific degree of buffering for mimicking the padding of helmets in contact sports. Injury severity can also be adjusted within our system by using different counterweights and heights (for various acceleration distances) within the pulley system, resulting in a change in velocity at impact. Therefore, our head impact system has the potential to be utilized in a wide range

of studies concerning head trauma that are focused on behavioral, structural, and functional consequences using adult flies of different ages and genetic backgrounds, helping to better understand the risk factors in the development of neurodegeneration and long-term dysfunction after head trauma.

2.4.3 Methods

Fly husbandry

Flies were maintained at 18˚C (activity-suppressing experiments) or 25˚C, with 60% humidity on a 12-hr:12-hr light:dark cycle and kept in vials containing fresh fly media consisting of cornmeal, yeast, molasses, agar, p-hydroxy-benzoic acid methyl

Table 3 *Drosophila* Fly Lines

Fly Line	Full Genotype	Source	ID
w^{1118}	w^{1118};;	Bloomington	RRID:BDSC_5905
UAS-CaLexA-LUC	w[*]; P"["w[+mC]=LexAop-LUC"]"attP40; P"["w[+mC]=UAS-mLexA-VP16-NFAT"]"H2/TM6B, Tb[1]	Dr. Kate Abruzzi (Rosbash Lab)	FBti0184974 [176]
OK107-GAL4	w[*]; P[w[+mW.hs]=GawB]OK107 ey[OK107]/In(4)ci[D], ci[D] pan[ciD] sv[spa-pol]	Bloomington	RRID:BDSC_854
nSyb-GAL4	w[*]; P"["y[+t7.7] w[+mC]=GMR57C10-GAL4"]"attP40;	Teri Ngo (Rubin lab)	FBtp0093974 [177]
nSyb-LexA	y[1] w[*]; PBac"["y[+mDint2] w[+mC]=nSyb-lexA::p65"]"VK00022/CyO;	Teri Ngo (Rubin lab)	FBtp0089965 [177]
LexAop-$Shibire^{ts1}$	y[1] w[*]; ; PBac"["pJFRC104-13XLexAop2-IVS-Syn21-Shibire-ts1-p10"]"attP2	Teri Ngo (Rubin lab)	FBtp0115094 [177]
nSyb>$Shibire^{ts1}$	w[*]; PBac"["y[+mDint2] w[+mC]=nSyb-lexA::p65"]"VK00022/CyO; PBac"["pJFRC104-13XLexAop2-IVS-Syn21-Shibire-ts1-p10"]"attP2	This paper	
Oregon R	Oregon R	Bloomington	RRID:BDSC_5

ester. Vials were changed every 3–4 days using sterile methods. The following stocks were used: w^{1118} (BDSC 5905), *UAS-CaLexA-LUC*[176] (from K. Abruzzi), *OK107-GAL4* (BDSC 854), *neuronal synaptobrevin-GAL4* (*nSyb-GAL4)*[177] (from T. Ngo), *nSyb-LexA*[177] (from T. Ngo), *LexAop-Shibire^{ts1}* [177] (from T. Ngo), *Oregon R* (BDSC 5). A stable line expressing *nSyb>Shibire^{ts1}* (*LexA>LexAop*) was created using the respective aforementioned stocks and used for pan-neuronal activity suppressing experiments. Unless otherwise noted, flies used were progeny from *nSyb-LexA* males crossed to female w^{1118}. For activity suppressing experiments, injured flies were progeny from *nSyb>Shibire^{ts1}* stably-expressing males crossed to female w^{1118}.

High-speed video recording

High-speed video recording of head impacts was performed using a Phantom Miro M310 controlled by a PC with Phantom Camera Control (PCC) software. Sequences recorded at either 3205 or 4106 frames per second were imported into Image J. Body orientation at impact was measured using the Angle tool.

Data Reporting and Statistical Analysis

No statistical methods were used to determine sample sizes but are consistent with sample sizes similar to those generally employed in the field [178]. Experimenters were not blinded as almost all data acquisition and analysis was automated. All flies in each vial were administered with the same treatment regimen. For each experiment, the experimental and control flies were collected, treated, and tested at the same time. A Mann-Whitney U test with Bonferroni or Holm correction for multiple comparisons was used for statistical analysis of behavioral data. All statistical analyses were performed using R (R v3.5, [rstatix]). P values are indicated as follows: **** $P < 0.0001$; ***

$P < 0.001$; ** $P < 0.01$; and * $P < 0.05$. For boxplots, lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. When all points are shown, whiskers represent range and boxes indicate lower quartile, median, and upper quartile, from bottom to top.

Chapter 3 Long-Term Function Following Repetitive Head Injury

3.1 Introduction

Although traumatic brain injury is usually self-limited (typically resolving within 10-14 days post-injury), recovery can follow a varied course and requires careful monitoring of signs and symptoms. This is especially important in the subset of patients who develop post-concussive symptoms. Behavioral signs and symptoms remain the mainstay method for gauging the recovery process because there is a dearth of reliable biomarkers, including neuroimaging in many cases (*see Chapter 4*). In fact, due to the subtle nature of mild TBI, conventional CT and MRI are often unable to detect structural changes seen following injury[179] and are seldom used for mild cases of trauma unless there is a concern for brain bleeding[180]. Advancements in fluid-based biomarkers and diagnostic imaging will surely provide more objective measures that aid in diagnosis and prognosis of head injury. Until then, symptomology remains the primary functional recovery measure.

Within human cases of mild head trauma, outcome measures are typically assessed using a variety of self-reported and interview-based assessments. This includes periodic assessment of post-concussion syndrome symptoms using a subjective checklist to measure physical, cognitive, emotional, and sleep-related function[181], the Glasgow Outcome Scale-Extended (GOSE) score which uses an interview-based approach, or the Rivermead Post-Concussion Questionnaire (RPQ) which gauges symptoms relative to the premorbid level. Additional assessments, such as the Brief Symptom Inventory-18 (BSI-18) involve psychological screening that measures somatization, depression, and anxiety[181]. More objective measures are currently under experimental investigation

including the Vestibular/Ocular Motor Screening (VOMS)[182]; a measure of vestibular function which is commonly perturbed following mild traumatic brain injury[183]. Another experimental approach involves a tablet-based platform using eye tracking techniques to measure subtle sensorimotor and cognitive deficits[184]. The development of more objective assessments following injury will improve diagnostic and prognostic measures, and aid in guiding therapeutic practices.

3.2 Mammalian Behavioral Paradigms

Experimental animal models of traumatic brain injury assess functional outcome measures using a variety of behavioral assays, oftentimes performed as a battery of tests. These assays test many of the same functional domains that are disrupted following human TBI, including those related to cognition, physical, neuropsychiatric and sleep-related domains. Each of these domains are discussed below, with an overview of their corresponding behavioral assays.

Physical Domain

Physical deficits, such as those involving sensorimotor integration are commonly disrupted following mild TBI, including: balance, gait, vestibular function, oculomotor function[185]. Because many existing rodent models employ the use of anesthesia during the injury induction period, they time to regain consciousness. As such, rodents recover on their back, and the latency for them to right themselves (righting reflex latency) is recorded. Milder injuries are typically associated with shorter latencies[186]. Other simple locomotor tasks involve activity monitoring of spontaneous movement, and grid walking, the latter of which involves measuring the rodent's ability to traverse atop an elevated wire surface and quantifying stepping errors[187]. The beam balance test

measures vestibular function while the rodent walks along an elevated thin beam[186]. One of the more commonly assayed sensorimotor function tasks is the rotarod test, which consists of a motorized rod that undergoes increasing rotational acceleration until the animal falls, akin to a unidirectional log roll[186, 188]. Interestingly, deficits following injury exist within the rotarod assay after functional recovery is achieved within the beam balance task, likely given that it is a more demanding task that requires a greater degree of sensory and motor integration[188]. Furthermore, given that the test involves learning, the rotarod assay can be used to not only measure functional recovery, but the ability to learn new skills[186, 188] following injury exposure(s).

Cognitive Domain

Testing cognition is an imperative process following head trauma being that one of the hallmark symptoms associated with head trauma exposure is amnesia, of which its varying degree is used to designate injury severity. Within rodents, maze tasks are the predominant memory and learning paradigm[186]. Mazes require visuospatial and working memory. Rodents utilize working memory as a means for exploratory behavior, often in the context of foraging for food[186]. Cognition is most often testing using the Morris Water Maze[186, 189]. Within the Morris Water Maze assay, rodents are placed into a circular water tank with opaque water that contains a submerged platform. The animal is released within the tank and swims until it finds the submerged platform. Acquisition of visual cues throughout the assay results in improved performance-shorter latency to find the platform[186, 189].

Neuropsychiatric Domain

Major depression and post-traumatic stress disorder can arise following mild TBI[72, 73]. Anxiety and depression-like behavioral phenotypes can be assessed in rodents following injury exposure. Commonly used behavioral assays include measures of behavioral despair (tail suspension test or forced swim test)[190, 191], anhedonia (sucrose preference test)[192], and anxiety-like behavior (open-field assay)[104].

Sleep

Sleep-wake disturbances are a common abnormality seen following all severities of head trauma[193]. This can manifest as insomnia (difficulty falling asleep or maintaining sleep), excessive daytime sleepiness, increased need for sleep and fatigue[193]. Sleep-related abnormalities are measured in experimental injury models using EEG/EMG and other devices that measure physical movement, including an actigraphy device and a piezoelectric sensor placed within the floor of the rodent cage[193]. The importance of healthy sleep function cannot be overstated as it represents both a symptom of injury exposure as well as an important recovery mechanism[193].

3.3 *Drosophila* Behavioral Paradigms

The use of *Drosophila* offers a wide variety of behavioral assays that can be paired with their highly-tractable benefits that enable precise investigation into anatomical correlates. Discussed below are several commonly used *Drosophila* behavioral assays that test functional deficits seen in mammalian models.

Negative Geotaxis assay

The negative geotaxis assay is a commonly used startle-induced climbing assay [139, 194-196] that is sensitive to age-related decline and neurodegeneration. It shares features that are comparable to the rotarod or beam walking assays used in rodent TBI

models to measure vestibulomotor and sensorimotor function deficits [188, 197, 198]. Typically, the assay involves a light tamping to elicit a startle response, which is immediately followed by flies moving upward against the force of gravity. Typically the negative geotaxis response is represented as the percentage of flies in the upper half of the test vial after 10s [139].

Olfactory learning

Cognition within the fly is typically assessed in the context of olfactory learning and memory. This is done using an aversive olfactory conditioning assay [199, 200]. Flies are trained in an optically sealed light:dark T-maze fitted with an aversive odorant, MCH (6-Mercapto-1-hexanol), placed in the lighted portion and no odorant in the dark portion. Flies are then tested the following day in absence of the odorant, and the light:dark preference is recorded [200]. Training can be performed at different timepointss relative to injury, either before or after to assess memory and learning respectively.

Drosophila Activity Monitor

Circadian locomotion is measured as a proxy for sleep-wake behavior in flies, which is done using the *Drosophila activity monitor (DAM)* [201]. To do so, flies are individually placed in glass tubes containing food, maintained in climate controlled 25°C incubators and allowed to habituate for one day, prior to six days of entrainment and measurement under a 12h light:dark. The number of beam crosses traversed by the fly in one-min intervals is scored and used to generate corresponding actograms. Differences in daily mean locomotor activity and circadian periodicity, (proxy measurements of sleep) is then analyzed[202].

3.4 Longitudinal Measurement of Motor Deficits in *Drosophila* Following Injury

3.4.1 Results

Acute recovery of climbing deficits following minimally lethal repetitive head impacts is sexually dimorphic

To emulate repetitive head injury exposure sustained across time, young adult (3 days post-eclosed) male and female flies were subjected to two sessions of repetitive impacts 24h apart; each session consisting of 15 iterative impacts delivered 10s apart (**Figure 3-1A**). Repetitively impacted flies exhibit minimal (<7%) acute mortality following both days of impacts (**Figure 3-1B**). To measure acute behavioral deficits following repetitive head impacts, startle-induced climbing was assessed using the negative geotaxis assay (NGA)[139, 194-196], which is a widely used assay sensitive to age-related decline and neurodegeneration. This assay tests both vestibulomotor and sensorimotor functions which are commonly assessed in traditional mammalian TBI models [188, 197, 198]. Flies were placed in plastic vials containing 2mL of 5% agar at the bottom and subjected to gentle tamping to induce a startle response. Videos were analyzed using an automated python-based tracking software, *idtracker.ai*[203] [204], to measure cumulative climbing distance (slope), total (final) and vertical distance traversed in a 10s span of time following the startle stimulus (**Video S3**). Testing was performed 1.5h after the first and second sessions of repetitive impacts ("1.5h" and "25.5h" with respect to the initial injury), as well as 24h after each session to assess recovery (24h and 48h with respect to the initial injury). While injured males showed an initial deficit in climbing after each session that resolved 24h later, female flies exhibited a progressive climbing deficit that was further exacerbated following the second session of impacts

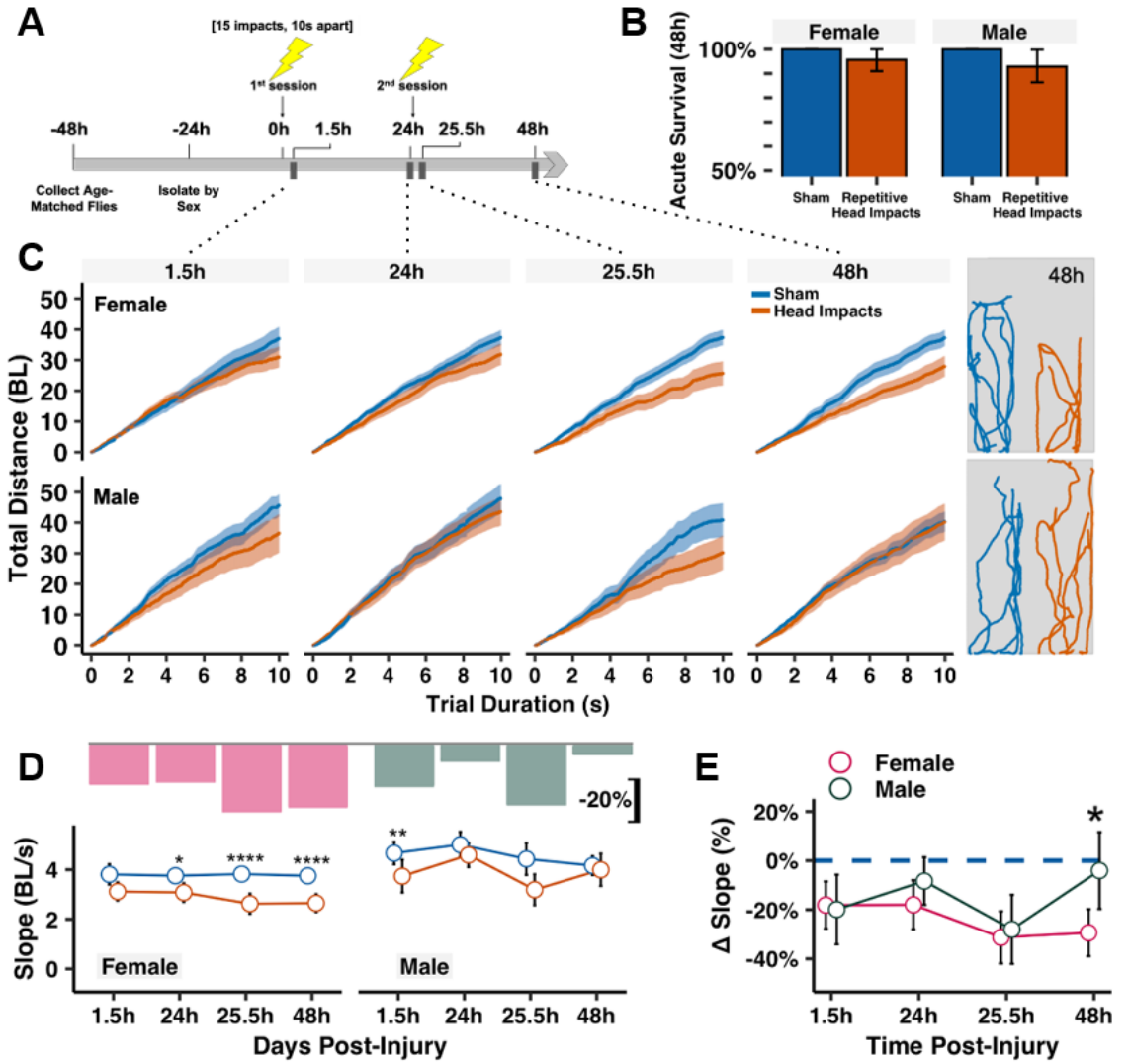

Figure 3-1 **Acute recovery of climbing deficits following minimally lethal repetitive head impacts is sexually dimorphic**

*(***A)** Injury timeline schematic showing that flies are subjected to two sessions of repetitive head impacts (24h apart). Each session consists of 15 iterative impacts spaced 10s apart. Behavior and mortality are longitudinally monitored throughout life. (**B)** Barplot of acute survival following repetitive impacts with 95% confidence intervals. n=56-69 flies per injured group/sex and n=42-50 flies per sham group/sex. **(C)** Total cumulative distance traversed during the negative geotaxis assay. Plotted values are median with 95% confidence intervals as shaded regions, (Right) Representative movement tracings from 5 representative flies/group of sham (blue) and injured (orange) flies during the 10s trial duration at 48h. Distance units are in body lengths (BL). (**D-E)** Plotted climbing slope showing that injured females exhibit a progressive decrease that worsens after the second impact session, while injured male flies show active acute recovery 24h after each session of impacts. Bar plots in (**D**) correspond to the median relative decrease in climbing performance between injured and sham groups (Δ slope= (Injured Slope-Median Sham

Slope)/Median Sham Slope). Plotted values are median with 95% confidence interval error bars in (**D-E)**. Mann–Whitney U test between (**D)** injured and non-injured groups, with Holm correction and **e** injured female and male performance relative to non-injured, with Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=25-35 flies per sex/time/injury group.

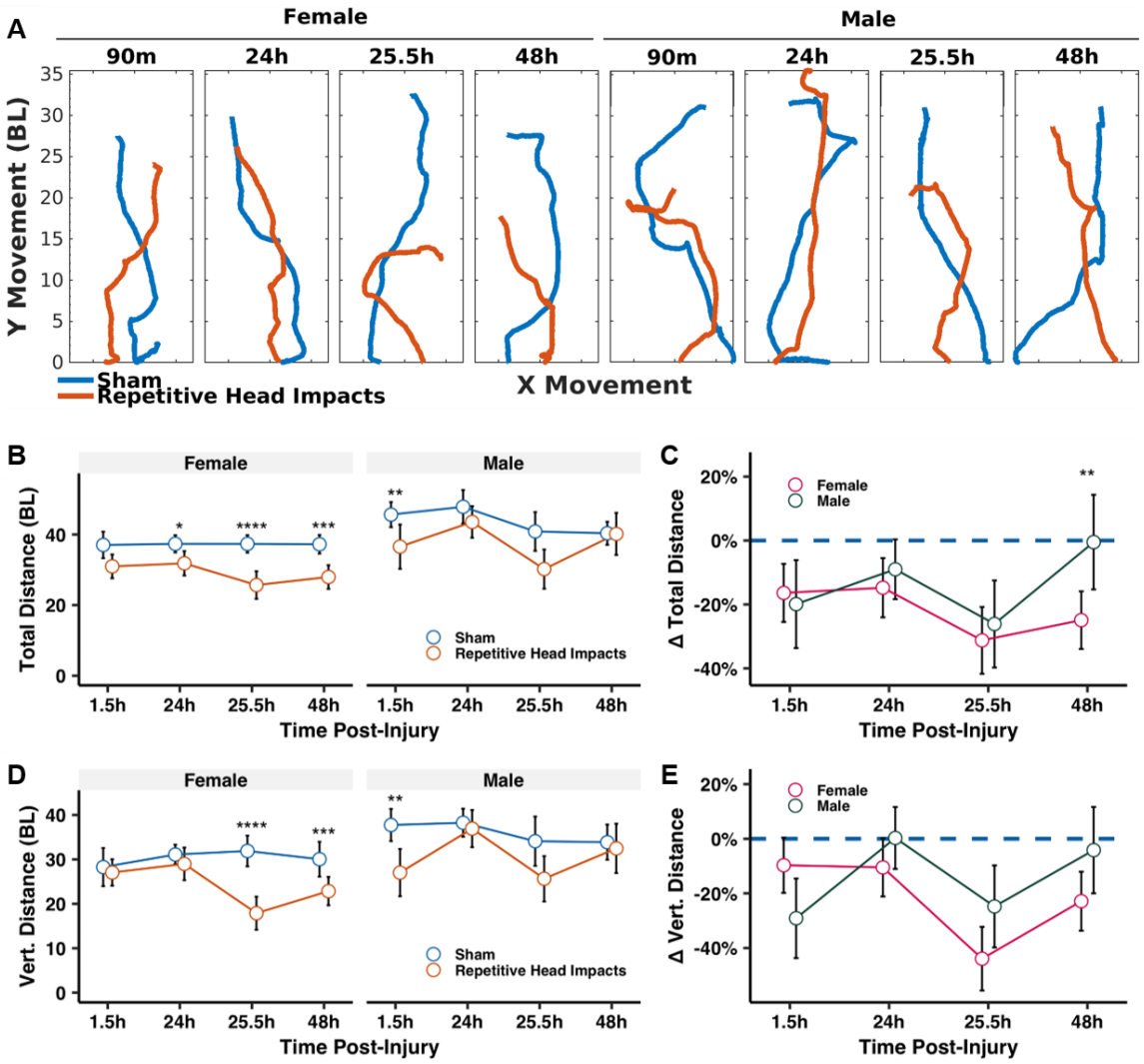

Figure 3-2 **Acute climbing deficits following repetitive mild head trauma**

(A) Representative (median) tracing of fly climbing. Repetitive head impacts elicit acute climbing deficits in both male and female, seen as a reduction in **(B)** total climbing distance and **(D)** total vertical distance traversed during the startle-induced climbing assay. Injured female flies show progressive relative behavioral deficits that worsen after the second impact session, while injured male flies show active acute recovery 24h after each session of impact. Plotted values are median **(B&D)** raw or **(C&E)** relative (to respective sex) values with 95% confidence intervals as error bars. Mann–Whitney U test between **(B&D)** injured and non-injured groups, with Holm correction and **(C&E)** injured female and male performance relative to non-injured, with Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=25-35 flies per sex/time/injury group.

(**Figure 3-1C-D** & **Figure 3-2**). 24h after the second session, a robust difference in climbing behavior was seen between injured sexes, with injured female flies showing a 30% reduction in climbing whereas injured males performed similar to their sham counterparts (**Figure 3-1E**).

Female flies experience a greater reduction in lifespan and long-term climbing deficit following repetitive head impact exposure

One key advantage of our *Drosophila* model of mild head trauma is that it enables the monitoring and examination of long-term effects on lifespan and brain structure and function in relatively large cohorts. We thus continued to routinely examine flies following repetitive mild head impacts over their lifespan. We first measured the mortality and found that repetitive head impact exposure resulted in a shortened overall lifespan that was more prominent in female flies (**Figure 3-3A&B**). To determine the effect of repetitive head injury exposure on long-term behavioral function, startle-induced climbing performance was routinely assessed until 98 days (14 weeks) post-injury. Flies subjected to repetitive head injury exposure exhibited long-term climbing deficits that were more profound in female than male flies (**Figure 3-3C-E** & **Figure 3-4**). Significant deficits in climbing performance of injured female flies were consistently observed up to 70 days post injury (**Figure 3-3C-D**). During this time period, injured female flies exhibited a greater reduction in climbing ability compared to injured males (**Figure 3-3E**). Sham female flies eventually achieve comparable performance to injured flies by 84 days post-injury, while a small decline in injured male climbing performance relative to sham existed up to 98 days post-injury (**Figure 3-3C-D** & **Figure 3-4**). Together, this data demonstrated that early exposure to repetitive mild head trauma

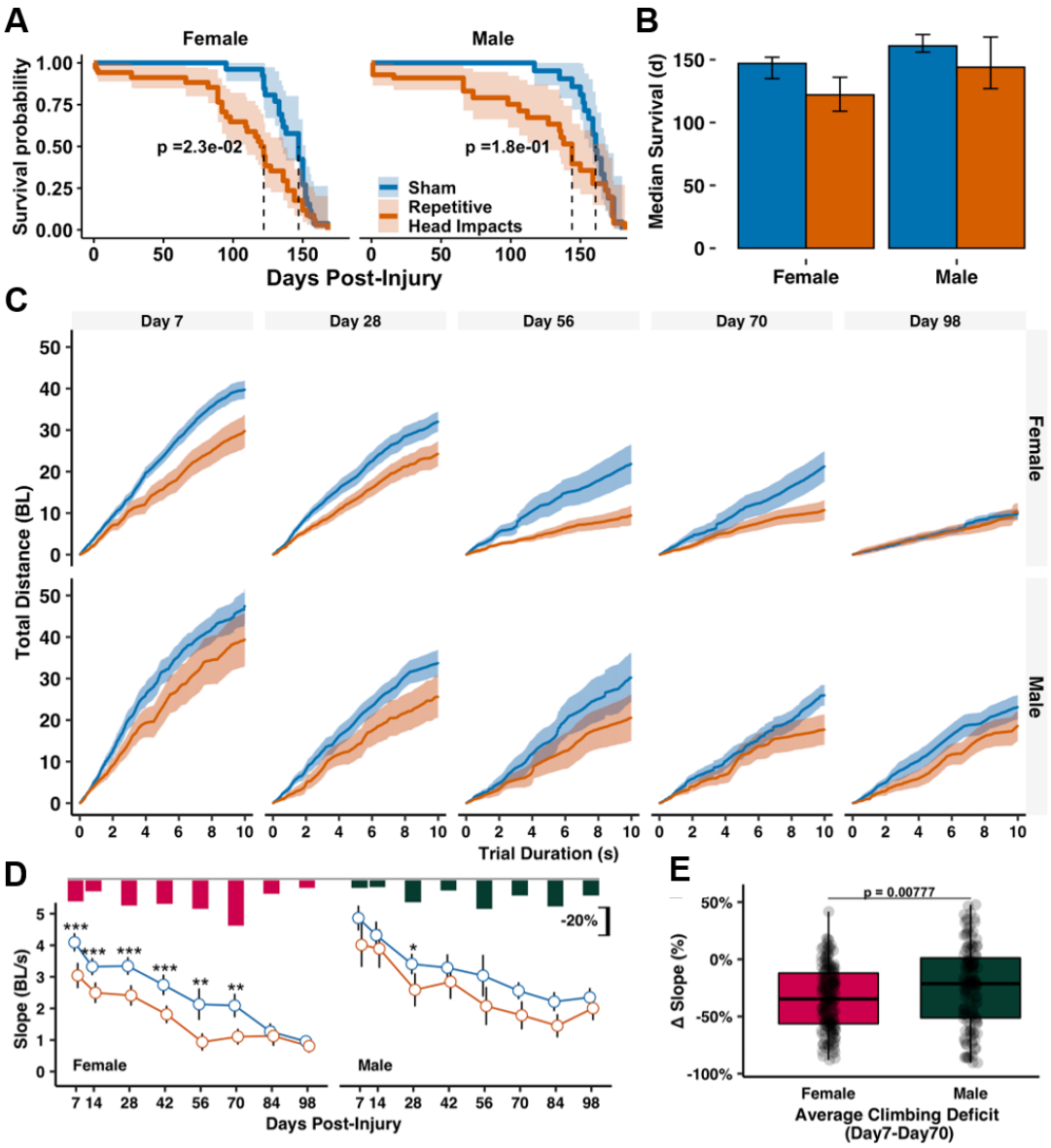

Figure 3-3 **Repetitive head impacts result in a shortened lifespan and long-term behavioral climbing deficits**

(A&B) Repetitive head impacts result in a shortened overall lifespan that is significantly different in injured female flies compared to sham injured flies. Kaplan-Meier p-values were determined using the Mantel-Cox log rank test with Bonferroni correction. **(C-E)** Repetitive head impacts exacerbate age-related climbing deficits that are more pronounced in female flies. Plotted values are median with 95% confidence intervals as shaded regions in **(C)** or error bars in **(D)**. Bar plots in **(D)** indicate median relative decrease in climbing performance between injured and sham groups (**Δ** slope= (Injured Slope-Median Sham Slope)/Median Sham Slope). Boxplots in **(E)** contain individually plotted **Δ** slope values with whiskers corresponding to the maximum 1.5 interquartile range. Mann–Whitney U test between **(D-E)** injured and non-injured groups, with Holm correction:

*p<0.05, **p<0.01, ***p<0.001, n≥22 flies per sex/time/injury group except day 56 (n≥11 flies).

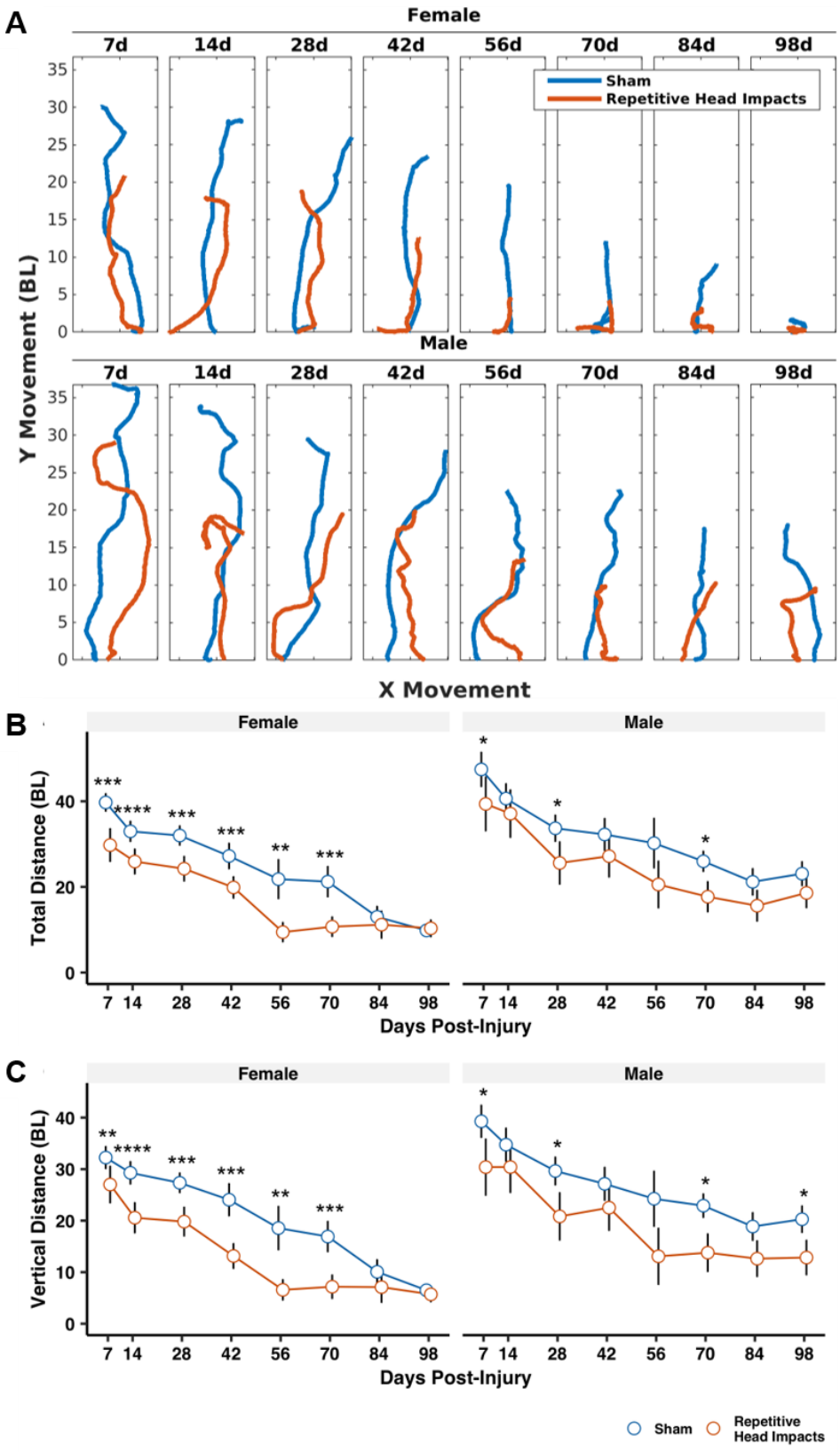

Figure 3-4 **Repetitive head impacts result in chronic behavioral climbing deficits** **(A)** Representative (median) tracing of fly climbing. Repetitive head impacts elicit chronic

climbing deficits that are more pronounced in female flies, seen as a reduction in **(A)** total climbing distance and **(B)** total vertical climbing distance traversed during the climbing assay. Plotted values are median values with 95% confidence intervals as error bars. Mann–Whitney *U* test between injured and non-injured groups, with Holm correction: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n≥22 flies per sex/time/injury group except day 56 n≥11 flies.

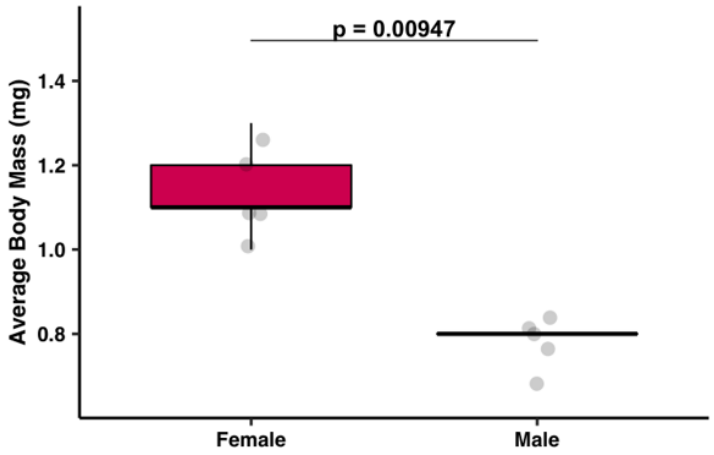

Figure 3-5 **Wet body mass of w^{1118} flies**

disrupts motor functions that lasts over a long period of time and disproportionally impact female flies. To partially account for physical differences in size (mass) that may explain the vulnerability to injury and sexually dimorphic behavioral effects, we measured the mass of female and male flies, which revealed a significant weight advantage (~40%) in favor of female flies (**Figure 3-5**). This difference in mass corroborates existing literature within the field [205, 206].

3.4.2 Discussion

While most affected individuals appear to recover from mild head trauma, the risk for developing long-term deficits in brain function and structure has been documented[16]. One interesting finding is that our data revealed that female flies were preferentially more affected by repetitive head impacts than males. This was seen behaviorally, as a greater reduction in climbing ability relative to sham injured flies, as well as a shortened overall lifespan. The greater persistent functional deficit found in female flies following injury parallels human data from the Transforming Research and Clinical Knowledge in Traumatic Brain Injury (TRACK-TBI) study in which the female sex was associated with decreased six-month functional outcome measured using the Glasgow Outcome Scale-Extended (GOSE)[88]. A recent cross-sectional human study revealed that females were more likely to report a less favorable health-related quality of life (HRQoL) during the chronic stage of TBI (10 years post-injury)[87]. Additional evidence demonstrates that young female athletes may take longer to become symptom free following sports-related concussion[90]. Taken together, this demonstrates sex differences following recovery from TBI and substantiates the importance of using both sexes in preclinical animal models. Interestingly, sex-dependent differences in function

(climbing behavior) in fly head injury models has only been examined once prior, in which no differences between sex were revealed[175]. However, flies were only measured for 72 hours during the post-injury period, and the method for measuring behavior was done using the traditional approach to measure startle-induced climbing performance[139, 194]. To provide greater sensitivity to potential subtle differences in climbing behavior, we used idtracker.ai[203], an automated deep-learning tracking algorithm that enables measurement of individual fly performance. Climbing function was measured until 98 days post-injury, which accounted for most of the overall lifespan for flies reared at 18°C (~150d median survival).

At this moment, the underlying mechanism for the greater vulnerability to mild head trauma for females remains unclear. It may be related to females being more likely to report mild TBI [100]. There are also a number of biological factors that may account for sex-dependent differences as well. Gender differences in head-neck anatomy result in greater head acceleration (angular acceleration and displacement) in females compared to males [207, 208], which may elicit a greater force acted upon the head and thus increase the injury risk found in females[208]. Female flies are known to have a relatively larger body size compared to males [205, 206], which may result in a greater impact force and worse outcomes following injury. Future high-speed camera studies are needed to address this consideration in greater detail.

3.4.3 Methods

Video-Assisted Startle-Induced Climbing Assay

High-speed video recording of head impacts was acquired using a Phantom Miro M310 high-speed camera controlled by a PC with Phantom Camera Control (PCC) software. Sequences recorded at either 3205 or 4106 frames per second were imported into Image J. Body orientation at impact was measured using the Angle tool.

Startle-induced climbing was assessed using a modified negative geotaxis assay. Flies were placed in vials containing 5% agar. Up to four vials were assayed at the same time, using a customized 3-D Polylactic Acid (PLA) printed rig. For each trial, the rig was lightly tamped three times, and fly movement was recorded using a Panasonic HC-V800 digital video recorder at 60 frames per second. Individual vials from each video were cropped, and the first 10s were trimmed in Image J and underwent automated fly behavior tracking using id.tracker.ai. All testing took place between ZT 3 and ZT 8 (ZT, Zeitgeber time; lights are turned on at ZT 0 and turned off at ZT 12) and testing occurred between 20-22˚C under normal lighting conditions.

Automated Fly Behavior Tracking using idtracker.ai

Automated fly behavior was performed using idtracker.ai, a deep-learning algorithm and software that permits individual tracking of flies (**Figure 3-6**). All videos were processed on a Google Cloud Compute Virtual Machine (VM) running a PyTorch Deep Learning VM Instance (c2-deeplearning-pytorch-1-2-cu100-20191005) equipped with an N1-Standard-16 (16 vCPUs, 60 GB memory) machine type, an NVIDIA Tesla T4 GPU, a 100GB SSD disk and Intel Haswell CPU platform. Subsequent data analysis was performed using custom Matlab scripts.

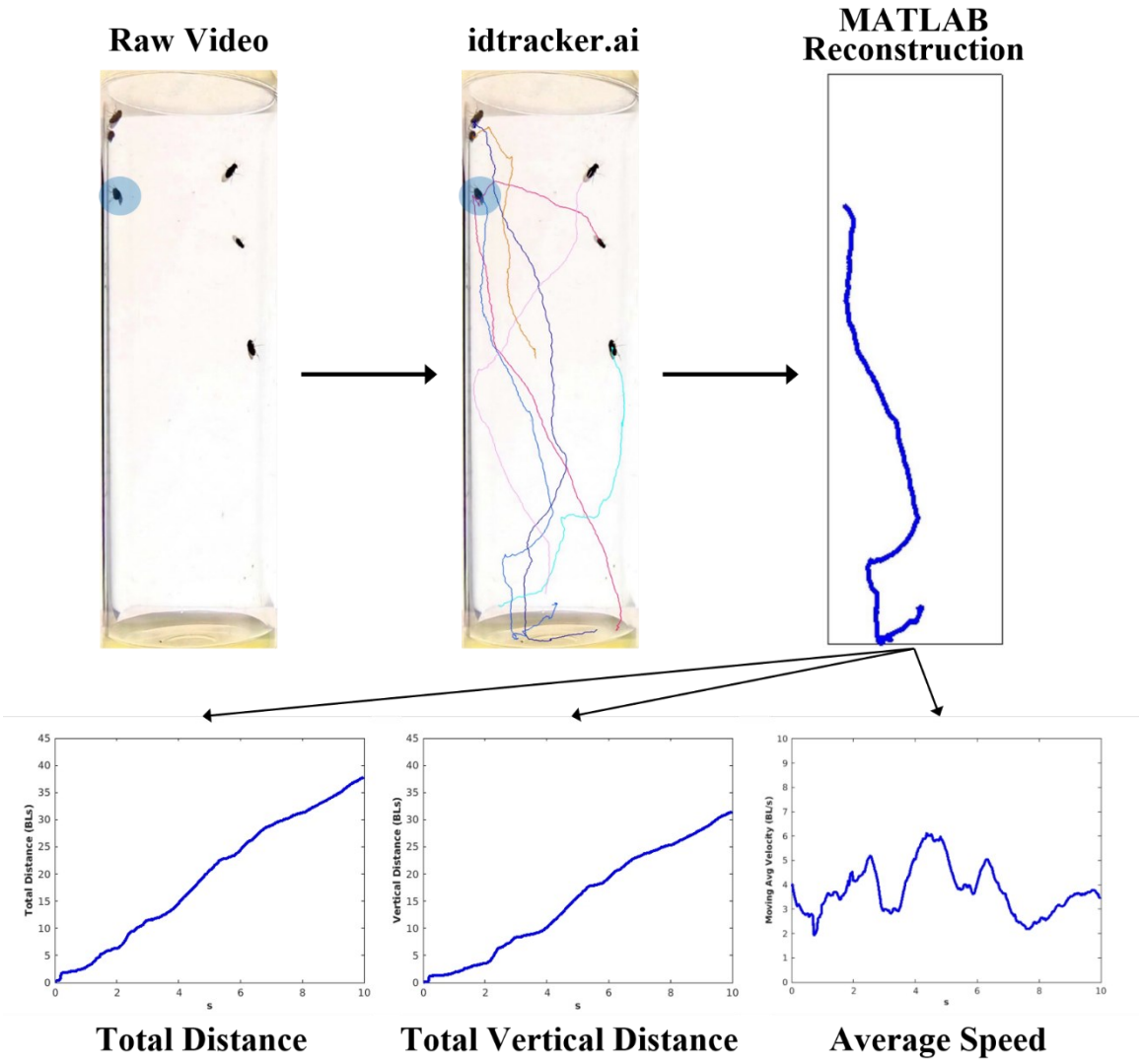

Figure 3-6 **Automated negative geotaxis assay workflow using idtracker.ai**

Raw video is processed using idtracker.ai, which provides continuous measurement of individual flies. Tracking data is imported within MATLAB where it is further processed for individual parameters of movement, including total distance, total vertical distance and average speed during the negative geotaxis assay. Reconstructed movement tracing and movement parameters are shown for the fly shaded in blue.

Chapter 4 Brain Degeneration Following Head Injury

A history of repetitive head trauma is a risk factor for developing several neurodegenerative disorders, including Alzheimer's disease (AD)[9, 23], Parkinson's disease (PD)[13], Amyotrophic lateral sclerosis (ALS)[19] and chronic traumatic encephalopathy (CTE)[15, 16, 22], whose onset appears years following injury exposure. Chronic pathology related to head trauma is often discussed in the context of chronic traumatic encephalopathy and dementia pugilistica (the latter of which heavily overlaps with CTE but is confined to boxers). As such, experimental models have focused on disease proteins implicated in CTE and dementia pugilistica and have begun to investigate their respective mechanisms. Several important factors that influence chronic neurodegeneration following head trauma include, but are not limited to, injury severity, injury frequency, and the age at which trauma is sustained [22]. Unless otherwise noted, this section will focus on neurodegeneration following exposure to mild repetitive head trauma.

4.1 Linking Head Trauma to Neurodegeneration

Within the acute stages of head injury, Osnato and Giliberti made note of small hemorrhages and a vacuolated, "moth-eaten" appearance of the cortex shortly following injury. Chronic neurodegeneration presumably related to head trauma exposure was more comprehensively studied by Brandenburg and Hallervorden in 1954 in the post-mortem brain of a 51-year old former boxer who developed progressive dementia and parkinsonism symptoms[35]. Described initially as a *"synaeretic syndrome"* that involved the aging of *"colloidal"* brain matter into a liquid and solid phase, it was presumed that

this process was associated with normal aging, but that it could be accelerated under pathological states, such as head trauma exposure, resulting in premature aging[35]. It was also during this time that senile plaques and fibril changes similar to those found in Alzheimer's disease were seen as well[35].

In 1958, Neubuerger et al. demonstrated further evidence in the brain of another former boxer with cerebral atrophy that was most pronounced within the frontal lobes[209]. Neubuerger et al. noted that, *"it is conceivable that multiple, partly subliminal concussions, repeated over a period of years, produce an altered state of the cerebral colloids, with inability to revert to the normal equilibrium"*, providing early support to the notion that exposure to *concussive* injuries could produce persistent, irreversible damage.

Shortly thereafter, in 1962, Spillane provided the first radiographic evidence of brain atrophy with corresponding ventricular enlargement and septum pellucidum changes in living patients with a prior history of repetitive head trauma in a longitudinal study of 5 boxers[210]. This was further confirmed in a larger cohort of retired boxers a few years later in 1966, showing both cortical and cerebellar atrophy[211].

In 1973, Corsellis et al. performed a retrospective study on the brains of 15 retired boxers which revealed the presence of neurofibrillary formation detected using silver impregnation, most pronounced within the substantia nigra and medial temporal lobe, and in some cases, accompanied by hippocampal scarring and atrophy [36, 212]. Within the same preserved tissue, years later in 1988, Roberts further characterized the neurofibrillary tangles found in the brains of Corsellis' study and showed that antisera

raised against the paired helical filament(PHF) of tau found in AD also detected the presence of neurofibrillary tangles in dementia pugilistica [37].

Tau, in its normal non-phosphorylated form binds to tubulin and promotes the assembly of microtubules. Upon phosphorylation, tau binds to and inhibits normal tau, thus causing destabilization of the microtubule cytoskeleton[213]. Abnormal aggregations of hyperphosphorylated tau are thought to result in degeneration by blocking critical axonal transport and depriving the neuron of vital intracellular trafficking[213], resulting in eventual cell death. At the time, Robert's described the appearance of neurofibrillary in head trauma patients as being found in the same affected regions to that of AD, including the lateral temporal cortex [37]. These neurofibrillary tangles were found to be ubiquitinated [214]. Given the similarities in pathology seen in both AD and dementia pugilistica, Roberts suggested that tangle formation may represent a final pathophysiological mechanism common to both repetitive head trauma and AD [37]. Additional follow-up by Roberts and others demonstrated involvement of senile plaque-containing β-amyloid deposition using, using what were at the time newer staining reagents, that was in close proximity to neurofibrillary tangles [215, 216]. Further investigation into the localization of neurofibrillary tangles found in dementia pugilistica revealed an inverse topographical arrangement of tangles compared with AD, which involved the superficial cortical involvement of neurofibrillary tangles in dementia pugilistica [217].

In 1999 Geddes et al. provided evidence of early similar neurofibrillary tangle formation without β-amyloid deposition in brains from five young adult males (ages 23-28) subjected to repetitive head trauma (two boxers, a footballer, an intellectually

disabled individual and epileptic who both repeatedly hit their heads)[218]. Neurofibrillary formation was most pronounced within close proximity to small cortical arterial vessels [218]. The absence of β-amyloid deposition found by Geddes et al. may reflect the difference in age of subjects, with their subjects all being young adults within the 20's while prior subjects were much older adults.

Most of the evidence linking repetitive head trauma to neurodegeneration had been largely limited to the study of boxers [219-221]. It was not until 2005, when Omalu et al. documented the first case report linking head trauma from American football players to chronic traumatic encephalopathy[32], which highlighted the importance of concussive and sub-concussive head impacts sustained in contact sports other than boxing to suspected degeneration. Neurodegeneration related to head trauma has since been linked to other contact sports, including soccer[16], wrestling[222], hockey[63], rugby[223], action sports[224] and is likely associated with other sports that involve repetitive sub-concussive blows such as those experienced in gymnastics, horseback riding, and others [225]. Interestingly, at the time of Omalu's 2005 report, the use of "CTE" to refer to neurodegeneration and neuropathology secondary to a past history of head trauma become more commonly used than "dementia pugilistica".

The progression of CTE is an insidious progressive process that develops years following injury exposure. It consists of 4 stages of progression, where the earliest stage consists of perivascular phospho-tau that remains confined within the sulci depths of the frontal cortices, and then gradually spreads to the superficial layers and adjacent lobes, eventually resulting in a reduction of brain weight and cerebral cortical atrophy [63, 226].

In 2016, a consensus panel of neuropathologists supported by the NINDS/NIBIB established the pathognomonic criteria for a diagnosis of CTE, requiring the presence of, "[phosphorylated]-tau aggregates in neurons, astrocytes, and cell processes around small vessels in an irregular pattern at the depths of the cortical sulci"[17]. Additional supportive evidence, although not required, included the preferential involvement of superficial cortical layers (II-III) over deeper layers (IIII-IV), the latter of which is more indicative of AD, as well as other subcortical structure involvement [17]. Other supportive pathology that is not tau-related includes signs of previous traumatic injury, such as septal abnormalities, as well as TDP-43 immunoreactivity [17]. While this was a concerted effort to establish a consensus definition for CTE, it was happened by a small sample size all derived from the same source, and was limited to moderate to late stage CTE severity [17]. A recent study performed a comprehensive quantification of tau pathology in CTE with particular emphasis on cell-specific spatial organization of tau-positive neurofibrillary tangles and thorn-shaped astrocytes. The findings from this study revealed that the pathognomonic perivascular tau-immunoreactive pathology seen in CTE was found in astrocytes which were more prevalent within the sulcal depths of the cortex, whereas neurofibrillary tangles assumed a more uniform distribution, similar to the late stages of AD[227]. Together this evidence provides more quantitative cell-specific CTE pathology and necessitates further effort to provide more objective diagnostic criteria for CTE. This is especially important to provide great insight into how CTE pathology differs from that of AD [23].

CTE has historically been diagnosed post-mortem, however, advancements in neuroimaging have enabled the ability to do so in living patients using PET ligands for

toxic tau and MRI-based measurements. In a case report of a former high school football player, longitudinal volumetric magnetic resonance imaging (MRI) of the brain showed progressive brainstem, diencephalic, and frontal lobe atrophy[228]. A separate study demonstrated [F-18] FDDNP (2-(1-[6-[(2-[fluorine-18]fluoroethyl)(methyl)amino]-2-naphthyl]-ethylidene)malononitrile) PET signals corresponding to neurofibrillary tangles within the brains of retired professional American football players with suspected CTE [229] with a spatial distribution pattern similar to neuropathology from confirmed CTE cases.

In addition to histological tau pathology, changes in plasma tau are also seen following injury[230]. Plasma tau, including plasma phospho-tau (phosphothreonine-231), plasma total tau, and the ratio of the two (plasma phosphor-tau:total tau) are all elevated following acute mild head injury[230]. Increases in plasma tau can persist into the chronic phase of TBI, which may be related to the tauopathy seen in CTE[230]. In addition to the phosphorylated status of tau, its particular stereochemistry may also play a role in tau-mediated pathology. The presence of *cis* conforming phospho-tau is detected early on in the course of injury exposure, in both clinical and preclinical studies [231-233]. A repetitive head injury model revealed that the production of a *cis* phospho-tau, also known as “cistauosis”, which is thought to arise before the onset of tauopathy. Blocking *cis* phospho-tau formation, using an antibody, prevents its formation, and mitigates subsequent trauma-mediated neurodegeneration and behavioral deficits in a weight-drop model of head injury [231-233].

Because the early stages of CTE are limited to abnormal tau accumulation in absence of frank atrophy, it is critically important to be able to diagnose disease early on, prior to subsequent degeneration, which likely provides the greatest potential for therapeutic intervention. Furthermore, understanding the antecedent events that potentiate neurodegeneration, including the cumulative effects of head trauma remain imperative towards understanding the overall pathogenesis of long-term sequelae related to degeneration.

4.2 Cellular and Molecular Pathology of Mild Traumatic Brain Injury

In addition to tau, which is the pathognomonic signature of CTE, several other disease-related proteins related to head trauma neuropathology include TDP and amyloid precursor protein, which are discussed in greater detail below.

Amyloid Precursor Protein

Amyloid precursor protein (APP) is a membrane bound protein whose diverse array of functions include nervous system development and plasticity, such as axonal growth/guidance, synaptogenesis and, dendritic and synaptic plasticity underlying learning and memory[234-236]. Full-length APP consists of a large extracellular domain and smaller cytosolic domain[237] that can undergo sequential proteolytic cleavage by α and γ-secretases to yield a non-amyloidogenic peptide, or undergo cleavage by β- and γ-secretases to yield amyloidogenic β-amyloid peptide that forms senile plaques found in AD[237]. Abnormal accumulation of β-amyloid is thought to begin during the preclinical stages of Alzheimer's disease[238], and continues to do so over time, through the onset of symptoms[239].

Interestingly, APP immunoreactivity, which is a marker of fast axonal transport and surrogate measure of axonal damage, is seen days to months following mild head injury in human patients[135, 240]. This is most commonly found within the fornix, a structure that communicates with the hippocampus and is involved in memory formation and retrieval[240]. APP immunoreactivity is specifically localized within axonal swellings[135]. APP immunoreactivity is also seen in brains from severely injured patients, where it is found in the medial temporal lobes [241]. It can be detected as early as 2h post-injury, and is found in axonal bulbs at 3h [242]. Within *Drosophila*, the APP homologue (APP-like or APPL) is acutely upregulated following a penetrating brain injury and is necessary for post-traumatic axonal regeneration within the fly[243]. Soluble APP (non-amyloidogenic) has been shown to regulate neurogenesis in the adult CNS[244], and promote stem cell neurite outgrowth [245]. Acute delivery of soluble APP in a severe experimental head injury model mitigated cell death and improved behavioral recovery, demonstrating neuroprotective effects of the non-amyloidogenic APP derivative[246].

Several reports have documented β-amyloid-containing plaque-like structures in brains from long-time professional boxers, providing evidence that its existence is seen during the chronic injury phase, and its formation can arise following milder, more repetitive head impacts[247]. Despite some studies reporting an absence of β-amyloid plaques in CTE [218, 248], a strong body of evidence has implicated APP within the axonal injury response [243, 249-251]. The formation of β-amyloid aggregates seen in some cases of head trauma may represent the loss of compensatory function of APP,

whose initial function may serve a neuroprotective role, but eventually results in its abnormal accumulation.

TAR DNA-Binding Protein 43

TAR DNA-binding protein 43 (TDP-43) is an RNA/DNA-binding protein involved in RNA processing and stabilization[252]. Under pathological conditions, TDP-43 is displaced from the nucleus, and can undergo hyperphosphorylation and ubiquitination, and subsequently form cytosolic aggregates[252]. TDP-43 pathology is implicated in amyotrophic lateral sclerosis and frontotemporal lobar degeneration (FTLD)-TDP. A past history of head trauma is associated with the development of ALS, of which ~90% of cases are considered sporadic in nature[253]. During the course of disease, loss of corticospinal and lower motor neurons degenerate. The neuropathology of ALS consists of motor neurons laden with inclusion bodies containing ubiquitin- and TAR DNA-binding protein of 43 kd (TDP-43). In 2010, King et al. reported the first evidence of TDP-43 pathology in dementia pugilistica in the brains of three former boxers who suffered from dementia[254]. The appearance of TDP-43 appeared similar to cases of frontotemporal lobar degeneration (FTLD)-TDP, specifically FTLD-TDP subtype 3, which features abundant intracytoplasmic inclusions, as well as intranuclear inclusions within the frontal and temporal lobes[254]. In 2010, McKee et al. surveyed a larger group of CTE-positive brains (former boxers, American football and hockey players), with 10 of 12 brains showing widespread TDP-43 pathology with glial cytoplasmic inclusions and brainstem nuclei involvement [253]. Interestingly, little to no involvement within the dentate gyrus was seen, and TDP-43-laden neurites and inclusions rarely co-localized with tau.

A greater understanding of the acute biology of tau, APP/β-amyloid, and TDP-43 will provide important clues that speak to why they abnormally aggregate during the chronic phase of injury.

Diagnostic Biomarkers of Head Trauma

In addition to chronic neuropathology associated with the long-term sequelae of repetitive head trauma, a number of fluid-based biomarkers have been studied within the acute injury setting, including S100 calcium-binding protein B (S100B), neuron-specific enolase (NSE), myelin basic protein (MBP), glial fibrillary acid protein (GFAP), and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) [255, 256]. In January of 2021, the FDA provided clearance for the imminent use of the first plasma-based rapid concussion test, Abbott lab's i-STAT Alinity TBI plasma test, taking only 15 minutes to complete and providing high sensitivity (95.8%) and negative predictive value (>99%). The test, which is designed to be used in cases of mild head trauma to aid in the decision to pursue computed tomography (CT) diagnostic imaging, measures UCH-L1 and GFAP within the blood. Further discussion of fluid-based biomarkers, including UCH-L1, GFAP, tau and other important biomarkers of concussion, and their relation to mechanisms of neurodegeneration are discussed below.

Ubiquitin C-Terminal Hydrolase L1 (UCH-L1)

Within the nervous system, synaptic plasticity involves a dynamic process of regulating abundance and location of various proteins involved in learning and memory. This process is mediated in part via ubiquitination, which is responsible for targeting various proteins, including misfolded or aggregated proteins, for degradation[257]. Ubiquitin C-terminal hydrolase L1(UCH-L1) is an incredibly abundant brain-specific

deubiquitinating enzyme (deubiquitinase) that is essential for maintaining axonal integrity[257]. UCH-L1 removes ubiquitin from substrates to maintain a pool of available ubiquitin within the cytosol, which is needed under homeostatic conditions[257]. Mutations and perturbations related to UCH-L1 are associated with neurodegeneration[257]. By virtue of its highly abundant expression that is largely exclusive to the brain, and rather limited expression elsewhere, it has served as a candidate marker for neuronal injury[257]. A discernable significant difference in serum UCH-L1 is detectable within an hour in individuals who sustain either mild or moderate head injury compared to healthy controls [258]. This finding is more prominent in affected individuals who possess detectable lesions on CT [258]. Serum UCH-L1 levels correlate with recovery, but it remains to be seen whether it provides prognostic value for measuring recovery following injury. A recent report revealed low specificity of serum UCH-L1 for mild CT-negative TBI, indicating that its reliance may result in unwarranted exposure to diagnostic imaging [259].

Serum S100B and GFAP

In addition to seeing cerebral atrophy in the brain of a former professional boxer, Neubuerger et al. reported findings of fibrillary gliosis and astrocytic proliferation in their 1958 study [209]. An emerging view of glia's role in health and disease may lend important insight into pathological, diagnostic and prognostic indicators following head trauma. Astrocytes undergo a reactive process in response to CNS injury, including mild cortical injury[260]. This can be seen as an increase in glial fibrillary acidic protein (GFAP) expression, an astrocytic-specific intermediate filament within the acute phase of injury, that eventually subsides [260, 261]. Serum GFAP can be used as a biomarker for

detecting intracranial lesions[262]. The use of GFAP enables detection of head lesions following mild head trauma that are CT-negative but MRI-positive [262]. This ability is most pronounced in plasma GFAP collected at 9-16h post-injury [262]. Acute serum GFAP levels taken on admission following mild head injury are positively-correlated with the presence of detectable lesions seen on CT and/or MRI three months post-injury, as well as those who are unable to return to work and who have suboptimal Glasgow Outcome Scale Extended (GOSE) scores six months following injury[263]. Together, this indicates that serum GFAP holds diagnostic and prognostic value in determining long-term recovery from mild head injury[263]. In addition to GFAP, S100 calcium-binding protein B (S100B) is another glial-derived protein, principally found in astrocytes implicated within head injury. Its presence within the blood is an indication of blood brain barrier disruption, which is thought to occur in response to mechanical head trauma [264]. In a study looking at acute changes in serum following sub-concussive head impacts sustained by American football players during a game, an elevated serum S100B was seen post-game, relative to pre-game, which was positively correlated with the number of repetitive sub-concussive impacts[264]. Unlike that of GFAP, acute serum levels of S100B do not differ between individuals with and without abnormal findings on neuroimaging[263].

Sport-related concussions provide a valuable platform to investigate potential biomarkers given the predictable exposures and risks to potential head trauma. It also provides opportunity for large-scale prospective studies that include adequate controls and adjusted measures (i.e. age, sex, sport, physical abilities, contact and non-contact

controls). It also provides opportunity to measure baseline measurements and the use of repeated-design experiments.

In one particular prospective cohort study involving professional hockey players, investigators measured plasma total tau, serum S-100B, and serum NSE prior to the start of the season, and at various timepoints in affected individuals who sustained a concussion[265]. The study found that only plasma total tau showed a statistically significant elevation, which peaked during the first hour after injury and declined within the subsequent 12h post-injury[265]. Furthermore, plasma total tau levels taken at 1h post-injury positively correlated with the time to recover and return to play, supporting its potential use alongside GFAP as a prognostic indicator[265].

Another prospective study involving NCAA athletes quantified serum UCH-L1, GFAP, tau and neurofilament light chain (NfL) before and after sustaining concussion[266]. All but neurofilament light chain were elevated relative to preseason baseline levels and compared to contact and non-contact athletic controls[266]. Furthermore, UCH-L1, GFAP and tau levels returned to near baseline when measured during the asymptomatic stage of recovery (~7 days post-injury; IQR, 4-11 days post-injury), and even more so when affected individuals returned to play (~21days post-injury; IQR, 17-28 days post-injury) [266]. Although UCH-L1, GFAP and tau were all seen acutely elevated (~3.5h post-injury) following concussion, they each assumed a varied time course to return to baseline levels[266]. GFAP remained elevated 24h post-injury, whereas UCH-L1 returned to baseline levels 24h post-injury[266]. The more gradual return to GFAP baseline levels may be indicative of a persistent activated glial state, which was seen even years after injury in the Neubuerger et al. study from the ‘50s,

which featured affected individuals who likely sustained far greater rounds of repetitive trauma [209]. Paradoxically, tau levels at 24h post-injury were decreased relative to both baseline and control levels [266].

An additional prospective study conducted in professional hockey players demonstrated that elevated serum neurofilament light chain was also correlated with the length of recovery and return to play [267]. Within the same study, the investigators also revealed an acute elevation (1h) in plasma tau, serum S100B, and serum NSE levels seen following a "friendly" match that eventually normalized 12h post-injury relative to baseline levels taken just prior to the game [267]. This latter finding was measured in concussion-free individuals. Although the authors concluded that "physical exercise" may confound findings showing elevated levels of these proteins, they did not acknowledge the effect of subconcussive impacts sustained during contact sports which may elicit an increase in these biomarkers without resulting in concussion or signs/symptoms.

Each of these respective fluid biomarkers deserves further investigation. Differences in their measured levels following injury may represent fundamental aspects of the acute and chronic injury response. Further investigation is required to understand how their expression within the acute setting of injury relates to potential chronic processes.

The precise mechanisms linking acute trauma to chronic neurodegeneration remains an active area of research. Preclinical models are especially important for understanding this process as many of the nuances and challenges that plague epidemiological human studies, such as injury severity/degree of exposure, age and other

unaccounted for potential confounding factors can be easily controlled within the lab setting. While repetitive head trauma is a risk factor for developing neurodegeneration, it remains a challenge to provide a causal link between the mechanisms underlying the early injury response and chronic cellular and behavioral sequelae. A more complete understanding of the brain's response to injury over time will lend new insight towards the development of neuroprotective strategies.

4.3 Measuring Neurodegeneration in *Drosophila*

Frank brain atrophy can be measured in the fly brain by detecting the presence of vacuoles[144, 268]. Traditionally, this is done using 4 or 7μm thick coronal sections from paraffin embedded fly heads[144, 146]. Sections can be stained with hematoxylin and eosin (H&E) and imaged with standard white light microscopy or left unstained and imaged under a standard epifluorescent microscope using blue light which shows the autofluorescence of brain parenchyma[144, 146]. The degree of vacuolization (both frequency and area) increases in aged *Drosophila*, which is further exacerbated in mutants that express proteins implicated in neurodegenerative diseases[144, 146].

4.4 Repetitive Head Impacts Exacerbate Age-Related Brain Degeneration

4.4.1 Results

To examine brain pathology related to repetitive head injury exposure, whole-brain gross neurodegeneration was longitudinally assessed by measuring vacuolization; a commonly used measure of neurodegeneration in *Drosophila*[146, 269]. We adapted two existing strategies to detect whole-brain vacuoles *in vivo* [141, 270]. To measure vacuolization, whole-brains were stained with phalloidin to detect actin-rich neuropil and DAPI to detect nuclei and imaged using two-photon microscopy. Regions devoid of both

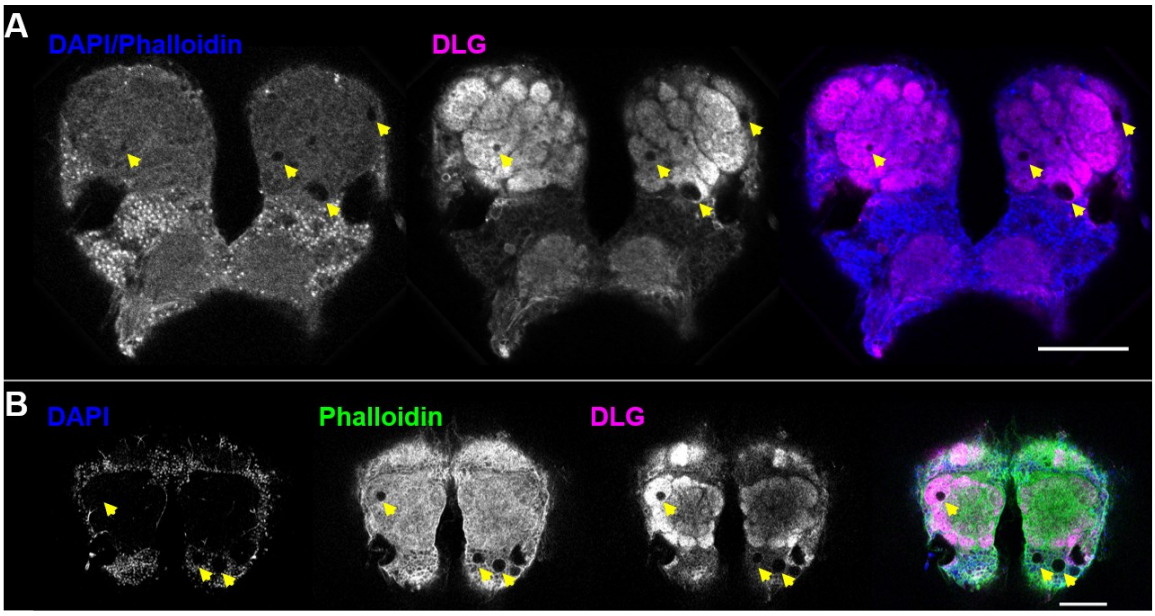

Figure 4-1 **Detecting neurodegeneration in *Drosophila* whole-brain mounts**

(A) Two-Photon Microscopy and **(B)** confocal microscopy of whole-brain mounts stained with DAPI and phalloidin to detect brain parenchyma and discs large 1 (DLG, magenta) to detect post-synaptic neuropil. Yellow arrows designate vacuoles (absence of signal), scale bar = 50μm.

signals were characterized as vacuoles[141]. To confirm that the loss of signal corresponded to a loss of neuronal parenchyma, post-synaptic labeling against Discs large (DLG) was performed to demonstrate regions devoid of DAPI and phalloidin specifically corresponded to loss of synaptic neuropil (**Figure 4-1**).

Brains from flies subjected to repetitive impacts were processed for neurodegeneration acutely (90 minutes) and chronically (5 weeks) post-injury. Although minimal to no vacuolization existed at the early timepoint across sexes and injury groups (**Figure 4-2**), aged brains exhibited a far greater degree of vacuolization, as measured by the number and area of vacuoles per brain (**Figure 4-3**). Both female and male flies subjected to repetitive head impacts resulted in an increase in neurodegeneration five weeks post-injury relative to sham (**Figure 4-3**), seen as an increase in overall number of vacuoles per brain (**Figure 4-3E**) and overall area of vacuoles per brain (**Figure 4-3F**).

4.4.2 Discussion

Our findings that fruit flies subjected to repetitive head impact exposure resulted in chronic frank brain atrophy recapitulates trauma-mediated neurodegeneration seen in both human cases of head trauma [15, 228], as well as in other existing fly models of injury [167, 170, 173]. Together, this provides convincing evidence that mechanical head trauma elicits an injury response that ultimately results in a conserved neuropathological hallmark finding of frank atrophy. This also validates the use of this model to probe potential upstream mechanisms of trauma-mediated neurodegeneration using our tractable fly model.

Katzenberger et al.'s initial fly TBI report demonstrated that flies exhibited brain degeneration 14d post-injury using their "HIT" device, as evidenced by the presence of

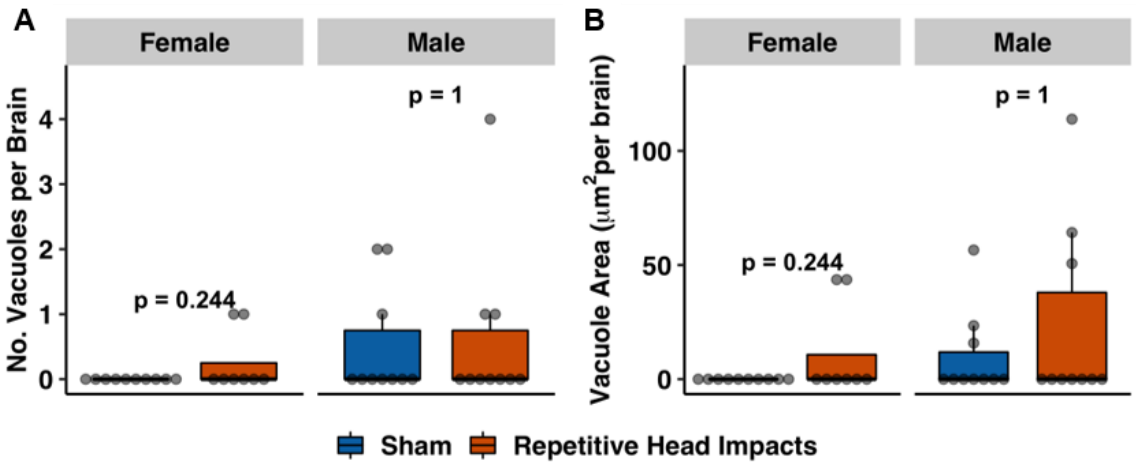

Figure 4-2 **Absence of acute neurodegeneration following repetitive head impacts** Repetitive head impacts elicit no acute (1.5h post-injury) neurodegeneration, neither seen as an **(A)** increased number of vacuoles and **(B)** vacuole area per brain. Boxplots contain individually plotted values with whiskers corresponding to the maximum 1.5 interquartile range. Within sex differences between sham and repetitive head impact conditions were analyzed with the Mann–Whitney *U* test with Bonferroni correction.

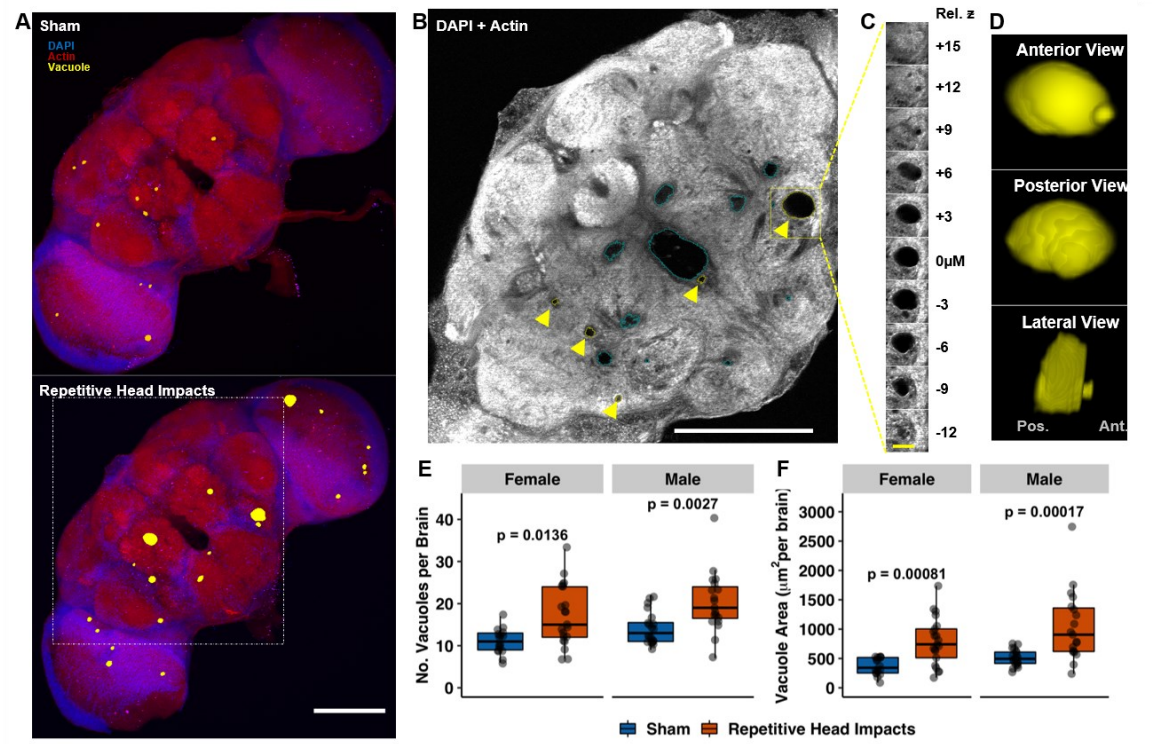

Figure 4-3 **Repetitive head impacts exacerbate age-related neurodegeneration five weeks after injury.**

(A) Representative max projection of whole-brain slices (1µM thick) imaged using two-photon microscopy with brain vacuoles (neurodegeneration) depicted as yellow overlays which correspond to regions devoid of DAPI (blue) and phalloidin (red) signal from a (Top) sham injury brain and (Bottom) repetitively injured brain, captured five weeks following repetitive head impact exposure (2 sessions of 15 impacts). **(B)** Representative two-photon microscopy slice from white square outline of injured brain in **a** where pathological vacuoles of varying size (enclosed within yellow outline with yellow arrow) are found throughout the midbrain while blue outlines designate physiologically normal holes. **(C-D)** Representative vacuole outlined in **(B)** is depicted in **(C)** a series of z-stack images and **(D)** 3-D reconstruction of the vacuole. **(E&F)** Quantification of **(E)** vacuole number and **(F)** vacuole area per brain from sham and repetitively injured brains. Boxplots contain individually plotted values with whiskers corresponding to the maximum 1.5 interquartile range. Within sex differences between sham and repetitive head impact conditions were analyzed with the Mann–Whitney U test with Bonferroni correction. White scale bar=100µM, yellow scale bar=20µM.

vacuoles found in brains from young flies[167]. The degree of degeneration was greater in flies that received a higher number of impacts[167]. Vacuole formation was further exacerbated in older flies receiving the same degree of impact exposure, together suggesting that repetitive impacts have a cumulative effect that is sensitive to age[167]. We have not yet begun to explore the effects of age on outcome measures within our repetitive injury model, but this is certainly feasible using our existing framework.

Using the same injury model developed by Katzenberg et al., with delivery of one impact to elicit a *milder* injury, Hill et al. recapitulated neurodegeneration following injury, seen 28d post-injury[170]. Hill et al. also demonstrated apoptosis, measured in dissociated fly brains using flow cytometry, 7d post-injury, but not 24h post-injury, indicating that programmed cell-death following injury took several days following injury[170]. We have not yet characterized the source of vacuole formation, whether it is a result of cell death, which could be further investigated with further staining for cleaved death caspase 1 (DCP-1, a caspase 3 homologue) and Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) to detect early and late stages of apoptosis, respectively. Alternatively, neuropil loss without cell death may contribute to the formation of vacuoles, which could be further quantified using markers of synaptic neuropil, like those found in **Figure 4-1**. Further characterization of vacuole location and their proximity to specific regions within the brain and their corresponding cell-type would also provide insight into which regions of the fly brain may be more vulnerable to degeneration following injury.

Within our model, which enables a much larger relative number of repetitive head impacts with a similar mortality profile to one “HIT” impact, we also saw no increase in

acute degeneration, measured by vacuole staining 90m post-injury. This may be indicative of the secondary injury response which occurs days to weeks following head trauma exposure. It would be of interest to perform a time-course study to longitudinally measure vacuole formation within our model to determine the earliest onset of neurodegeneration seen following injury exposure. This would be particularly important for determining the potential therapeutic window for interventions targeted at mitigating trauma-mediated neurodegeneration.

Interestingly, Hill et al. also demonstrated cell-specific neurodegeneration by quantifying the absolute cell count of a subpopulation of dopaminergic neurons (protocerebral posterior lateral 1 cluster, PPL1), which decreased 28d post-injury[170]. Furthermore, loss of *highwire (hiw)*, which is protective against transection-mediated axonal degeneration[161], attenuated PPL1 cell loss, but not frank degeneration[170]. Together, this data provided evidence of progressive degeneration following head trauma that is in part mediated by *hiw*. This work also substantiated the importance of an axonal-death signal in the novel context of closed head injury, which had not previously been examined. Using our model, we can apply a similar approach to study proteins of interest that are implicated in axonal injury but have not yet been explored within models of closed-head injury.

Saikumar et al. also documented neurodegeneration within their head-specific model [173]. Flies that sustained a single mild injury incurred degeneration 10d post injury (but not 24h post-injury), while injuries of greater severity elicited acute neurodegeneration 24h post injury[173]. While gross degeneration is seen across all severities of injury, it appears to be accelerated in injuries of greater severity. Again, our

findings demonstrating no acute vacuole formation correspond to the mild injury phenotype seen in Sakumar et al.'s piezoelectric head-specific model. It would be interesting to determine whether delivery of a greater number of individually sub-lethal impacts within our model can accelerate and/or exacerbate vacuole formation. It would also be of particular interest to vary the timing in-between delivery of our repetitive head impacts to determine the effect of recovery on the cumulative effects of injury exposure. This is a critically relevant aspect to human cases of trauma, where the brain is thought to be particularly vulnerable immediately following injury[271].

Perhaps of greatest value in terms of future work is looking at disease proteins of interests that are thought to be involved in chronic degeneration related to head trauma exposure. Using the immense genetic tractability of fruit flies, future work using our model can dissect molecular pathogenesis related to tau signaling. Both acute and chronic injury involves phosphorylated tau signaling. Having established that the fly incurs trauma-mediated neurodegeneration, we can begin to probe various pathways of tau signaling that may be implicated in atrophy. It has not yet been determined whether the fly can recapitulate tau pathology seen in CTE. Within future work, tau pathology can be assessed biochemically and histologically in a *Drosophila* knockin fly line whose endogenous tau orthologue is replaced with a wildtype human tau gene, which preserves similar levels of protein expression and exhibits no baseline behavioral, cellular or molecular phenotype[272]. To assess whether the fly brain recapitulates aspects of CTE, brains from injured flies can be stained for disease-associated phosphorylated tau epitopes (AT270, and AT8)[273]. The presence of neurofibrillary-like tangles[158] can also be quantified. For biochemical studies of tau pathology, Western blot analysis can be

performed on head extracts of injured flies and probed with the same aforementioned tau antibodies. Additional studies could interrogate tau function and assess corresponding changes in neurodegeneration following repetitive head injury. Tau is shown to be implicated in both the acute and chronic injury response, its precise role in neuronal injury remains unclear. *Drosophila* tau-null mutants show no obvious baseline deficits[142]. It would be interesting to assess whether the loss of tau may affect vulnerability to injury. The loss of tau may preclude degeneration and abnormal aggregation seen in CTE and other neurodegenerative diseases alike, however, its role in microtubule stability may provide some level of protection against mechanical insults. Additional related future work can elucidate the role of tau phosphorylation towards neurodegeneration seen following injury. To do so, tau phosphorylation can be experimentally reduced, either through overexpression of the tau phosphatase PPA2[274] (nSyb>PPA2-overexpression), or knockdown (via RNA interference) of the tau kinase GSK3β homologue, *shaggy*[275] (nSyb>shaggy-RNAi), followed by measurement of whole-brain neurodegeneration. PPA2 and GSK3β are the most prevalent phosphatase and kinase, respectively, related to tau phosphorylation[276], and are both implicated in disease. This work demonstrates that the fly brain undergoes neurodegeneration following repetitive exposure to mild head trauma. Future biochemical and histological analyses could further validate the use of *Drosophila* to study precise mechanisms of neurodegeneration related to head trauma exposure.

The study of neurodegeneration secondary to head trauma remains an active area of research, fueled by both scientific curiosity and public mainstream attention, as professional sports and members of the military represent some of the most high-profile

examples of individuals who sustain repetitive head trauma. Earlier detection and more objective diagnostic methods are needed to better understand CTE and pathology related to head trauma. Much in the same way that other neurodegenerative diseases like AD and PD have garnered efforts to diagnose individuals during the earlier stages of disease, CTE and pathology related to head trauma. This is critical especially considering that like PD and AD, symptomatic onset typically appears later in life in each of these aforementioned diseases, yet neurodegeneration is believed to begin years in advance [277, 278]. Interestingly, even in absence of overt symptoms, underlying pathology has been documented radiographically[221] and pathologically[15] in asymptomatic individuals with a past history of repetitive head trauma.

It is also important when assigning risk of neurodegeneration following head trauma to account for differences in the nature of head trauma exposure. Even within contact sports, there is variability in the type of head trauma sustained. Critchley, who provided early behavioral characterization of CTE, made it a point to say that while other sports involve physical contact, one thing that separates boxing (and now combat sports) from other contact sports is the goal of leaving the opponent *"hors de combat"* or out of action[279]. Delivery of head blows is a deliberate aspect of combat sports, where it is an unfortunate and unavoidable risk in other sports that occurs by happenstance. Another factor that is difficult to account for in terms of CTE is related to convenience sampling for collecting CTE specimens. The brain banks used to study CTE should not come only from the families who suspected pathological concern[21].

4.4.3 Methods

Immunohistochemistry and Two-Photon Microscopy

Whole flies were fixed for 3 hours in 4% paraformaldehyde (PFA) in phosphate-buffered saline containing 0.5% Triton-X (PBS-T). The flies were rinsed 4 times for 15 minutes each with PBS-T and brains were subsequently dissected in PBS-T. Brains were permeabilized overnight in 0.5% PBST at 4 °C with nutation then blocked in 5% normal goat serum (NGS) in PBS-T for 90 minutes at room temperature with nutation. Brains were stained with DAPI (1:1000; Invitrogen D1306) (nuclear marker) and Alexa Fluor 594 (AF594) phalloidin (1:400, Invitrogen A12381), which binds to filamentous actin and stains the whole-brain parenchyma). Regions devoid of DAPI and phalloidin are considered vacuoles (Video S4). Stained brains were rinsed 4 × 15 minutes with PBS-T, followed by one wash in PBS for 90 minutes and then mounted on glass slides within SecureSeal Imaging Spacers (Grace Bio-Labs, Bend, Oregon, USA) containing SlowFade Gold Antifade mounting medium (Life Technologies, Carlsbad, CA, USA S36937). Whole-brain imaging was performed using a two-photon FV1000 laser-scanning confocal (Olympus) to acquire 1μM thick sections. Image analysis was performed using ImageJ (Fiji) software.

To demonstrate that vacuoles detected using this strategy correspond to neuropil loss, additional confirmatory staining was performed on a subset of brains for the post-synaptic marker mouse monoclonal α-discs large 1 [1:50; DSHB 4F3] (**Figure 4-1**). The secondary antibody was AF-568 labeled goat anti-mouse Ig (1:400; Invitrogen A11031). Alexa Fluor 488 (AF488) phalloidin (1:400, Invitrogen A12379) replaced the use of AF594 phalloidin. Whole-brain imaging of 1μM thick sections was performed using either a two-photon FV1000 laser-scanning confocal (Olympus) or a Nikon C2 laser-scanning confocal.

Chapter 5 Neuronal Activity in Long-Term Deficits of Repetitive Head Injury

5.1 Aberrant Excitability in Brain Aging and Neurodegeneration

Aberrant neuronal hyperexcitability is implicated in several prominent neurodegenerative disorders, including AD[280-282], ALS[283, 284] and PD[285], as well as ischemia[286]. Hyperexcitability in AD is apparent in the asymptomatic early stages of the disease[287], and may serve as a driving force in the development of pathological hallmarks of AD, including, amyloid beta deposition[288, 289] and tau accumulation[70] that ultimately result in neuronal atrophy. A number of FDA-approved pharmaceutics that antagonize glutamatergic signaling exist to combat neurodegenerative disease, including amantadine (Parkinson's disease)[290, 291], riluzole (ALS)[292], and memantine (AD)[293]. Changes in neuronal excitability are also associated with physiological brain aging[294]. Age-related increases in neuronal activity are thought to serve in a compensatory manner in the aging brain, likely in an attempt to thwart anatomical and physiological changes overtime that would otherwise decline in motor and cognitive functions [295, 296]. In short, to achieve the same level of performance or function, the aging brain may have to utilize a greater degree of activity.

5.2 Hyperexcitability Following Traumatic Brain Injury

Neuronal hyperactivity is also implicated in TBI [48, 297-301]. Rapid neuronal depolarization in response to mechanical stimulation was first demonstrated within the giant axons of the lobster Julian and Goldman in 1962 [302]. This phenomenon was revealed to be a sodium-dependent process, as blocking sodium either via administration of procaine (a pharmacological sodium-channel blocker) or removal of extracellular sodium abolished mechanically-induced depolarization [302]. Subsequent *in vitro* axonal

injury models provided further evidence of cationic influx, which occurs within seconds following injury and initially involves Na^+ influx via mechanosensitive sodium channels, followed by subsequent depolarization and activation of voltage-gated Ca^{2+} channels (VGCC)[51]. Ca^{2+} influx following stretch injury is dependent on Na^+ entry through tetrodotoxin-sensitive Na^+ channels[51] (this model induced 70-75% strain). Within a milder *in vitro* stretch injury model, a single low-strain stretch (<5%) resulted in an increased axonal expression of sodium channels (NaChs) 24 hours after injury[68]. This milder stretch injury also elicited increased intracellular Ca^{2+} that was further exacerbated following a second stretch insult 24h later[68]. Blocking sodium channels using tetrodotoxin prevents exacerbated calcium influx following injury re-exposure[68], together suggesting that cationic influx is proportional to injury exposure and is elicited by sodium-dependent depolarization. Interestingly, intracellular calcium following stretch injury drives protease-mediated degradation of the sodium channel (NaCh) α-subunit[107], whose function is important for channel inactivation[303, 304]; resulting in a disinhibition of the sodium channel and further prompting sodium influx in a feed-forward process[107]. Together, these fundamental experiments highlight the early ionic disturbances seen following mechanical injury.

In response to elevated cationic influx, there is a concomitant increase in the release of excitatory neurotransmitters [48, 297-300], resulting in further downstream excitation. A sudden surge in elevated extracellular glutamate was first documented by Vink et al. in 1989 using the fluid percussion injury model. Using microdialysis with high-performance liquid chromatography, a peak increase of 282% (moderate injury) and 940% (severe) was detected within 10 minutes of injury collected near hippocampal

regions CA2 and CA3[298]. Extracellular glutamate remained elevated for over one hour, indicating sustained release and/or impaired neurotransmitter recycling [298]. Pretreatment with a competitive NMDA antagonist, antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) improved long-term behavioral outcome following moderate injury[298].

A subsequent study using the Feeney weight-drop model to deliver a milder cortical contusion injury recorded a nearly 4-fold increase in extracellular cortical glutamate detected within 10 minutes of injury, which returned to baseline ~1h later[300]. A transient elevation in taurine and aspartate, as well as several metabolites (lactate, inosine and adenosine) was detected as well[300].

In addition to sodium and calcium, extracellular potassium (K^+) is acutely elevated following injury. This was seen as a transient 2-fold increase in extracellular K^+ detected within the area of the hippocampus following mild injury within the fluid percussion injury model[299]. Interestingly, pretreatment with tetrodotoxin or a continuous infusion of kynurenic acid, an N-methyl-D-aspartate (NMDA) glutamate receptor antagonist mitigated extracellular potassium release following injury, indicating that neuronal discharge was in part responsible for the increase in extracellular potassium[299]. In a follow up study, a rapid increase (~80%) in cortical glucose utilization was seen acutely following injury. This trauma-induced hyperpolarization was dependent upon glutamate release, as kynurenic acid dampened the increase in glucose utilization seen following injury [297]. Energy failure is thought to be an underlying component of TBI, where the indiscriminate release of neurotransmitters and persistent

hyperexcitability results in an energy crisis as the brain attempts to restore homeostasis[62]. Furthermore, the decrease of cerebral blood flow seen shortly following injury results in a paradoxical exacerbation of energy crises[62]. Blocking the early surge in neurotransmitter release precludes a hyperglucose state. Clinical MR spectroscopy data from mild head trauma patients revealed an elevated glutamate-glutamine level that is detected within white matter, with no difference seen in gray matter[305]. This was assessed during the subacute injury period (~10days)[305]. Follow up (~4months) later resulted in a return to baseline for white matter, but an elevated glutamate-glutamine level seen in gray matter, indicating that changes in excitability may undergo spatiotemporal dynamics following injury. To date, it has not yet been studied how this process affects long-term mechanisms related to trauma, specifically, neurodegeneration related to injury exposure.

5.3 Activity-Mediated Effects of Repetitive Mild Head Trauma

5.3.1 Results

Elevated brain activity by repetitive mild head impacts and the protective effects by acute suppression of neuronal activity are sex-dependent

We next began to explore the mechanisms underlying the development of long-term behavioral and brain dysfunction after repetitive mild head impacts. Therapeutic hypothermia[46, 47, 306, 307] and medically induced comas[308] are two existing neuroprotective strategies used acutely to treat severe forms of neurotrauma. We sought to understand whether these same neuroprotective mechanisms exist within the fly, specifically examining the potential neuroprotective benefits of hypothermia-induced coma, known as *chill coma* in flies[309], following repetitive mild head impacts. To do

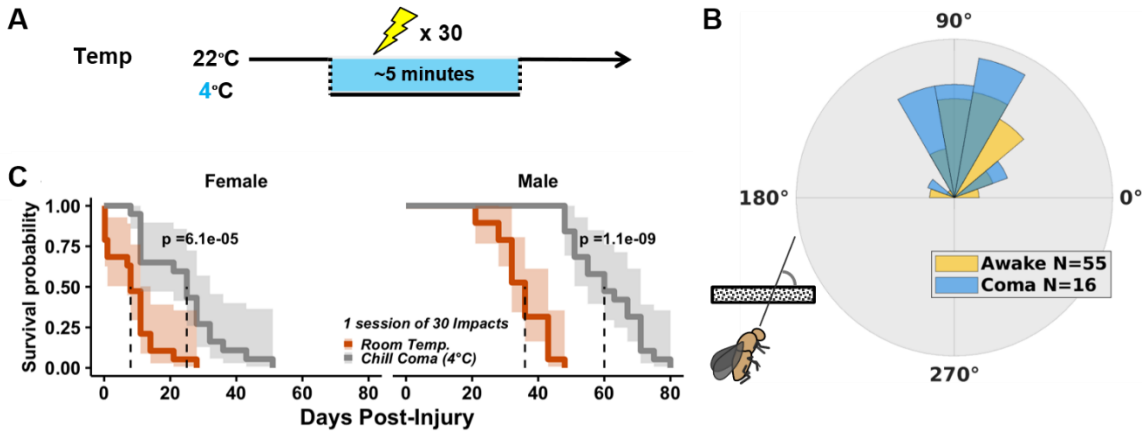

Figure 5-1 **Chill coma is protective against repetitive head trauma**

(A) Chill coma is delivered just prior to injury by transferring flies to a pre-chilled injury vial and then maintained during the injury induction period (~5m), after which flies are returned to ambient temperature. **(B)** Angular histogram of head orientation at impact in awake and chill-coma (temporary flaccid paralysis) flies **(C)** Long-term survival of Oregon R flies subjected to a high number of iterative head impacts (30 impacts). Kaplan-Meier p-values were determined using the Mantel-Cox log rank test with Bonferroni correction.

so, we first subjected adult flies to a high number of iterative, successive injuries (30 impacts) in one session to elicit a high rate of acute mortality (**Figure 5-1)**. We found that this 30-impact regimen caused a ~20% acute female mortality at room temperature (22°C) within 48h after head impacts, which accelerated over subsequent days (**Figure 5-1**). Injured males did not experience any acute mortality, but they did have a shorter overall life expectancy.

We then examined the potential protective effects of chill coma. To induce chill coma, flies were quickly transferred to a pre-chilled 4°C injury vial, resulting in immediate chill coma-induced flaccid paralysis. Flies were then subjected to one session of 30 iterative successive head impacts delivered within a 4°C cold room. After delivery of the injuries, flies were then immediately transferred back to ambient room temperature vials. Strikingly, flies subjected to 30 head impacts when in chill coma did not exhibit any acute mortality after the impacts and the overall survival was also substantially improved in both sexes (**Figure 5-1B**). Since the acceleration and impact speed of flies at both temperature groups are maintained, this result suggests that death induced by 30 impacts was not a result of gross physical damage to the fly head and body, which was also confirmed by gross survey under a dissection microscope. Furthermore, the headfirst orientation at impact is not affected in incapacitated flies subjected to chill coma at the time of injury (**Figure 5-1C**). These results indicate that it is not the motionless incapacitated state of flies, but rather the biological nature of chill coma that contributes to the protection against the 30 head impacts.

As chill coma represents a global inactivation of bodily processes including neuronal activity, we sought to investigate the brain-specific changes in activity related to

repetitive head trauma exposure. To do so, we measured neuronal activity using a non-invasive *in vivo* luciferase-based transcriptional indicator of calcium signaling, CaLexA[176, 310] (**Ca**lcium-dependent nuclear import of **LexA**); a Nuclear factor of activated T-cells (NFAT)-LexA chimera whose transcriptional activity is based on the Ca^{2+}-regulated dephosphorylation and nuclear translocation of NFAT which then drives luciferase expression. Pan-neuronal expression of UAS-CaLexA using the neuronal synaptobrevin-GAL4 (*nSyb-GAL4*) is weak and sparse [178], and in our hands, results in sick and sluggish appearing flies (data not shown). Instead, we measured neuronal activity in an essential midbrain structure, the mushroom body [311], using the robust mushroom body driver to express CaLexA (*OK107>CaLexA-LUC*). The mushroom body contains roughly 2% of the total fly brain neurons [311] and features a dense organization of projection neurons that communicate with various regions of the fly brain, making it a center for high-level integration of functions related to learning/memory [311] and startle-induced climbing [312]. Flies expressing the neuronal activity reporter were subjected to repetitive impacts (1 session of 30 impacts) and collected for fly head lysate preparation 1.5 hours following injury. Bioluminescence (luciferase activity) was measured from lysates. Injured female flies exhibited a 20% increase in measured activity relative to their sham female counterparts, whereas no difference was seen between injured and sham male flies (**Figure 5-2B**). This finding demonstrates that neuronal activity elicited by repetitive head injury within the acute phase of injury is sex-dependent. We then took a tailored approach to conditionally suppress neuronal activity at the time of injury, using the temperature sensitive dynamin homologue mutant, *shibire^{ts1}* (*shi^{ts1}*)[177, 313, 314] expressed pan-neuronally (*nSyb>shi^{ts1}*).

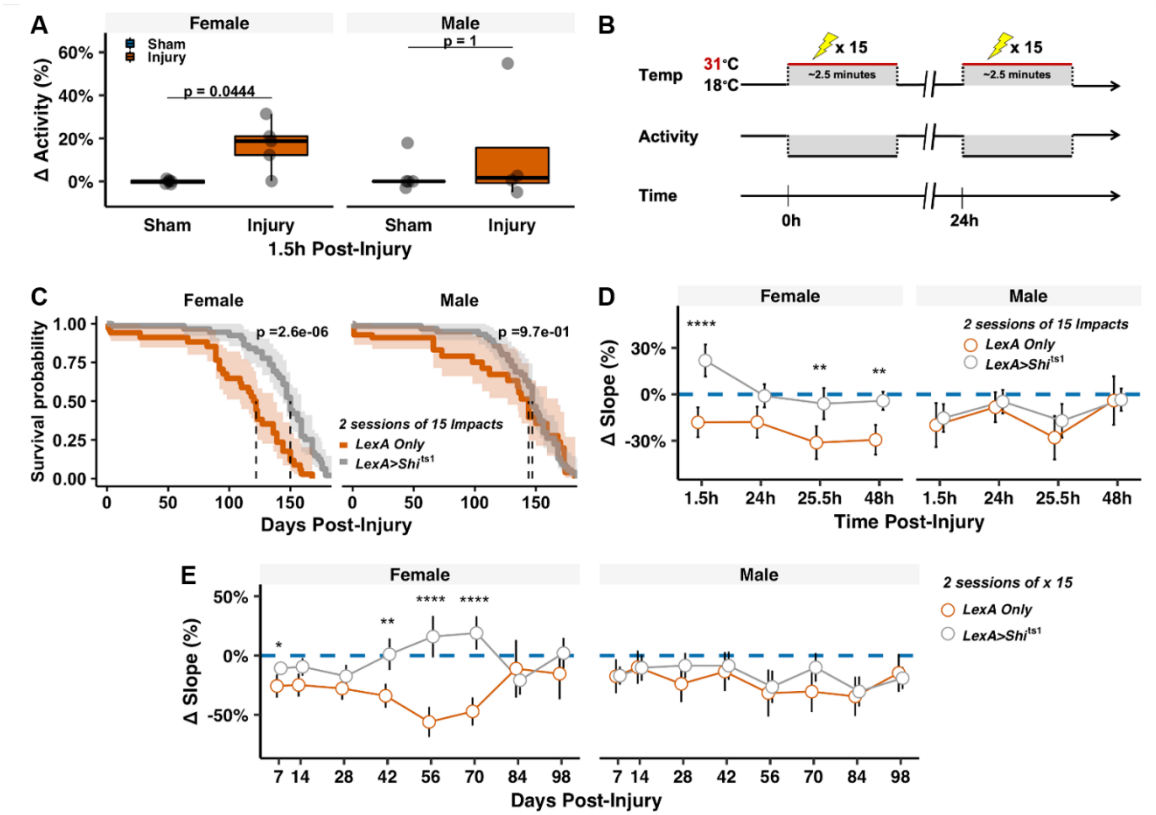

Figure 5-2 **Long-term benefit of suppressing acute injury-induced neuronal activity**

(A) Quantification of neuronal activity using mushroom body in vivo calcium monitoring (OK107>CaLexA-LUC) revealed an acute increase in neuronal activity 1.5h following repetitive head impact exposure in lysates collected from injured female brains, but not males. Within sex differences between injury and sham were analyzed using the Mann-Whitney U test with Bonferroni correction, n=4-7 lysates per group. **Δ** activity= ((Sample luminescence)- (Median Sham Luminescence))/ (Median Sham Luminescence). Boxplots contain individually plotted values with whiskers corresponding to the maximum 1.5 interquartile range. **(B)** Schematic overview of strategy to conditionally silence neuronal activity using the pan-neuronally expressed temperature-sensitive hypomorphic null allele, Shibire ^{ts1} during the repetitive injury induction period. 18°C → 31°C shift conditionally suppresses neuronal activity for 2.5 minutes during delivery of repetitive head impacts in pan-neuronally shi^{ts1}-expressing flies (LexA>shi^{ts1}). **(C)** Kaplan–Meier survival curves showing that blocking neuronal activity at the time of injury protects against shortened lifespan following injury exposure which preferentially benefits female flies. p-values were calculated using the Mantel-Cox log rank test with Bonferroni correction and correspond to within sex differences between injured LexA only and Shibire^{ts1}-containing flies (LexA>shi^{ts1}). **(D&E)** Blocking activity protects against **(D)** acute and **(E)** chronic climbing deficits related to repetitive head impact exposure that are preferentially found in females. Plotted values in **(D&E)** are relative

median slopes (relative to respective uninjured sham) with 95% confidence interval error bars. Differences in climbing behavior were analyzed using the Mann–Whitney U test with Holm correction, between injured LexA Only and Shi^{ts1}-containing flies (LexA>shi^{ts1}). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

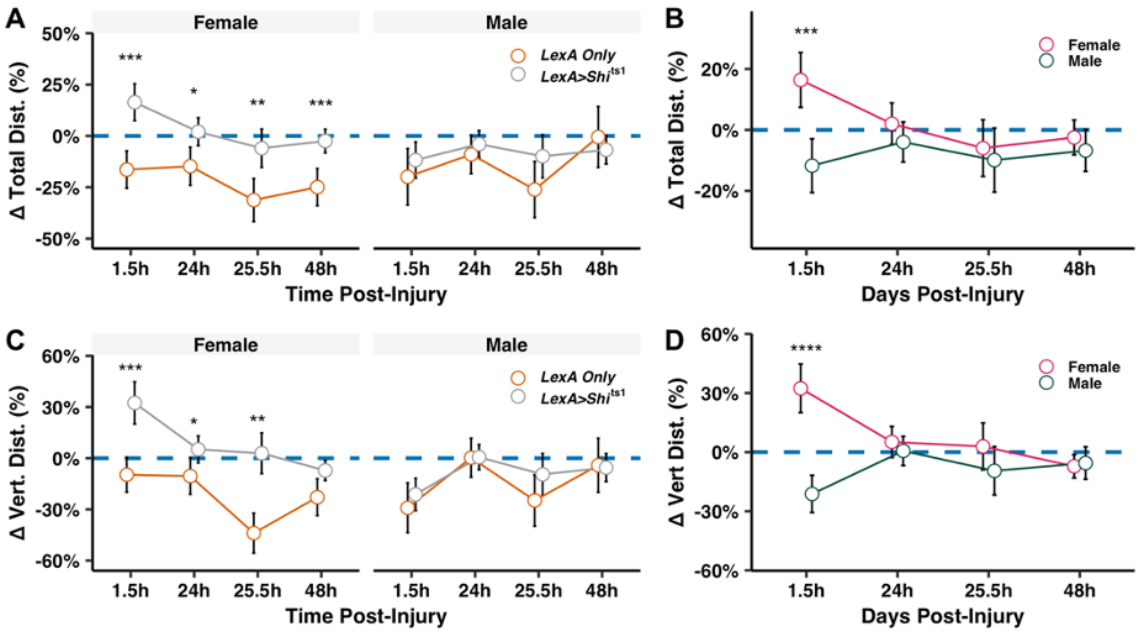

Figure 5-3 **Acute effect of suppressing injury-induced neuronal activity**

Blocking activity protects against acute climbing deficits in female flies, specifically **(A&B)** relative total distance and **(C&D)** relative vertical distance traversed. Plotted values are relative median values (compared to respective genotype sham) with 95% confidence interval error bars. Differences in relative climbing behavior were analyzed using the Mann–Whitney *U* test with Holm correction, between injured *LexA Only* and Shi^{ts1}-containing flies. *p<0.05, **p<0.01, ***p<0.001.

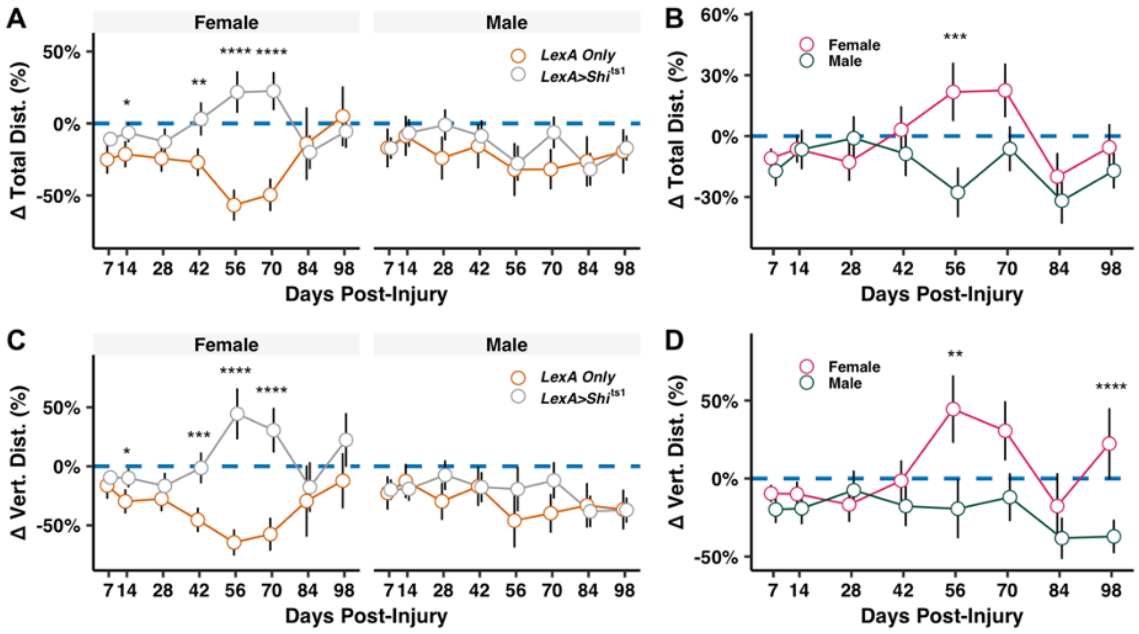

Figure 5-4 **Chronic effect of suppressing injury-induced neuronal activity**

Blocking activity protects against chronic climbing deficits in female flies, specifically **(A&B)** relative total distance and **(C&D)** relative vertical distance traversed. Plotted values are relative median values (compared to respective genotype sham) with 95% confidence interval error bars. Differences in relative climbing behavior were analyzed using the Mann–Whitney *U* test with Holm correction, between injured *LexA Only* and *Shi*^{ts1}-containing flies. *p<0.05, **p<0.01, ***p<0.001.

Pan-neuronal expression of shi^{ts1} enables conditional suppression of synaptic activity by halting synaptic vesicle release, via interruption of the readily releasable pool of synaptic vesicles, thereby inducing a reversible state of flaccid paralysis **(Video S5)** [177, 313, 314]. Conditional suppression of synaptic activity occurs rapidly when exposed to the non-permissive temperature threshold of 30°C, which is initially achieved through transfer to a pre-warmed vial and maintained via exogenous heat delivered by a heat lamp.

Control flies expressing either pan-neuronal *LexA* only (*nSyb-LexA)* or pan-neuronal *shibire^{ts1} (nSyb>shi^{ts})* flies were injured during two sessions of 15 impacts delivered 24h apart. During each injury session, flies were subjected to the non-permissive temperature for only the extent of the injury induction period (~2.5 minutes long) (**Figure 5-2C**). Suppression of neuronal activity at the time of injury nearly abolished the acute mortality found in both injured *nSyb-LexA* flies and restored normal female lifespan (**Figure 5-2D** & **Figure 5-3**). Suppression of neuronal activity at the time of injury restored acute female climbing performance relative to *nSyb-LexA* injured performance, whereas injured *nSyb>shi^{ts1}* males showed no significant improvement compared to injured *nSyb-LexA* (**Figure 5-2E** & **Figure 5-4**). Moreover, long-term climbing performance of injured *nSyb>shi^{ts1}* females better resembled their sham *nSyb>shi^{ts1}* counterparts compared to injured *nSyb-LexA* flies (Figure 5F). This finding was most prominent during Days 42-70 post-injury. Interestingly, the long-term relative performance of injured male *nSyb>shi^{ts1}* flies appeared no different than injured *nSyb-LexA* flies, which may be due to the milder initial climbing deficit seen in injured males (**Figure 3-3**).

We next examined if blocking acute activity at the time of injury is protective against long-term neurodegeneration following repetitive head impacts. Similarly, repetitive head impacts were delivered to *nSyb>shi^{ts1}* flies at the non-permissive temperature and injured *nSyb>shi^{ts1}* flies were processed using the same methods shown in **Figure 4-1** for vacuole assessment following the aforementioned acute-silencing regiment. Acute silencing of activity at the time of injury was found to substantially reduce the number and size of vacuoles in female brains, while no obvious reduction in injury-induced vacuole formation was found in injured male brains from *nSyb>shi^{ts1}* compared to *nSyb-LexA* (**Figure 5-5**). Together with the mortality and climbing data, this data suggests that acute aberrant neuronal activity elicited by repetitive head trauma may potentiate long-lasting dysfunction that preferentially affects female flies. In accordance with this finding, blocking acute activity through the expression of the dominant negative transgene, *shibire^{ts1}* provides long-term neuroprotection against repetitive injuries that preferentially benefits females.

5.3.2 Discussion

Here in this study, we were able to measure acute neuronal activity *in vivo* following exposure to repetitive mild head trauma, which revealed elevated activity preferentially within female flies. We then targeted neuronal activity, specifically neurotransmitter release, at the time of injury using the transgenic temperature-sensitive *shibire^{ts1}* mutant fly line as a tool to conditionally suppress whole-brain neuronal activity and monitored mortality, lifelong functional deficits and brain degeneration. Our data provides the first evidence that silencing neuronal activity at the time of injury was able to largely mitigate the long-term detrimental effects of mild repetitive brain trauma in

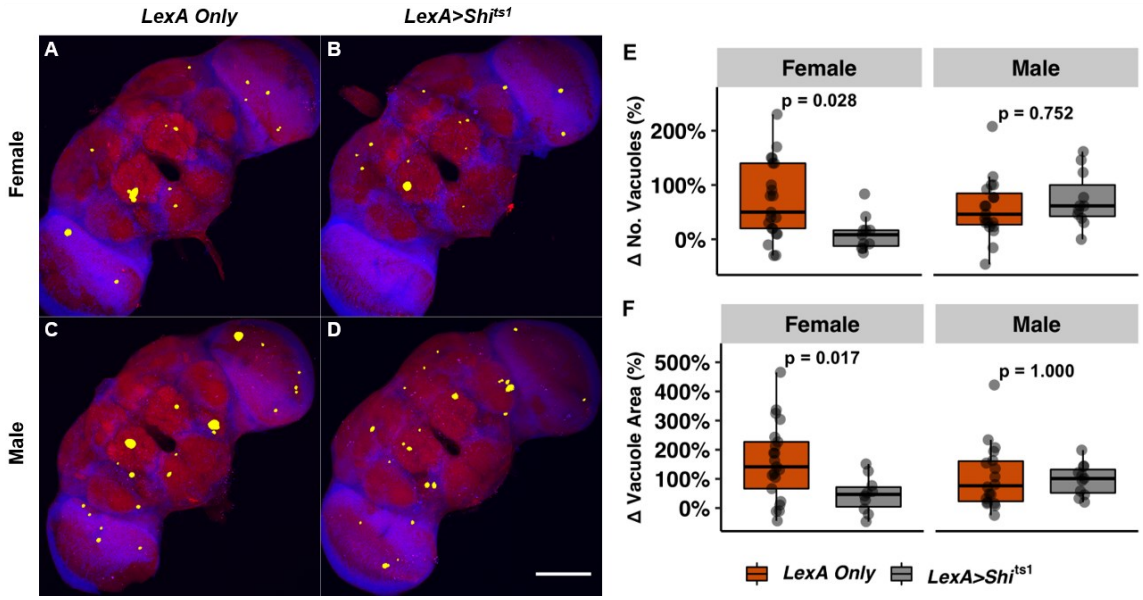

Figure 5-5 **Acute suppression of neuronal activity mitigates injury-induced chronic neurodegeneration in a sex-dependent manner**

(A-D) Representative max projection of whole-brain slices (1µM thick) imaged using two-photon microscopy with brain vacuoles (neurodegeneration) depicted as yellow overlays which correspond to regions devoid of DAPI (blue) and phalloidin (red) signal from injured **(A)** female nSyb-LexA brain, **(B)** female nSyb>Shi^{ts1} brain, **(C)** male nSyb-LexA brain, and **(D)** male nSyb>Shi^{ts1} brain. White scale bar=100µM. **(E&F)** Boxplot of relative quantification of **(E)** vacuole number and **(F)** vacuole area (relative to respective uninjured sham) with whiskers corresponding to the maximum 1.5 interquartile range. Within sex differences between injured nSyb-LexA (LexA Only) and nSyb>Shi^{ts1} (LexA>Shi^{ts1}) were analyzed with the Mann–Whitney U test with Bonferroni correction. White scale bar=100µM.

female flies. Together, this supports a mechanism involving synaptic plasticity and neuronal excitability related to trauma-induced degeneration. Future work using our system can assess the potential benefit of silencing activity at various timepoints following repetitive injuries to determine the long-term therapeutic window of active rest, which is a commonly prescribed treatment following head injury. Additional work will also longitudinally examine how early repetitive head impact exposure affects neuronal excitability later in life, especially given that increased neuronal excitability is associated with brain aging[294, 315], for which injury exposure may accelerate.

Although blocking activity seemed to preferentially benefit injured females to a greater extent than injured males, this may be related to the timing and duration of the activity suppression. Also, it is more difficult to discriminate potential therapeutic effects in males given that they show a much milder phenotype. Within our current paradigm, neuronal activity suppression was limited to the time of the injury exposure (~2.5 minutes), after which flies were quickly returned to the permissive temperature for the remainder of their lives. Existing neuroprotective strategies like therapeutic hypothermia[46, 47, 306, 307] and medically induced comas[308] may exert their beneficial effects through mitigation of aberrant neuronal activity that persists following injury exposure. Metazoan species, including *Drosophila*, enter into a reversible state of hypothermia-induced coma (a flaccid paralysis) known as chill coma when exposed to sufficiently low enough temperatures; presumably an evolutionarily conserved mechanism to thwart the negative effects of extreme temperature exposure on neurophysiological processes[309]. Chill coma provided a neuroprotective effect in flies against mortality following head injury exposure.

Additional strategies could be used within our current system that provide chronic suppression of changes in hyperexcitability using glial cells involved in excitatory neurotransmitter recycling by specifically targeting excitatory neurotransmitter transporters[316-318]. The inability to properly regulate excitability may be related to the loss of excitatory neurotransmitter buffering capacity in glial cells, primarily astrocytes, which have been shown to be perturbed in severe isolated scenarios of head trauma[319, 320]. While this remains to be seen in repetitive mild head trauma exposure, decreased buffering capacity may potentiate chronic changes in hyperexcitability that then result in eventual neuronal degeneration. Interestingly, the early stages of Alzheimer's disease may involve abnormal changes in glutamatergic buffering, which results in persistent excessive neuronal activation, excitotoxicity, and eventual cell death[321, 322]. Activity-dependent changes related to repetitive exposure to trauma may represent an early mechanism that potentiates AD and CTE pathology, as well as other neurodegenerative diseases.

Following injury, aberrant localization of tau is not limited to the axon. Following experimental axonal injury in an *in vitro* cell culture stretch model that delivers either a single high (20%) or repetitive low (2%) strain (with a strain rate of 1,000%/s) to primary hippocampal neurons resulted in a gradual mislocalization of phosphorylated tau to dendritic spines[108]. This is seen as early as 1h post-stretch, resulting in subsequent synaptic dysfunction 24h post-stretch [108]. Interestingly, functional deficits are blocked in cultured cells from tau-KO deficient mice, and inhibiting tau phosphorylation either through genetically modified tau-constructs or pharmacologically also mitigates

functional deficits, suggesting that phosphorylated tau is critical for functional deficits following stretch injury [108].

Removal of tau, whose increase is seen acutely following injury[108, 230] and is chronically deposited within brains of CTE patients[15, 32], is neuroprotective within models of excitotoxicity, including ischemic infarct[323] and epilepsy[70, 324], and preclinical AD models[71]. Potentiating NMDA-mediated neuronal activity rapidly increases extracellular tau [325]. Hyperexcitability as a driver of pathology has also been implicated in ALS, where cortical hyperexcitability can drive TDP-43 pathology[326]. Tau and TDP-43-mediated changes in excitability, and vice versa, following repetitive injury may represent a phenomenon linking acute mechanisms to chronic neurodegeneration.

At the systems level, region-specific changes in brain excitability relative to the site of injury occur following mild head injury. Using a large-scale multielectrode measurement system, Ding et al. demonstrated suppression of activity within a cortical region (primary somatosensory cortex) distal to the site of injury (motor cortex) during the acute injury phase in a focal cortical compression rodent injury model[327]. This was followed by an eventual period of hyperactivation 2h post-injury. A separate study examined hippocampal function following mild traumatic brain injury and revealed impaired gating of cortical input by the dentate gyrus, resulting in increased CA3 excitability within the subacute phase of injury [301].

Future experiments using our novel injury model can investigate the relationship between tau dysfunction and corresponding changes in neuronal excitability following injury. In particular, we can examine whether tau knock-out(KO) mutant flies[142] are

protective against injury-induced hyperexcitability. We can then examine how hyperexcitability regulates tau phosphorylation following injury and how these processes contribute towards long-term dysfunction and neurodegeneration.

5.3.3 Methods

Neuronal Activity Luciferase Reporter

To measure luciferase activity, representative groups of 2-4 *OK107>CaLexA-LUC* sham and injured flies were quickly immobilized using CO_2, decapitated and heads collected in 100µL 1x Promega Glo Lysis Buffer (Promega #E2661), and 4-7 independent samples were collected for each treatment group. Fly heads were homogenized using an automated pestle motor mixer (Argos Cat. No. A0001) for 1 minute at room temperature, then incubated at room temperature for 1h, centrifuged for 10 min at 13.5k RPM. The supernatant was transferred to a new tube. For luciferase assays, 60 µL of each sample was mixed 1:1 with Steady-Glo Luciferase system (Promega #E2510) following the manufacturer's protocols, and then added to a white-walled 96-well plate at room temperature and incubated in the dark for 10 minutes. Luminescence was measured using a BioTek Synergy HTX Multi-Mode Reader.

Chapter 6 Future Directions and Conclusions

6.1 Sexually Dimorphic Effects

At this moment, the underlying mechanism for the greater vulnerability to mild head trauma for females remains unclear. Female flies are known to have a relatively larger body size compared to males [205, 206], which could affect the force sustained at impact within our system. Future work will more comprehensively study differences in impact force between male and females. Even if impact forces differ between male and females as a result of their differences in masses, we can use different counterweights for each sex to either accelerate males to elicit a comparable impact force, or slow down females to dampen the impact force, thus equilibrating the force sustained at impact between both sexes. Additionally, genetic strategies can be used to equalize body size between male and female flies by masculinizing female flies through modulation of sex-lethal signaling, which restricts tissue growth to that of the male size [328].

It is also plausible that male and female brains and their circuits may have different thresholds and/or respond differentially to physical insults. For example, male brains may tolerate a greater degree of physical impact before generating the hyperexcitatory response needed for brain deficits. Sexually dimorphic vulnerability to injury may in part be attributed to neuroanatomical differences, which have been shown to exist *in vitro*[89]. In response to mechanical trauma, sudden stretching of the axon can increase membrane permeability, resulting in the temporary loss of ionic homeostasis, an influx of cations and excessive excitatory neurotransmitter release, and subsequent excitotoxicity, metabolic dysfunction and cell death[24, 48-51]. Cultured neurons from female human and murine sources feature axons with fewer microtubules per axon,

resulting in a thinner axon diameter compared to males[89], making it potentially more vulnerable to trauma. Moreover, in response to injury using a cell stretch injury model, female axons exhibited a greater acute influx of calcium compared to males[89], which may explain the finding of more persistent deficits in hippocampal synaptic plasticity following mild TBI, seen in preclinical data from female rats[329]. Our study provides corresponding *in vivo* data demonstrating that repetitive injury elicits acute neuronal activity within the injured female fly brain. Although this surge of excitation is thought to be short-lived, on the scale of minutes to hours[46, 47], this action may potentiate long-lasting effects on synaptic plasticity and neuronal function and affect the brain's vulnerability to further injury, especially following repetitive exposures, however this theory remains untested in part to the lack of animal models for long-term examination. Additional sexually dimorphic factors that may account for differences in vulnerability to injury may involve immune-based mechanisms as well, which have been shown to exist [95]. Further study using our tractable model will provide additional insight into activity-mediated and immunological-based mechanisms of injury and their role in subsequent degeneration.

6.2 Glial Contributions

As components of the active synapse, astrocytes regulate glutamate signaling through reuptake, a process responsive to elevated synaptic glutamate that occurs on the order of minutes[318, 330-333]. This is performed by the glutamate transporter-1 (GLT-1), also known as the Excitatory Amino Acid Transporter-2 (EAAT-2). Impaired reuptake function in astrocytes can exacerbate the injury response[334] and is perturbed in brains from neurotrauma[319, 320, 335] and AD patients[322, 336]. Loss of EAAT2

function in AD is associated with corresponding increases in excitotoxicity and neurodegeneration[336], and increasing astrocytic glutamate transporter function mitigates tau pathology and rescues symptoms in preclinical AD models[317, 321, 337]. Together, this suggests that glial regulators of excitability may underlie the development of neurodegeneration in AD and CTE.

6.3 Mitochondrial Function Following Injury

Excitotoxicity is an early mechanism of axonal injury that is thought to drive mitochondrial dysfunction and intracellular free radical generation [338-340], however the role of these processes towards long-term dysfunction following mild head injury remains unclear. Mitochondria are highly dynamic cellular organelles that respond to acute injury [271, 341], and are implicated in neurodegenerative diseases. Frequent and repetitive exposure to head trauma may promote cumulative mitochondrial dysfunction and persistent elevated reactive oxygen species that drives long-term neurometabolic impairment and eventual degeneration. Our injury model, together with the tractability of *Drosophila* would enable precise investigation into mitochondrial mechanisms implicated in relaying the injury response.

6.4 CNS Remodeling Following Repetitive Mild Head Trauma

In severe forms of head trauma, axonal fibers can undergo a process known as diffuse axonal injury (DAI)[44, 60, 68, 342-346]. DAI includes a host of widespread axonal pathologies following TBI, such as severing or stretching of the axon immediately following injury[347], as well as subsequent processes that result in impaired axonal transport, swelling[61], and retraction[44]. Although severe DAI can result in complete severance of the axon, much less is known about axonal integrity in response to milder

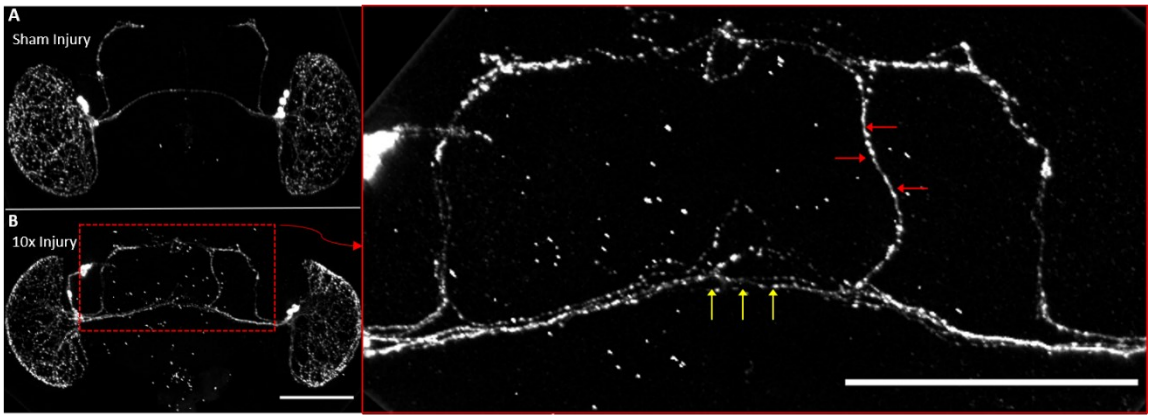

Figure 6-1 **Axonal outgrowth in response to mild repetitive head trauma**
Whole-brain mounts stained for pigment dispersing factor from **(A)** Sham injured and **(B)** 10x-injured flies showing aberrant axonal outgrowth of the posterior optic tract containing pigment dispersing factor neurons 24h following injury. Red arrows indicate aberrant outgrowth, yellow arrows indicate defasciculation of the posterior optic tract. Scale bar= 100µm.

injury, especially repetitive mild trauma[14]. Additional cellular responses of the axon to TBI include the defasciculation of axon bundles, increased local sprouting and outgrowth from damage axons[250, 348-350], all of which are noted in our novel injury model (***Figure 6-1***). Axonal sprouting and outgrowth are usually limited to the peripheral nervous system as a number of inhibitory cues exist within the central nervous system impede axonal regeneration, including Rho1/Rho-associated coiled-coil containing protein kinase (ROCK) signaling[351, 352]. Inhibition of Rho1/ROCK signaling promotes axonal regeneration following axonal injury[353, 354]. Other members of the Rho GTPase family are essential regulators of growth cone motility, axonal migration and spine dynamics via their direct effects on the actin cytoskeleton, and have antagonizing actions on Rho/ROCK signaling[355], which may be important for mediating remodeling following injury. Several genetic screens using axotomy models of injury have identified critical axonal injury sensing pathways that are involved in whether axons regenerate or degenerate, including dual leucine zipper kinase (DLK)[161, 356-360]. DLK is a major upstream activator of JNK signaling following axotomy[359]. Its role in axonal injury without physical breaking is unknown. Because mild and subconcussive forms of head injury are thought to preserve physical axon integrity it is imperative to have a representative model of injury that accurately recapitulates axonal injury in absence of physical tearing or severing[14]. Further application of our model can provide insight into the changes in axonal morphology in response to trauma that may underlie cellular reorganization in response to trauma that is responsible for long-term deficits.

6.5 Mitigating Risks Associated with Head Trauma

Mainstream attention to mild head trauma is largely attributed to the popularity of American football. It was only in 2005, when the first case of CTE was found in the brain of a retired NFL player[32]. During that same year, the "Mild TBI" committee representing the National Football League published a report justifying same-day return to play guidelines for concussed individuals, claiming that those who, "[returned] to play within the same game faced no additional risk of secondary injury either in the game or during the season"[361]. While it remains to be seen why some individuals experience greater repercussions following mild head trauma than others, it is undeniably certain that a central focus to combat complications related to head trauma should be on limiting exposure and risk to trauma. In the case of American college football, the incidence of concussion is disproportionally greater in the preseason, where nearly 50% of the overall concussion incidence is sustained during this time, which is only 20% of the overall season[362]. During the preseason, players sustain the greatest number of impacts, nearly twice as frequently per practice, with more practices conducted within a given time frame[362].

6.6 Conclusions

An emerging body of evidence supports the view that people with a past history of repetitive mild head trauma are at increased risk for developing cognitive and motor impairment and brain degeneration later in life[15, 16]. However, it remains unclear how the early exposure to mild head trauma leads to the development of long-term neurological dysfunction. This dissertation focused on our recently developed *Drosophila* head injury system to investigate the long-term detrimental effects of repetitive mild head trauma and their underlying mechanisms.

Our data show that repetitive mild physical impacts delivered to the adult fly head result in acute concussion-like responses and impairments in startle-induced climbing behavior. Importantly, when monitored over an extended period of time (throughout the entire lifespan), the early repetitive head trauma was found to shorten lifespan and elicit a decline in behavior, particularly in female flies. Whole-brain imaging also revealed increased brain degeneration in flies that received early repetitive head trauma. Finally, we provided evidence that neuronal activity plays a critical role in mild head trauma-induced long-term deficits in behavior and brain pathology within female flies. These findings not only support the use of *Drosophila* as a model system to study the long-term brain degeneration and dysfunction developed by environmental insults, but also provide crucial insight into the underlying mechanisms involving excitatory neuronal activity in the development of brain deficits induced by head trauma. The finding that silencing neuronal activity during head impacts can effectively mitigate the development of long-term deficits in female flies suggests it as a potential therapeutic target for treating chronic sequelae of repetitive mild head trauma exposure.

The basis of this work calls to question whether treating *milder,* potentially unassuming injuries, should receive greater attention as a means to thwart long-term complications. Our novel injury model, together with our non-invasive automated behavioral testing paradigm and method for assessing neurodegeneration, are amenable to future high-throughput genetic screening studies[162]. Understanding the natural course and lifelong implications of head trauma exposure is critically important for developing long-term neuroprotective strategies. We will continue to use our tractable fly model to investigate genetic and environmental factors that affect neurodegeneration

secondary to repetitive head trauma exposure and provide insight into the fundamental mechanisms that lead to the progression of lifelong neurological dysfunction.

Figure Legends for Supplementary Videos 1-5:

Supplementary Video 1 (VideoS1)

Demonstration of headfirst impacts using our novel *Drosophila* model Multiple unrestrained awake flies contained within a plastic injury vial are accelerated upward until the vial reaches the top of the apparatus, after which forward momentum of the flies carries them further upward until they impact the upper surface of the vial where they sustain headfirst impacts.

Supplementary Video 2 (Video S2)

Head impacts elicit immediate concussive-like behavior Flies sustain acute signs of neurological injury immediately following head impacts, such as temporary loss of consciousness and uncoordinated behaviors that become more prevalent with increasing number of repetitive head impacts.

Supplementary Video 3 (Video S3)

Automated tracking of fly climbing using idtracker.ai Raw video is processed using idtracker.ai, which provides continuous measurement of individual flies. Tracking data is imported within MATLAB where it is further analyzed for individual parameters of movement, including total distance.

Supplementary Video 4 (Video S4)

Novel approach to measure frank neurodegeneration in *Drosophila* whole-brain mounts Two-photon microscopy stack of whole-brain mount stained for DAPI (nuclei) and phalloidin (actin for brain parenchyma). Regions devoid of DAPI/phalloidin that are outlined in green correspond to physiologically normal holes while yellow outlined regions correspond to pathological vacuoles.

Supplementary Video 5 (Video S5)

Conditional suppression of neuronal activity via pan-neuronal expression of *Shibire^{ts1}*
Demonstration of conditional suppression of neuronal activity in pan-neuronally expressing *Shibire^{ts1}* flies (*nSyb>Shi^{ts1}*). Flies are exposed to the non-permissive temperature using a warm water bath.

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