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Drosophila as a Model to Explore Secondary Injury Cascades after Traumatic Brain Injury

Lori M. Buhlman[a,*], **Gokul Krishna**[b,c], **T. Bucky Jones**[d], **Theresa Currier Thomas**[b,c,e,*]

[a]Biomedical Sciences Program, Midwestern University, Glendale AZ, USA

[b]Department of Child Health, University of Arizona College of Medicine - Phoenix, Phoenix AZ, USA

[c]Barrow Neurological Institute at Phoenix Children's Hospital, Phoenix, AZ, USA

[d]Department of Anatomy, Midwestern University, Glendale, AZ, USA

[e]Phoenix VA Health Care System, Phoenix AZ, USA

Abstract

Drosophilae are emerging as a valuable model to study traumatic brain injury (TBI)-induced secondary injury cascades that drive persisting neuroinflammation and neurodegenerative pathology that imposes significant risk for long-term neurological deficits. As in mammals, TBI in *Drosophila* triggers axonal injury, metabolic crisis, oxidative stress, and a robust innate immune response. Subsequent neurodegeneration stresses quality control systems and perpetuates an environment for neuroprotection, regeneration, and delayed cell death via highly conserved cell signaling pathways. Fly injury models continue to be developed and validated for both whole-body and head-specific injury to isolate, evaluate, and modulate these parallel pathways. In conjunction with powerful genetic tools, the ability for longitudinal evaluation, and associated neurological deficits that can be tested with established behavioral tasks, *Drosophilae* are an attractive model to explore secondary injury cascades and therapeutic intervention after TBI. Here, we review similarities and differences between mammalian and fly pathophysiology and highlight strategies for their use in translational neurotrauma research.

*Corresponding Authors Theresa Currier Thomas, Ph.D., theresathomas@arizona.edu (T.C.T), Associate Professor, Translational Neurotrauma Research Program, BARROW Neurological Institute at Phoenix Children's Hospital, Department of Child Health, University of Arizona College of Medicine – Phoenix, 425 N. 5th St., 322 ABC-1 Building, Phoenix, Arizona 85004-2127, USA; Lori M. Buhlman, Ph.D., lbuhlm@midwestern.edu (L.M.B), Professor, College of Graduate Studies, Midwestern University Glendale, USA.

All authors contributed equally

Author contributions

Lori Buhlman: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Gokul Krishna:** Methodology, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration. T. **Bucky Jones:** Conceptualization, Methodology, Formal analysis, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration. **Theresa Currier Thomas:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition

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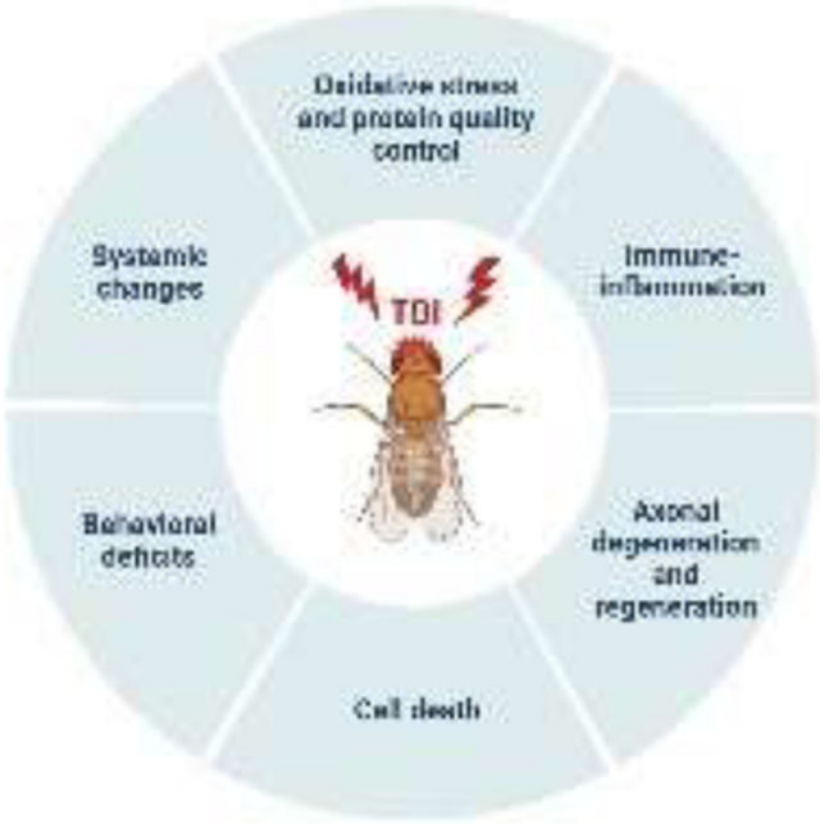
Graphical Abstract



Keywords

traumatic brain injury; *Drosophila* ; secondary injury; inflammation; innate immunity; oxidative stress; mitochondria; apoptosis; neurodegeneration

1. Introduction

Traumatic brain injury (TBI) is currently estimated at 69 million cases per year worldwide (1), contributing to high health care expenditures, lost productivity, and diminished quality of life for the patient. TBI has become a leading cause of death and disability, with some TBI survivors experiencing problems with cognitive, sensory, and emotional processing, requiring long-term care and rehabilitation (2). Despite knowing several mechanisms involved in the development of TBI-initiated neurological and psychiatric deficits, neuroprotective post-injury treatments to lessen or prevent these deficits remains largely unrealized.

Classification of human TBI as mild, moderate, and severe is traditionally based on symptoms using the Glasgow Coma Scale (GCS) score (3). Closed head trauma can further be classified using several clinical factors involving the duration of loss of consciousness, post-traumatic amnesia, altered mental state (disoriented or confused),

neurological symptoms, and neuroimaging findings. Eighty to ninety percent of TBIs are categorized as mild, primarily resulting from blunt non-penetrating head injuries due to falls, vehicle accidents, violence, contact sports, and combat activities (4). Diffuse TBI is caused by an external mechanical impact or rapid acceleration-deceleration of the head that can cause diffuse neuron, glia, and cerebrovascular injury, initiating complex and prolonged sequelae of biochemical and physiological secondary injury processes. Prolonged secondary injury cascades can promote acute and delayed neuron death, such that TBI presents more like a neurodegenerative disease than a static event (5). The progressive loss of neurons and degenerating axotomized processes initiate immune responses to clear debris; promoting chronic neuroinflammation that is purported to accelerate the onset of genetically predisposed and age-related neurodegenerative diseases, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and chronic traumatic encephalopathy (CTE) (6, 7). TBIs have been challenging to treat because the pathways, complexity, and time course of secondary injury processes directing acute and delayed disease pathogenesis are poorly understood.

TBI animal models have guided important discoveries in identifying discrete mechanisms, mapping pathological events, and testing potential disease-modifying therapies. Mammalian models allow tight control over injury severity, and they faithfully recapitulate particular aspects of clinical TBI due to the conservation of the neuro-glial-vascular unit across species. Such discoveries have led to significant advances in understanding the mechanisms leading to neurological deficits by employing a range of state-of-the-art technologies that provide exquisite detail of ongoing pathology (8). A well-established body of evidence indicates that secondary injury processes mediating chronic neuroinflammation are a primary culprit in persisting and late-onset post-TBI symptoms.

Rodent models are the primary animal model for studying TBI, where their shorter lifespan facilitates longitudinal tracking. While mammalian models are essential, the translational process, length of study duration, drug and drug delivery costs, vivarium maintenance, and requirement of highly trained personnel present financial barriers to testing both acute and longitudinal impact of secondary injury processes and long-term effectiveness of therapeutic strategies. While genetic modification is available for mice and some rats, the process requires substantial time, skill, funding, and oversight. Further, mammalian systems have redundancy in their signaling pathways that can confound outcomes due to compensation. Translating outcomes from mammalian research to clinic care remains challenging due to adaptive and maladaptive signaling cascades associated with chronic neurodegeneration and neuroinflammation. Our knowledge of secondary injury mechanisms can be expanded by complementary use of novel models to unravel the complex interactions of a wide variety of pathophysiological processes that instigate and perpetuate neurological deficits after diffuse TBI.

Drosophila offers the unique advantage of a model system with an array of genetic and biological approaches that can be utilized to rapidly uncover longitudinal pathophysiological processes associated with a heterogeneous TBI that underlie neurological diseases. Herein we discuss the secondary pathomechanisms that play a role in the long-term effects of TBI. We review what is currently known about central nervous system (CNS) primary

injuries that drive secondary reactions due to alterations in signaling cascades that operate after TBI. We introduce current methods used to induce TBI in *Drosophila* and summarize new developments that highlight the utility of *Drosophila* as a model for TBI as cellular and molecular players in neuroinflammation and immune response, axonal degeneration and regeneration, cell death mechanisms, and peripheral effects. Finally, we present future perspectives on how studies in fly models will reveal crucial functions of genes that regulate underlying secondary injury cascades to infer translatability of these invertebrate studies to rodent and human pathophysiology. Unless otherwise stated, we focus on diffuse TBI.

2. TBI pathophysiology

2.1. Primary injury and secondary injury cascades

Primary injury during TBI is caused by biomechanical forces such as direct contact, rotational acceleration/deceleration, compression, torsion, or blast wave. In mammals, diffuse TBI is multifocal and imparts shear, tensile and compressive strains. It commonly produces damage to long-distance white matter connections via diffuse axonal injury (DAI), which includes damage to the neurofilament subunits and the axolemma adjacent to the nodes of Ranvier. Regions of gray-white matter interface and associated capillaries are vulnerable to shear forces resulting in petechial hemorrhage. Other forms of primary cellular injury include dendritic and synaptic damage and disruption. Movement of the brain within the skull can cause mechanical depolarization of neurons and release high concentrations of excitatory neurotransmitters that exacerbate cellular and tissue damage and contribute to blood-brain barrier permeability (8). TBI also causes dysregulation of cerebral blood flow and metabolism, which typically decreases after brain injury contributing to a mild ischemic effect (9). Together, these primary injuries instigate a number of secondary injury cascades implicated in the chronic morbidity associated with TBI (see Figure 1).

Increased extracellular levels of glutamate cause uncontrolled and excessive stimulation of glutamate receptors, leading to hyperexcitability and spreading depolarization, increasing the global energy demand. At the same time, decreased cerebral blood flow can cause a deficit of oxygen, resulting in an energy deficiency and metabolic crisis, where glycolysis is reduced, and anaerobic glycolysis reciprocally increases for days post-injury (10). Massive depolarization increases extracellular potassium and intracellular calcium and sodium, causing increased levels of Ca^{2+} in mitochondria that trigger the release of cytochrome C and apoptosis-inducing factors (AIF) into the extracellular space and initiates cell death signaling (11). As mitochondrial respiration increases to meet the energy demand, it produces excess reactive oxygen species (ROS) beyond the ability of antioxidants (e.g., glutathione peroxidase, superoxide dismutase) to neutralize (12). Calcium also activates numerous cellular pathways that stimulate the activation of protein kinase signaling, calpains, caspases, and enzymes, including nitric oxide synthase (NOS), which produces free radicals. Free radicals and ROS damage nucleic acids, inhibit the electron-transport chain, and cause oxidative damage to phospholipids, proteins, and some enzymes (11). Damaged biomolecules and organelles increase and offset protein homeostasis impacting the ubiquitin-proteasome and autophagy-lysosome pathways, where reduced proteasome activity and increased autophagic flux are impaired after experimental TBI (13-16). Despite

the survival of axotomized and damaged neurons, secondary molecular cascades, immune responses, and altered afferent/efferent signaling can further compromise cellular stability and tip the balance towards cell death for months and years post-injury (17). Delayed cell death prolongs neurodegeneration and the activation of microglia and astrocytes associated with the neuroinflammatory response (18).

Neuroinflammation, which involves local and peripherally-derived immune responses, is an important aspect of secondary injury after TBI (19). Moreover, growing evidence indicates a strong link between neurological deficits and abnormalities in immune mechanisms that most likely involve both innate and adaptive immunity. The innate immune response is responsible for the rapid initial response to the primary injury and facilitates activation of the adaptive immune response (20). Cells involved in adaptive immunity include T- and B-lymphocytes, which have been implicated in both neuroprotection and neurodegeneration depending on the context in which they are activated (21). Yet, the mechanisms underpinning innate immunity following TBI are confounded by the complexity of the cell-mediated adaptive immune component, in part because they overlap. Therefore, it is of great therapeutic relevance to determine the effects of TBI on mediators of innate immunity in pathways that are highly conserved across species. Because the cellular effectors of innate immunity can determine the inflammatory phenotype, their involvement in secondary pathological processes has implications on the chronic responses after injury.

In mammals, there is still much to learn about the innate immune response to TBI. TBI causes DAI (hallmark pathology) that is characterized by impaired axonal transport leading to progressive axonal swelling, disconnection, and Wallerian degeneration of the distal axon (22, 23). Within minutes to hours post-TBI, these stressed, injured, and dying cells can release damage-associated molecular patterns (DAMPS; aka alarmins, IL-1α, S100 proteins, IL-33) and pathogen-associated molecular patterns (PAMPS). DAMPS and PAMPS are ligands for pattern recognition receptors (PRRs), including Toll-like receptor (TLR), NOD-like receptor (NLR), AIM2-like receptor (ALR), RIG-I-like receptor (RLR), and C-type lectin receptor (CLR) families, which are also important for activation of microglia and astrocytes, stimulating infiltration of monocytes, and recruitment of microglia (24). DAMPS and PAMPS also stimulate pathways that lead to the activation of nuclear factor-κB (NF-κB), AP-1, and IL-1β to mediate inflammation, inflammasomes, and apoptosis in response to brain injury (25). In addition to phagocytosis of dead tissue, infiltrating monocytes and activated microglia release tumor necrosis factor-α (TNF-α). Levels of the pro-inflammatory cytokine, TNFα, increase after TBI and are associated with other neurodegenerative disorders with prolonged activation of microglia and astrocytes (26). Along with activating similar pathways as PAMPS and DAMPS, TNF can stimulate the c-Jun N-terminal Kinase (JNK) pathway with the adaptor protein TRAF6, which also positively regulates signaling cascades for NF-κB and AP-1 and promotes apoptosis and autophagy after TBI (27-30).

The Janus kinase/signal transducer and activator of transcription proteins (JAK/STAT) pathway is important for both pro-inflammatory and anti-inflammatory signaling, where it has been implicated in the promotion of neurite outgrowth, cellular differentiation, proliferation, cell survival, and inhibitory neurotransmission (33, 34).

Following experimental TBI, the JAK/STAT pathway activated within 3h. Pharmacological manipulation that increased activation of the JAK/STAT pathway supported benefits, including reduced apoptosis and less tissue loss for acute cellular events (34, 35). However, inhibition demonstrated improvement in epilepsy and a prolonged improvement in vestibular function in other brain injury models (36). A better understanding of this pathway and the longitudinal impact on activation and inhibition after TBI may be valuable to guiding circuit reorganization and repair.

2.2. Secondary injury mechanisms increase the risk for neurological deficits

The high degree of complexity of multiple processes involving molecular and circuit-level components after TBI has long-term implications for the development of neurological deficits. ROS are responsible for processes that lead to an accumulation of protein aggregates involved in secondary pathogenic processes that form hallmark deposits after TBI that are major risk factors for developing chronic neurodegenerative diseases like CTE, frontotemporal dementia, and AD (37-39) . Dopamine neurons are vulnerable to oxidative stress, with implications for loss of dopamine neurons associated with PD (40). Multiple secondary injury mechanisms serve as mediators of apoptosis, resulting in delayed apoptosis. Delayed onset of neuron degeneration increases the temporal window for neurodegenerative processes and prolongs the activation of glia and other associated neuroinflammatory processes, corroborating clinical pathology (41, 42). Activated astrocytes and microglia also mediate adaptive and maladaptive regenerative and compensatory processes post-injury (43-45). Neuroplastic regenerative responses manifest as dendritic and synaptic sprouting (46), indicating axonal and dendritic growth and connection in surviving axotomized neurons (47). This novel connectivity does not reconstitute the same level of pre-injury circuit integrity, contributing to the appearance of novel or increased severity of symptoms reported pre-TBI (8). Damage and reorganized circuits can prevent or decrease the effectiveness of feedback regulation of hypothalamic-pituitary axes, preventing the return to homeostasis and giving rise to affective and other neuroendocrine-related symptoms (44, 48-52). Increased circulating inflammatory mediators can also influence peripheral organs, like the gut, contributing to leaky gut syndrome that can instigate peripheral inflammatory responses and microbiome dysbiosis, which forms precursors for neurotransmitters and hormones (53, 54). These distinct processes occur in parallel, where biomolecules from secondary injury cascades often overlap or have multiple targets. The interacting secondary injury factors give rise to an array of acute and chronic behavioral deficits that include impairments of cognitive, affective, sensory, motor functions, and sleep-wake rhythms. Clinically, this creates challenges for identifying TBI-induced symptoms, diagnosing appropriate treatment, and distinguishing TBI-induced symptoms from other non-TBI related neurological deficits, where a better understanding of the relevant signaling cascades may be relevant to preventative treatments or better symptom management for improved long-term outcomes and treatment.

3. Modeling TBI in *Drosophila*

As a human TBI model, the fly offers a simplistic platform to recapitulate head injuries at controlled levels of force and acceleration to study injury mechanisms that are not yet

well understood. The advantages of the fruit fly as a human disease model have been well recognized in modern biology, as approximately 75% of human genes have a fly ortholog. *Drosophila* has emerged as an excellent model system for several neurological disorders with a broad measure of validity in the context of genetics (construct validity) and simple and complex phenotypic behaviors (face validity) to test potential candidates for therapeutic efficacy (predictive validity). A range of recent technological advancements allow genetic and molecular manipulations that isolate pathophysiological pathways, thus providing a more comprehensive understanding of complex disorders (55). Thousands of genetically manipulated *Drosophila* stocks are commercially available, facilitating observation of cell-specific gene function/activity and organelle markers (https://bdsc.indiana.edu/). Flies are inexpensive to maintain and have identifiable, behaviorally conserved brain circuitry. A relatively short lifespan facilitates longitudinal aging studies, making them particularly attractive models of neurodegenerative disease. Given these useful features and to address specific scientific questions, flies are uniquely positioned to fill the knowledge gaps left out in mammalian TBI models. To this end, multiple TBI models have been developed for the assessment of TBI in *Drosophila*.

3.1. Head and body injuries

The high impact trauma (HIT) device inflicts an impact acceleration-deceleration injury to flies by deflecting and releasing a plastic vial rapidly onto a polyurethane foam pad, modeling head and/or body injuries that occur in motor vehicle accidents and sports injuries (56). The HIT model reproduces closed head injury characteristic of blunt non-penetrating head trauma. The angle of spring deflection can be changed to alter impact velocity, and repetitive strikes can be administered to increase injury severity. HIT induces temporary incapacitation, locomotor dysfunction, mitochondrial protein oxidation, and brain neuropil vacuole formation (56-58). Heads of young and older flies exposed to HIT injury have increased Relish (*Relish* is the fly NF-κB ortholog) and antimicrobial peptide (AMP) expression within twenty-four hours of injury, and AMP expression levels correlate with mortality (56, 59-62). Interestingly, changes in larval length, early eclosion, and behavioral deficits have been observed in offspring of HIT-injured flies (63). The HIT model induces high variability in injury severity and lacks control of rebound injury. A caveat for this model is that variability in reproducibility across laboratories can be introduced by components of the HIT device, including the type of spring used and the thickness and consistency of the foam padding, thus requiring optimization of each HIT device. A major advantage of the HIT model is its relatively low price, ease of setup and operation, and high throughput.

The bead ruptor platform model incorporates a homogenizer platform to induce repetitive impact to a large number of flies in screwcap tubes (64). The model is well suited to study the functional consequences of repetitive brain injury in the absence of anesthesia. Video acquisition allows calculation of fly velocity and frame-by-frame analysis detection of head, thorax, and abdominal contact with the tube. The degree of injury can be controlled by adjusting the rotation speed and injury exposure time. Bead ruptor platform-induced TBI damages neural processes, induces sleep disturbances, and increases acute and sustained AMP expression (64). As with the HIT model, the bead ruptor platform causes a

combination of head and body injury. This model also allows observation of the structural and functional consequences of repetitive injuries. While both head-body injury models generate variable injury severity within a single experiment, the force of injury can be more tightly controlled than with the less expensive HIT device.

3.2. Head-specific injury models

For the Strike Device injury model, direct head impact is delivered when pressurized CO_2 is applied to the head of an anesthetized fly that is immobilized by a fly holder attached to a syringe barrel (65). The burst of gas creates a very rapid, localized acceleration-deceleration and replicates diffuse injury commonly observed in humans. This technique reduces damage to other parts of the body and allows for delivery of measurable impacts restricted to the head, while controlling for severity, time between impact, and the number of impacts. Injured flies have locomotor deficits and reduced life span (65). Because the direct impact to the head is more controlled and reproducible in this model, it allows more precise mapping of the CNS response to injury. This model is relatively expensive, requires more technical equipment, and has relatively low throughput.

Another closed head injury device that reduces body injury and models human diffuse TBI consists of a pull-type solenoid powered block to inflict a precisely controlled strike to the head of individually restrained flies (66). Flies are collected using an aspirator and transferred into a restrainer (pipette tip) to expose the fly's head. The head is subjected to impact by a controlled current which drives the brass trapezoid-shaped block into the head. Injury-induced phenotypes include increased mortality and motor deficits. This closed head injury model provides the opportunity to avoid anesthesia, but the procedure is labor-intensive, low throughput, time-consuming, and requires specialized equipment.

The controlled compression head-specific injury model mimics compression loads with a blunt impact as used in rodent TBI models. Here, variable voltage to a power amplifier causes a piezoelectric actuator to bend rapidly, creating a linear displacement and compression of an immobilized fly head against a metal plate (67, 68). The compression impact can be adjusted for mild, moderate, and severe injury levels to induce ataxia, neuronal degeneration, and reduced lifespan. Severe injury induces cognitive and memory acquisition deficits as well as transient blood-brain barrier permeability and decreased macrophage-mediated debris clearance in the brain. Additionally, the controlled compression injury can induce transient increases in lysosome number, proteasome activity, and antioxidant mediator glutathione S transferase, all of which indicate oxidative stress (68).

4. Insights gained from *Drosophila* TBI models

4.1. Oxidative stress and protein quality control system

Mitochondrial respiration is the primary source of ROS in cells. Oxidative stress occurs when antioxidant systems fail to control levels of ROS, which react with and damage phospholipids, nucleic acids, and proteins. The accumulation of free radicals and oxidative modification of fatty acids generates mitochondrial lipid peroxides that can trigger deadly

changes in membrane permeability and a feedforward production of ROS by the electron transport chain, which itself is susceptible to oxidation (reviewed in (69)). Oxidative stress is implicated in the propagation of neurodegenerative diseases via secondary injury processes following TBI (reviewed in (12)). Cyclooxygenase disruption has been reported in 24 hours post-TBI fly brain, leading to oxidative stress and decreased ATP production (70). Increased mitochondrial aging and a transient increase in antioxidant demand, both symptoms of oxidative stress, have also been reported in flies exposed to TBI (68, 71). Similar events have been identified in neurodegenerative diseases and disease models (40, 72-76).

Intracellular protein turnover reduces oxidative stress by eliminating damaged proteins and depolarized mitochondria, promoting cell function and survival. After TBI, oxidative stress disproportionately increases levels of oxidized dysfunctional proteins (misfolded) that can evade degradation systems and promote toxic protein aggregate formation (77). Disruptions in the ubiquitin-proteasome system, chaperone-mediated autophagy, and macroautophagy have been heavily implicated in neurodegenerative processes, including TBI (14, 78). Interestingly, mild TBI triggers a sustained increase in autophagosome marker autophagy gene 8a (atg8a), the *Drosophila* ortholog of mammalian GABA type A receptor-associated protein (GABARAP) and autophagosome substrate receptor ref(2)p (fly ortholog of p62) (64). Moderate and severe TBI can transiently increase the presence of LysoTracker®-positive lysosomes and degradation of CL1-GFP, which is recognized by E3 ubiquitin ligases (68), while mild TBI has no effect on LysoTracker® staining. Cytoplasmic aggregates called “stress granules” containing the fly homolog of ALS-associated transactive response DNA-binding protein-43 (TDP-43) appear in the fly brain within 24 hours after repetitive TBI, and their presence increases with the number of hits (79). Taken together, these reports suggest that autophagy demand could increase with the severity of brain injury. Rapamycin administration or overexpression of atg8a reduces stress granule formation; thus, autophagy machinery may be overwhelmed after TBI or inhibited by it (79). Additional studies featuring the wide variety of available fly models of neurodegenerative diseases have the potential to strengthen our understanding of the mechanisms by which TBI predisposes patients to a variety of neurodegenerative diseases (80). While *Drosophila* TBI can trigger autophagy, severe brain injury may produce an insurmountable burden on autophagy machinery, warranting therapeutic strategies promoting autophagy, particularly in early disease stages.

4.2. Immune response and inflammation

Inflammation is a highly conserved cellular response to stressful stimuli such as infection and tissue damage that plays a critical role in the clearance of the pathogen or tissue debris, followed by the re-establishment of tissue homeostasis. The acute inflammatory response is initiated by so-called ‘danger signals,’ including PAMPs and DAMPs released by injured neurons. These molecules interact with PRRs on a variety of cells to generate early inflammatory mediators, that if appropriately controlled, play an important role in the eradication of the pathogen and/or clearance of cellular debris. If not appropriately controlled, however, the inflammatory response can become chronic, which may be associated with exacerbation of tissue injury rather than promoting tissue repair. Several clinical and experimental studies have shown that TBI induces a sustained

neuroinflammatory response and overactivation of microglia that continues years after the insult - leading to chronic neurodegeneration, progression of dementia, and traumatic encephalopathy (32, 81, 82).

The fact that *Drosophila* is devoid of adaptive immune reactions offers a simplistic yet well-characterized model of highly conserved innate immunity relative to the human. Pioneering studies identified several multifaceted innate defense responses involving systemic and cellular reactions as players in the first line of defense in *Drosophila*, with most components of innate immunity evolutionarily conserved across species (83-85). In Table 1, the homologs for molecules of these pathways are defined for *Drosophilae* and mammals. In *Drosophila*, the two main systemic innate immune response pathways include the immune deficiency (Imd) and the Toll pathway. In the Imd pathway, binding of the cell surface receptor, Peptidoglycan recognition protein LC (PGRP-LC) activates Imd, which is a death domain-containing protein sharing homology with the mammalian receptor-interacting protein (RIP) (86). In mammals, RIP functions in a complex with the TNF-α receptor; thus, the Imd pathway in *Drosophila* is considered the significant equivalent of the TNF-α receptor signaling in mammals, while the Toll signaling pathway is similar to the mammalian TLR pathway involving myeloid differentiation primary response protein 88 (MyD88) (87, 88). These Imd and Toll signaling pathways function in parallel and induce the nuclear translocation of NF-κB homologs which activate the transcription of overlapping but different effector proteins, including AMPs, which are ubiquitously expressed in all multicellular organisms from insects to humans (89-93). In *Drosophila*, chronic expression of NF-κB-induced effector proteins in the absence of infection or overt trauma has been associated with the development or progression of neurodegenerative phenotypes (94-97). In mammals, NF-κB signaling is an important regulator of TBI-induced changes in the phenotypic plasticity of microglia and macrophages that results in the release of proinflammatory factors and free radical formation (81, 98-100).

Numerous studies in *Drosophila* suggest that TBI activates both the Imd and the Toll pathways. The study of innate immune pathway activation in flies after TBI has begun to elucidate the potential role of AMP gene expression on various behavioral factors and pathological injury outcomes. Although there is some discrepancy across studies, there is general agreement that AMP genes related to both Toll (e.g., *Drosomycin*, *Metchnikowin*) and Imd (e.g., *Diptericin*, *Attacin*) pathway activation are upregulated in the heads of TBI flies as early as one to two hours post-injury (56, 60, 64, 101). The majority of studies suggest that expression is maintained for at least 24 hours post-injury and potentially as long as 72 hours post-injury before returning to baseline (56, 66, 102). Furthermore, these injury-induced effects appear to be independent of injury model as similar observations have been reported with the HIT device (56, 60, 101), the Omni bead homogenizer (64), a head-only model (66), and a penetrating injury model (102). Few studies have evaluated long-term changes in AMP gene expression following TBI in *Drosophila*, and those that have yielded conflicting results with one study reporting that expression of all AMP genes returned to baseline at 7 days post-injury (66) and others reporting a secondary increase in AMP genes at 7 days post-injury (64). Overexpression of *Metchnikowin* increases TBI-induced mortality and the absence of *Relish*, the NF-κB homolog in the Imd pathway, reduces TBI-

induced mortality (62, 66), which implicates injury-induced activation of NF-κB signaling in secondary injury processes with deleterious consequences.

Two additional innate immune pathways present in *Drosophila* include the JNK and the JAK/STAT pathways (Figure 2). Much less is known regarding the role of these pathways in *Drosophila* TBI (dTBI), although they are both activated in response to axonal injury in the *Drosophila* CNS. In *Drosophila*, the highly conserved, canonical JNK pathway is activated when Eiger, the *Drosophila* homolog of mammalian TNF, binds to its cognate receptor, Wengen. With the help of the adaptor protein, TNF receptor-associated factor 6 (Traf6), *Drosophila* transforming growth factor activated kinase 1 (dTAK1; homologous to the mammalian MAP kinase kinase kinase 7) (103) is activated. *Drosophila* TAK1 is also activated downstream of Imd as a separate branch of the Imd pathway. Activation of dTAK1 results in the phosphorylation off *hemipterous* (homolog of mammalian JNKK, or MKK7), which then phosphorylates *basket* (JNK homolog), that translocates to the nucleus to activate transcription of AP-1-related genes. The dAP-1 is a heterodimer formed by Jra (the homolog of mammalian c-Jun) and kayak (homolog of mammalian c-fos). Jra expression increases following dTBI (101), indicating that the JNK pathway is indeed activated by dTBI. Several factors influence the effects of this pathway on the outcome from TBI. If the innate immune response is adequately controlled, low levels of ROS will result in transient activation of JNK signaling, which is important for wound healing and may serve a neuroprotective function. An increase in glutathione-S-transferase (Gst) genes has been reported following diffuse TBI (68), suggesting that an antioxidant response is initiated following CNS injury. Furthermore, *Relish* mutant flies have a downregulation of Gst genes, suggesting a role for *Relish* in mediating the antioxidant response (104). Conversely, if tissue injury is such that high levels of ROS are produced (e.g., oxidative stress, which is known to occur with chronic inflammation), there is sustained JNK activation, which likely contributes to inflammation-induced apoptosis as has been observed in mammalian models (105-107). In addition to oxidative stress, JNK signaling is negatively regulated by *Relish*. Activation of *Relish* attenuates JNK activity by promoting the proteasomal degradation of dTAK1 (108). In the absence of *Relish* activation, JNK signaling is sustained, resulting in a pro-apoptotic response. Although it has not been directly evaluated in TBI, such a mechanism could account for enhanced survival observed in *Relish*-deficient flies (66).

Several genes that regulate activity in the JAK/STAT pathway, including *Socs36E* and *Tep2*, are induced following injury, which suggests that the JAK/STAT signaling pathway is activated in flies as has been reported in mammals (Figure 2) (36, 60, 109). The JAK/STAT pathway is implicated in regenerative inflammatory signaling in response to TBI. *Tep2* is a member of the thioester-containing protein (TEP) family of genes, which are complement-like proteins that share homology with the α2-macroglobulin/complement C3 family. *Drosophila* expresses a single STAT protein, Stat92E, that, although not directly evaluated in dTBI models, is a critical element in the axonal response to injury (see below). In addition, Stat92E, in association with dAP-1, regulates the production of AMPs (110). The AP-1/JNK and JAK/STAT pathways interact to negatively regulate *Relish* activation, which plays an important role in terminating immune responses. Specifically, dAP-1 (Jra) and Stat92E combine with the *Drosophila* HMG protein (a homolog of mammalian HMGB1, the prototypic DAMP), dorsal switch protein (Dsp1), and histone deacetylase

to inhibit the transcription of the diverse immune effector genes activated by *Relish* (110). This is important to avoid the potentially negative effects that would occur with prolonged activation of the innate immune response (e.g., chronic inflammation). Whether this regulatory mechanism is operative after dTBI in insects or mammals is not known.

4.3. Axonal degeneration and regeneration

Axonal degeneration in the CNS and the peripheral nervous system have been observed in *Drosophila* and mammalian models of TBI and neurodegenerative disease. The response to axotomy in *Drosophila* resembles the responses observed in mammalian models. Specifically, within minutes, the formation of retraction bulbs and filopodial sprouts is observed in the proximal axonal fragments, with vesiculation and fragmentation apparent in the distal segments (111). These characteristic features of Wallerian degeneration indicate that this is a conserved evolutionary process. Similar to the limited regeneration observed in mammalian CNS axons, the sprouting response in *Drosophila* is transient with only limited regrowth of axons.

The clearance of axonal debris generated in response to axotomy is a prerequisite for axonal regeneration. Several studies have aimed at identifying the signaling pathways that promote clearance of axonal debris in the CNS. Early work indicated a requirement for Draper in the clearance of axonal debris in the CNS (Figure 2) (112). Later studies identified ensheathing glia as the predominant CNS phagocytic cells expressing draper following axotomy and established that Draper-mediated engulfment of axonal debris requires *dCed-6*, *Src42a*, and *Shark* (113, 114). DAMPs bind to Draper and activate the dJNK pathway and the JAK/STAT pathway (115) and JNK signaling and Stat92E in glial cells are both required for upregulation of Draper after axotomy (116). In addition, Eiger/TNF signaling via dTRAF6 activates Dorsal/NF-κB to induce proliferation of ensheathing glia following stab injury of the ventral nerve cord (117), and transcriptional reporters for JNK pathway signaling to dAP-1 are increased in glia after axonal injury (116). Collectively, these data provide several mechanisms by which activation of JNK and Jak/STAT signaling in response to axotomy can provide cross-regulation of NF-κB signaling pathways that regulate the innate immune response to injury.

Axonal regenerative responses in *Drosophila* can be enhanced by activating several signaling pathways, including PKA, JNK, and the JAK/STAT pathway (111). Similar to cAMP-based improvements in axonal regeneration observed in mammals (118, 119), increased PKA activity enhanced axonal sprouting in flies (111). The JNK pathway also plays a key role in axonal regeneration after injury. Activation of JNK signaling by overexpression of a constitutively active form of *hemipterous*, the *Drosophila* homolog of mitogen-activated protein kinase kinase 7 (*MAP2K7*), increased CNS axonal regeneration after injury, an effect that was blocked by overexpression of a dominant-negative allele of *basket*, the *Drosophila* homolog of JNK (111). Larvae from adult *Drosophila* that were previously given a closed head TBI exhibited axonal and dendritic regeneration via activation of the dual leucine zipper kinase (DLK) and the JNK pathway (120). While the mechanisms of action are not yet well understood, researchers hypothesize that inactivation/activation of these

pathways is mediated in part by the enzymatic and cellular pathway changes that occur post-TBI.

4.4. Cell death

Cell death after TBI results from mechanically induced primary injury and secondary neuroinflammation. While apoptosis, necroptosis, pyroptosis, and necrosis are all implicated in TBI patients and mammalian models, cell death reported in fly TBI models is primarily limited to brain vacuole formation, where the contributions of apoptosis, necroptosis, or necrosis are not clear (56, 68, 121-124). *Drosophila* studies have advanced our understanding of conserved apoptotic pathways (Figure 3) (125, 126). Interestingly, while intrinsic apoptosis machinery in flies and mammals comprises many of the same components and mechanisms of regulation, its contribution to cell death has been primarily observed during development. Whether the lack of evidence of apoptosis in dTBI is due to its limited contribution to cell death in the fly model or to scarcity of research conducted in this area remains to be determined. If the latter, powerful genetic manipulation/expression tools and highly conserved apoptotic homologs are untapped resources. For example, the fly adaptor protein Dark is a functional homolog of mammalian intrinsic pathway-mediated cytoplasmic apaf-1 (mediated by NLR signaling in mammals) that promotes the formation of apoptosomes in conjunction with the initiator caspase Dronc (mammalian homolog of caspase-9; Table 1) (127-129). This subsequently activates effector caspases DrICE (caspase 3 homolog) and Dcp-1 (127-131) promotes apoptosis. Whether cytochrome c is involved in fly apoptosome formation is unclear (132-136). Dark shares cytochrome c interacting domains WD40 and CARD, suggesting that it may (127, 128, 133); however, fly apoptosome formation can occur in the apparent absence of cytochrome c (132-134).

As in mammals, fly mitochondria swell in response to stress (72), suggesting that mitochondrial membrane permeabilization may be involved in initiating intrinsic apoptotic signaling. Apoptosis regulation also seems to be conserved as flies express homologs of apoptosis regulating proteins including anti-apoptotic protein Buffy, and pro-apoptotic Debcl, which are Bcl-2 and Bok homologs, respectively. Interestingly, Buffy appears to bind to both endoplasmic reticulum and mitochondrial membranes to suppress the unfolded protein response, while Debcl functions more like Bok by binding to mitochondrial membranes (131, 137-141). *Drosophila* inhibitor of apoptosis (IAP) protein, Diap1, regulates DrICE caspase activity via ubiquitination and competitive binding (142), and dOmi, a fly pro-apoptotic protein that functions like its mammalian counterpart, HtrA2/Omi in its inhibition of IAPs (135, 136, 143). Diap2 may also provide protection against inflammation as Imd pathway components are also Diap2 ubiquitination substrates (144-146). Conversely, IAP antagonist Hid transcription can be triggered by JNK signaling upon Eiger binding of its receptor Grindelwald (GRND) (147, 148). GRND is the fly homolog of mammalian TNF-α receptor, and its endogenous agonist Eiger is the fly homolog of TNF-alpha. It is important to note that developmental apoptosis pathways may differ from degenerative ones; where only the former is well characterized. Reaper, Hid, Grim, and Sickle are functional homologs to pro-apoptotic proteins SMAC/DIABLO in that they inhibit Diap1; however, unlike their mammalian IAP antagonist homologs, Reaper, Hid,

Grim, and Sickle activities are transcriptionally regulated (149). Reaper, Hid, Grim bind to mitochondria to promote membrane permeabilization during developmental apoptosis (150).

While flies have fewer orthologs of mammalian necroptosis proteins, functional studies suggest homologous pathways that signal necrotic cell death exist in flies. Indeed, there are numerous reports of changes in innate immune protein levels following dTBI; thus, necrosis or necroptosis may be primary contributors to cell death. For instance, as discussed above, *Drosophila* Imd and PGRP-LC pathways share functional homology with mammalian RIP1/TNFR1 and RIPK2 nucleotide-binding oligomerization domain 1/2 (NOD1/2) mammalian necroptotic pathways, respectively (151-154). Fly necroptosis appears to be mediated via Imd and JNK signaling pathways. Additionally, inhibition of target of rapamycin (TOR, the *Drosophila* ortholog of mammalian TOR) increases lifespan via downregulation of conserved substrate cyclic AMP response element-binding protein coactivator transducer of regulated CREB activity (TORC) (155). Flies harbor a single isoform of TORC (crtc) that seems to perform the functions of mammalian TORC1 and TORC2 (155, 156). Although not yet demonstrated in flies, disrupted TOR activity may trigger inflammatory pathways resulting from decreased autophagy (157-159).

5. Improving translatability of *Drosophila* in TBI research

The heterogeneity of TBI involves multiple entities that influence pathogenesis and rate of disease progression. Indeed, several factors, including age, injury location, injury severity, previous head injuries, genetics, sex, drug abuse, comorbid psychological disorders, and sub-optimal post-morbid conditions contribute to continuing or late-onset TBI symptoms (160). Current TBI classification is primarily based on duration of loss of consciousness, GCS score, and length of post-traumatic amnesia, along with several neuroimaging measures used for diagnosis and prognosis; however, the ongoing pathophysiological processes are not considered. The present situation surrounding the management of the secondary injury phase of TBI reflects the current lack of success for therapeutic intervention. Animal TBI models have helped to reproduce clinically relevant injuries and generate behavioral, functional, and structural consequences of human TBI. These models provide assessments of acute neurological responses and evaluation of chronic behavioral morbidities.

Despite the encouraging discoveries that have paved the way for understanding the consequences of TBI, the full translational benefit has not been achieved. An unanswered question as to how and why TBI leads to persistent morbidity can be partially attributed to an incomplete understanding of the self-propagating secondary injury mechanisms driving chronic pathophysiological discord long after TBI. Moreover, it is unclear how these injury mechanisms further confer interindividual vulnerability toward various neurological deficits. While animal models are essential to translational progress, the roles of the intact nervous system, presence of vascular, and immunologic components contribute unaccounted complexity to result interpretation. Some of the challenges to modeling TBI in rodents and larger mammals include longer study duration, drug development, drug delivery, vivarium maintenance, sample size, and trained personnel, which contributes to costly experiments (albeit less expensive than clinical trials). In addition, technical aspects such as the requirement of pre-injury surgical procedures and the use of anesthetics, which

are not representative of “real-life” human TBI, can influence some outcome measures. Other factors include over-optimistic pre-clinical interpretations, lack of inclusion of both sexes, the absence of valid biomarkers, and the use of inbred animals that lack genetic diversity possessed by the human population (161). Therefore, utilizing multiple TBI models that serve as an excellent *in vivo* system to delineate secondary injury mechanisms that underlie TBI pathology likely sets the stage to substantially improve our understanding of the pathogenesis and promote translational confidence.

5.1. Validity in Drosophila

As discussed previously, great progress has been made with fly TBI models to recapitulate important neurological, biochemical, pathological, and behavioral analogies of human brain injury. There are several acute and subacute pathological and behavioral phenotypes that fly TBI models collectively display that are remarkably similar to key features of human TBI, including axonal shearing and motor deficits possessing face validity. For example, injured flies exhibit temporary suppression of reflexes causing loss of the righting reflex and start moving after a delay, the duration of which was found to be associated with the injury severity (Figure 4)(68, 162). The flies remain momentarily disoriented and often demonstrate problems with balance, including circling, ataxia, incoordination, and loss of equilibrium, similar to difficulties in coordination, posture and steadiness of movement observed in humans after TBI (Figure 5; Movie S1-S4). In addition, TBI has been associated with the development of epilepsy, including the occurrence of early seizures (162). Similarly, flies subjected to TBI exhibit spontaneous seizures involving twitches and uncontrolled jumps (68). Another observation from our lab using the HIT model is the temporary splaying of wings. The splaying occurs in the absence of overt damage to the wings, and wing position returns to normal within minutes to hours post-injury. The splaying of wings has similarities to the ‘fencing response’ noticed as temporary abnormal flexion or extension posturing of the extremities in rodent studies of TBI (163) and tonic posturing after sport-related human head injuries. The fencing response occurs without convulsions, immediately after injury, and during a loss of consciousness. Similar to the Parachute and Moro reflexes in human infants (in response to rotation or startle – infants arms extend or flex as if to break the fall), the response is thought to be a phylogenetically conserved spinal reflex involving activation of vestibular nuclei (164). In mice, Math1 gives rise to spinocerebellar and cuneocerebellar systems involved in fine motor control, proprioception, and posture (165). While this requires further evaluation, the *ato* homolog of *Math1* is conserved across species and involved in the development of *Drosophila* chordotonal organs, which are associated with the joints of wings, legs, and the second antennal segment and thought to be involved in proprioception and required for geotaxis (166, 167). The rest-activity cycle in *Drosophila* also has apparent similarities with human sleep-wake rhythm and has been particularly useful to study post-traumatic sleep changes (66).

To shed light on TBI pathogenesis, it is necessary for a thorough understanding of the behavioral consequences that emerge longitudinally after injury. Such outcomes can be exploited to test therapeutic approaches capable of modulating multiple downstream secondary injury pathways to promote behavioral recovery. The fly has been utilized for studying various simple and complex behaviors such as locomotion, sleep patterns, circadian

rhythm, courtship, and mating relevant to several human diseases. Flies can be visually scored, timed, and measured by negative geotaxis and visual closed-loop flight (56, 63, 101, 104, 168). Locomotor disabilities are commonly observed after TBI in humans and animal models, and the HIT model has been shown capable of reproducing movement deficits with decreased spontaneous locomotor activity (101). Testing for negative geotaxis reveals a loss of 50% more of normal progression toward light. When using a dark/light box exploration, flies, which normally are phototaxic, tend to stay in the dark side of the box, indicating photosensitivity after TBI (169) or increased anxiety-like behavior. Motor disturbances similar to those observed in humans after TBI have been reported in flies using the test for climbing behavior in response to gentle mechanical stimulus. Chronic phenotypic behavior deficits detected in flies have the potential to be evaluated for circuit-related disruption, where altered neurotransmission and maladaptive circuit reorganization contribute to the persistence of symptoms (170). For instance, sleep/wake cycles impairment was observed out to 5 days post-injury (171), and aggressive behavior has been observed in brain-injured flies and offspring (121), where early modulation of secondary injury cascades could improve behavioral deficits. Further, there is the potential to test for other phenotypic TBI symptoms, including depression and anxiety-like behavior, diapause, equilibrium, grooming (indicative of stress), learning and memory, phototaxis (circadian rhythms, sensory sensitivity, or anxiety), response to novel and familiar stimuli, and visual discrimination (indicative of TBI-induced optic neuropathy). Collectively, these endpoints suggest *Drosophila* carries a good face validity to longitudinally study TBI pathophysiology associated with TBI-like symptoms.

5.2. Systemic effects

It is now recognized that TBI compromises systemic physiology causing substantial changes in the functioning of peripheral organs. Several recent clinical and preclinical reports have supported the importance of brain-peripheral interactions mediated by several direct and indirect pathways involving microbiome, hypothalamic-pituitary-adrenal (HPA) functioning, immune signaling, neurotransmitters, hormones, and other neuropeptides. There is now emerging evidence that the influence of TBI on the systemic organs reverts on the brain and contributes to exacerbating the pathogenesis of TBI. Experimental data have implicated the role of bidirectional interaction in promoting secondary post-injury neuroinflammation, particularly associated with changes to systemic immunity. Other findings have indicated that alterations to the systemic immune system promote changes in immune signaling within the brain, and enhanced peripheral inflammation has been found in several neuropsychiatric disorders. Post-TBI infections and the initiation of the inflammatory response can continue chronically and promote neurodegeneration (172, 173). Among several comorbidities, gastrointestinal (GI) disturbances have been reported in TBI patients, including intestinal barrier dysfunction, changes in gut motility, and gastric emptying (174, 175). It has been reported that intestinal barrier dysfunction is associated with mortality in *Drosophila* after TBI (59). It appears that alterations of genes involved in tissue barrier function and homeostasis were primarily responsible for a higher risk of death after TBI (176). From the metabolic standpoint, TBI contributes to metabolic dysfunction characterized in peripheral organs, which can promote secondary brain damage.

Fly TBI models have expanded the animal findings to include changes in metabolic components after TBI. Indeed, findings have indicated that post-injury metabolic derangements with glucose dysregulation promotes mortality (176). In addition, transcriptomic analysis of w^{1118} flies injured with the HIT device showed conserved long intron sequences in genes involved in mitochondrial energy metabolism (70, 177). As gene regulatory mechanisms have emerged as important determinants of cellular functions such as metabolism, these results support the utility of the fly TBI model to uncover translationally relevant physiological processes and provide more mechanistic evidence for secondary cellular injury. Overall, continued research on this frontier will facilitate more important systemic changes on the pathophysiological outcomes of TBI.

5.3. Challenges in modeling TBI in flies

Several common challenges remain inherent to the modeling of TBI in flies and require consideration in interpretation. Lacking a conserved closed cardiovascular and respiratory system in *Drosophila*, complications and contributions from TBI-induced elevated intracranial pressure, arterial blood pressure, irregular breathing, and reduced heart rate are not a factor of outcomes. Inherent differences in the vascular milieu potentially limit the utility of flies to understand vascular injury that promotes local hypoxia and ischemia, secondary hemorrhage that has negative consequences on cerebrovascular autoregulatory function, and metabolic function. Also, differences in the immune strategies between flies and humans offer limited insights into the contribution of the adaptive immune system. A thorough understanding of the adaptive immune responses role in injury, repair, and restoration of tissue homeostasis requires the use of a mammalian system to integrate the information derived from molecular entities and pathways studied in flies. While these factors differentiate fly versus mammalian experiments, they also provide opportunities to isolate relevant pathways without the influence of cardiovascular alterations and adaptive immunity.

5.4 Gaps in knowledge

Despite a small brain, small body size, and the presence of an exoskeleton, *Drosophila* have an intact sophisticated and well understood nervous system and innate immune system. Several fly models of TBI have been developed and modified over the years for direct relevance to human TBI. The remarkable progress made in recapitulating principal features of TBI in flies is supported by the understanding that cellular pathways involved in TBI pathogenesis are present in both flies and humans. To address how acute TBI-induced injuries can transition to persisting and late-onset morbidities relevant to human post-TBI symptoms, multiple fly TBI models have been developed and show conserved behavioral and secondary cellular changes that are analogous to human TBI. Fly TBI models use mechanical force and a continuum of injury severities to induce brain trauma, similar to human TBI and mammalian models.

6. Strategies for using *Drosophila* in TBI research

The mechanics and methods of injury associated with primary injuries are well documented and can be replicated in animal models. Secondary injuries after TBI are

challenging to study, as mammalian models have redundant signaling pathway proteins, interactions between innate and adaptive immunity, complex local circuits, and higher-order interconnected networks. Mammals also have a substantially longer lifespan, complicating essential longitudinal evaluations required for a temporal and comprehensive understanding of beneficial vs. detrimental downstream cascades. *Drosophilae* are gaining traction as a model for TBI research as novel insights into the causative mechanisms involved in secondary injuries can be better studied due to the relatively simple brain, control of gene expression, and conserved brain function and neurotransmitters (71, 178). *Drosophila melanogaster* models of AD, PD, and ALS are already established and contributing useful information on neurodegenerative diseases (reviewed in (179, 180)). Genetically modulating gene expression to isolate targets implicated in playing a role in secondary injury is relatively simple in flies due to the lack of redundant signaling pathway proteins. Findings can then be validated in vertebrate animal models that are physiologically more similar to humans.

6.1. Utility of Drosophila as a model for TBI and neurodegenerative disease

While etiologies of neurodegenerative diseases are largely unknown, in rare cases, disease-causing mutations are inherited. Observing effects of disease-causing mutations offers clues to pathophysiology, but complex neuroanatomy and genetic redundancy complicate brain injury studies in mammals. The relatively small fly brain controls shared fundamental behaviors that are facilitated by activity of functionally homologous structures and conserved neurotransmitters, receptors and signaling pathways (181-183). Flies have orthologs or homologs of many human genes implicated in neurodegenerative diseases including, *APP* (*Appl*), *MAPT/TAU* (*tau*), ATM (tefu), *BACE1* (*bace*), *LRRK2* (*Lrrk2*), *PRKN* (*park*), PINK1 (*Pink1*), DJ1 (*DJ-1alpha*, *dj-1beta*), *TDP-43* (*TBPH*), *SOD1* (*Sod2*), Frederick's ataxia-associated *FXN* (*fh*), and spinal cerebellar ataxia-associated *ATXN1* (*atx-1*), (184-195). The ability to control gene expression, and the ease of conducting high throughput measurements of stereotypical behaviors make *Drosophila* an attractive model for elucidating contributions of conserved signaling pathways to neuropathology (71, 178). Fly TBI studies also provide additional insight into putative neuroprotective compound screening.

Drosophila models of AD, PD, ALS, and ataxia-telangiectasia implicate aberrant mitochondrial dynamics, oxidative stress, inflammation, disrupted protein turnover, altered autophagy events, and apoptosis in their pathology, all of which are consistent with TBI pathology (72, 76, 179, 180, 196-206). For example, tau phosphorylation increases under cell stress in *Drosophila,* although it does not seem prone to aggregation in flies (64). A variety of genetic reporters of oxidative stress are available, including MitoTimer and redox-sensing GFPs (roGFPs) that can be targeted to the cytosol or mitochondria (207-210). Fluorescent markers like mitoGFP and mCherry-atg8a can be used to observe mitochondrial dynamics and autophagy initiation, respectively; the two can be used in conjunction to quantify autophagosome-to-mitochondria recruitment as an initial event in mitophagy (72, 209). Available at the Bloomington Drosophila Stock Center in Bloomington, IN, are fly stocks that harbor genetic markers of innate immunity/inflammation, including those for circulating macrophages (hemocytes; no. 30140), CD36 member Cr12 (no. 25041), nitric

oxide synthase (no. 76766), toll receptor-ligand spaetzle (no. 83269), and anti-microbial peptides (no. 36558; https://bdsc.indiana.edu/index.html). A wide variety of mutant stocks and stocks harboring RNAi, CRISPRCas9 machinery, or overexpressing constructs for a wide variety of genes involved in neurodegenerative disease studies are also available. Protocols measuring deficits in fly behaviors associated with neurodegeneration include assessments for memory, sleep behavior, aggression, courtship, gut dysbiosis, lifespan, and mobility (211-216).

6.2. Sex, development, and peripheral effects after TBI

Since the use of *Drosophila* as a model organism for TBI is relatively novel, many gaps in knowledge are ripe for exploration. Historically, clinical and preclinical neurotrauma research did not include females or treat them as an independent variable. Recent reports indicate profound sex differences in neuroinflammation, response to treatment, fertility, and long-term morbidity; where valuable information can be discovered using flies (217). Clinical and preclinical studies indicate that estrogen has neuroprotective and anti-inflammatory effects, with evidence that females may have a higher tolerance to stress and injury. Flies express estrogen related receptors and synthesize the ligand, ecdysone, from their diet (218), indicating several approaches to evaluating the lineal roles of this receptor-mediated pathway in TBI-induced secondary injury cascades. TBI in adult women can lead to an increased risk of preterm labor/delivery and their infants developing neurologic disorders. Flies exposed to TBI (HIT method) have decreased pupation, longer larval length, faster pupae formation, and metamorphosis rates (63). Offspring from brain-injured females also had decreased negative geotaxis and implications of impaired social interaction (63). Together, these data indicate that the secondary injury cascades activated after TBI during pregnancy can directly impact first-generation offspring in flies, mice, and humans (219). TBI in 0-7 days post-eclosion *Drosophila* caused intestinal barrier permeability associated with secondary damage to the septate and tight junctions. Permeability was demonstrated by increased passage of bacteria and dietary glucose across the intestinal barrier, which activated the innate immune response and was associated with a higher incidence of death within 24 hours of injury. Antibiotic administration decreased the number of endogenous bacteria, but did not improve survival after TBI, indicating that increased mortality is not directly due to increased permeability to bacteria, but primary or other associated secondary injury cascades (59).

7. Concluding remarks

TBI in *Drosophila* provides the opportunity to isolate and evaluate specific secondary injury cascades for a deeper understanding of the development of chronic neurological deficits. Injuries in flies recapitulate pathological pathways associated with oxidative stress, innate neuroinflammatory response, axonal degeneration/regeneration, and cell death, providing feasibility and essential groundwork for future research (Figure 6). Loss of responsiveness, discoordination, disorientation, wing splay, and post-TBI activity indicate good face validity compared to rodents and clinical observations. Full body and head-specific injury models and established behavioral paradigms similar to those currently used in rodent models are optimized and available for the longitudinal evaluation of behaviorally relevant circuit

function. Genetically modifying *Drosophila* serves as a complementary tool to isolate and modulate pathophysiological mechanisms and assess the impact on gene expression, molecular function, and behavioral outcomes. High throughput compound screening is feasible due to rapid reproduction (breeding), short lifespan, and affordability of the flies to identify therapeutic compounds to test in mammalian TBI models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Traumatic brain injury (TBI)-induced secondary injury cascades are simplified and conserved in flies
- Fly TBI models trigger oxidative stress, innate immunity, cell death, and neurodegenerative pathways
- Permits the isolation and modulation of pathways responsible for phenotypically similar behavioral deficits in mammals
- Short lifespan allows detailed longitudinal tests to identify therapeutic windows and test interventions
- Validation of environmental, biological, and genetic factors can guide and accelerate translational TBI research

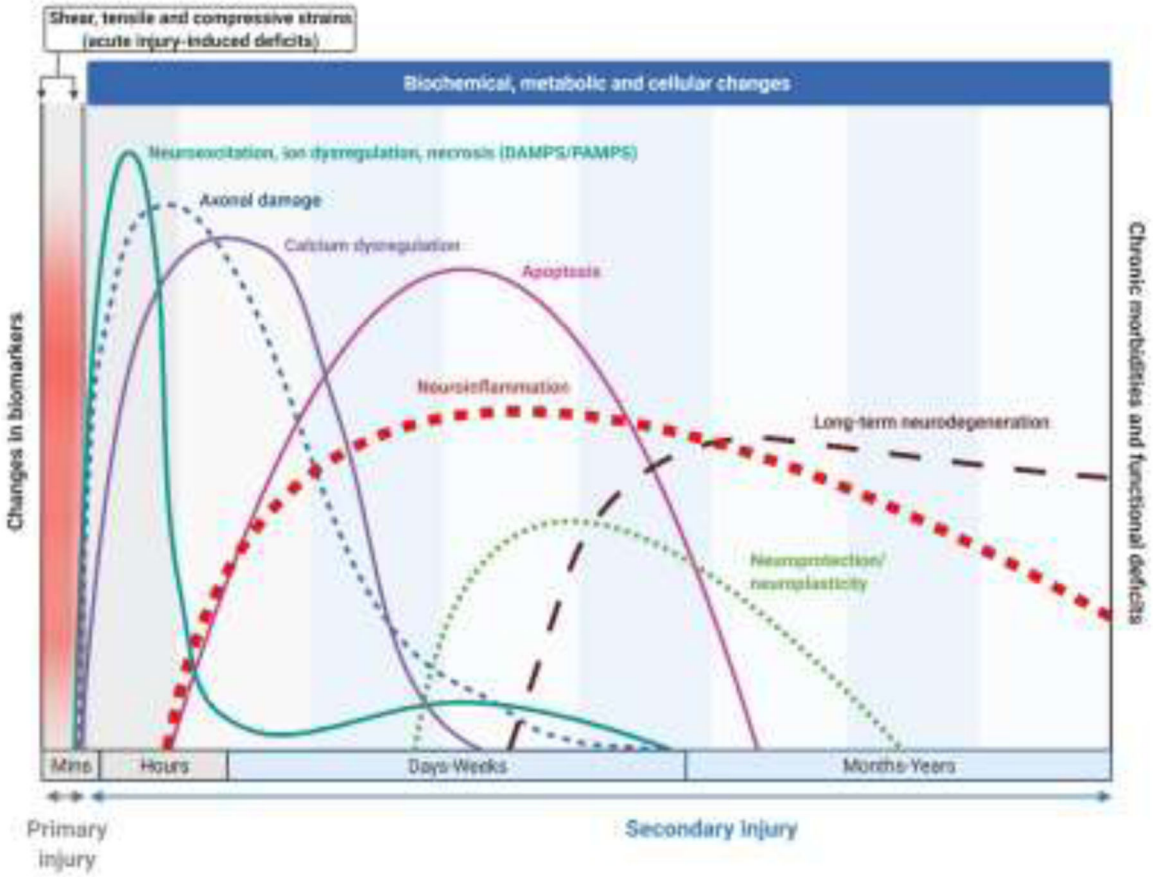

Figure 1.

Changes in TBI pathophysiological biomarkers over time post-injury. Graphs are not representative of actual fold changes (modified from Bramlett and Dietrich, 2015 and Simon et al., 2017) (31, 32). Created with BioRender.com

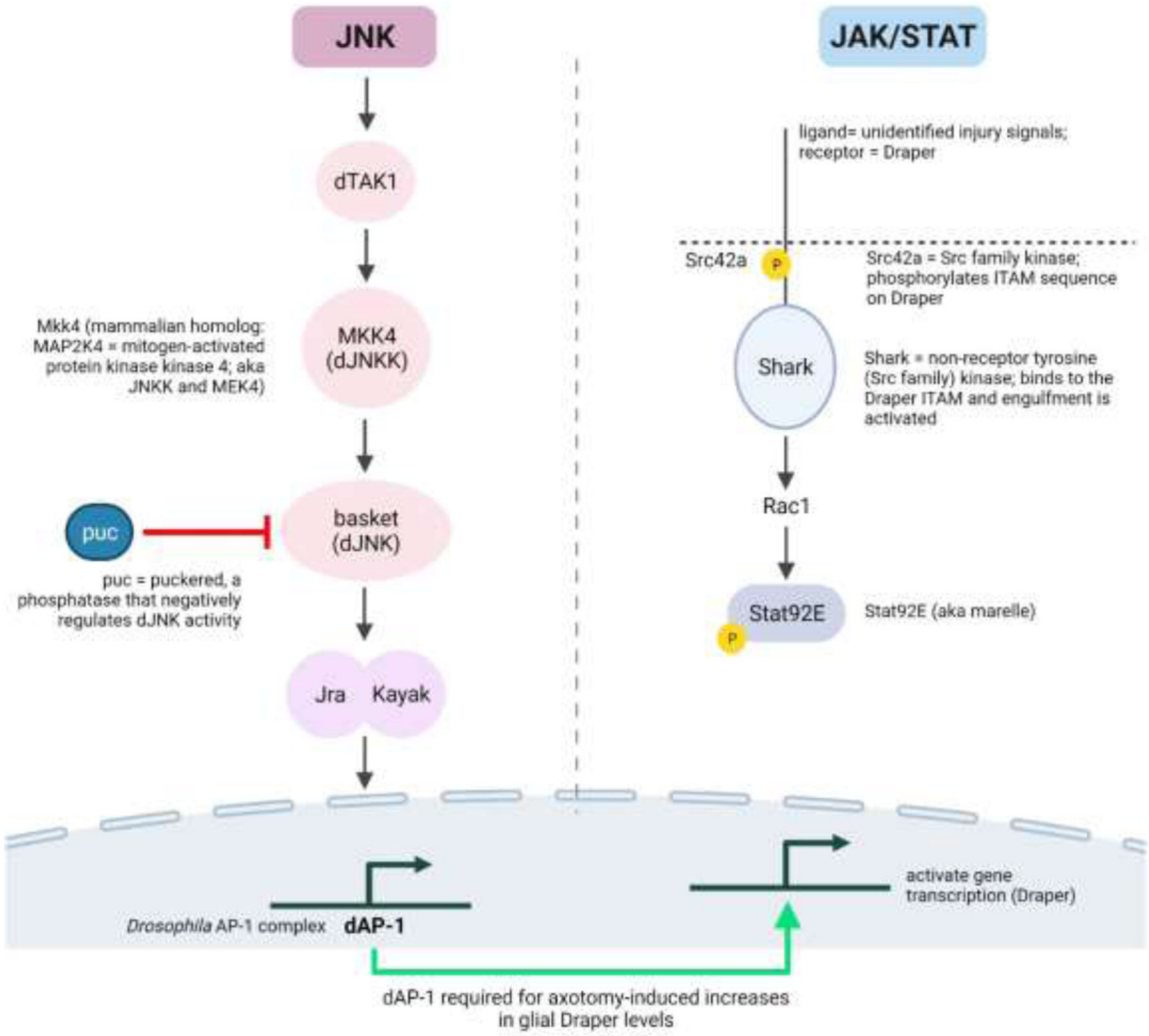

Figure 2.

Immune signaling pathways in *Drosophila* that are activated in response to CNS injury. The red line indicates negative interactions between the indicated pathways. Created with BioRender.com

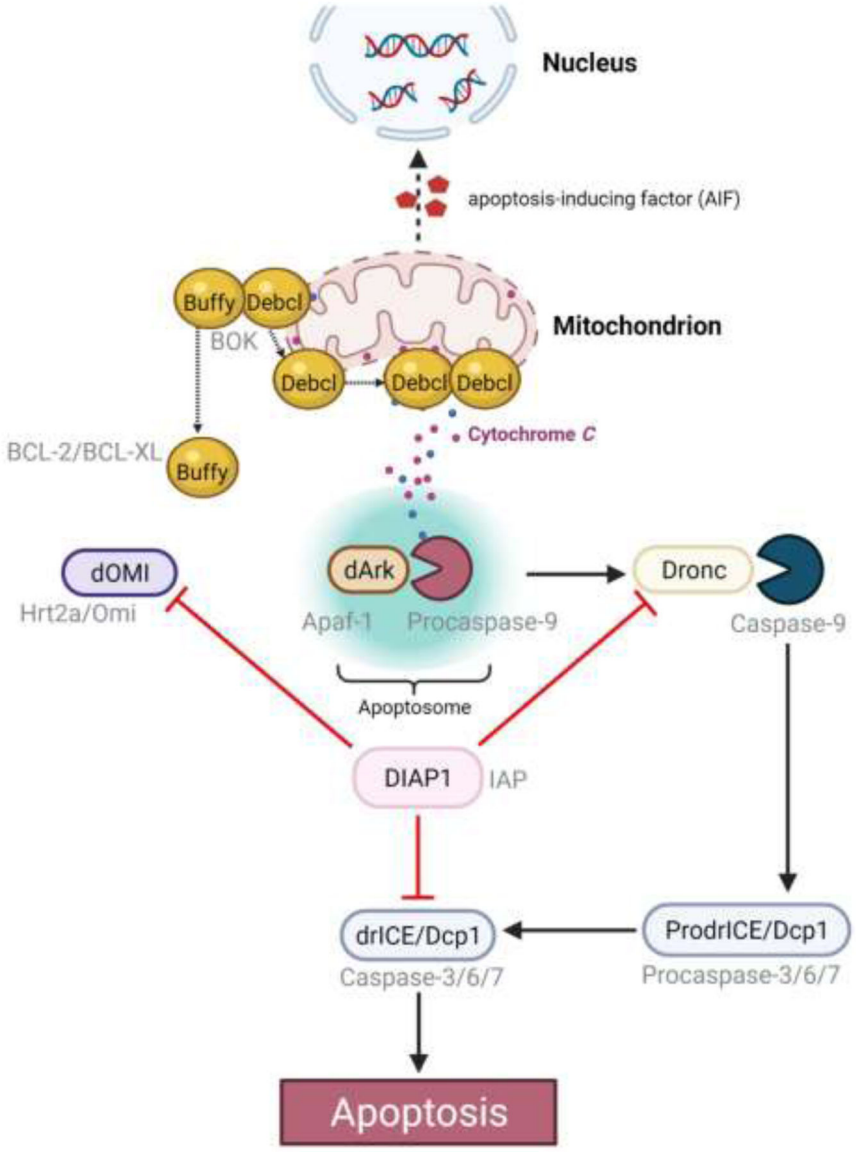

Figure 3. Intrinsic apoptosis in *Drosophila*.

The scheme above depicts interactions of *Drosophila* apoptosis players and their mammalian (in grey font) orthologs and homologs. Dashed arrow (⇢) indicates translocation. Created with BioRender.com

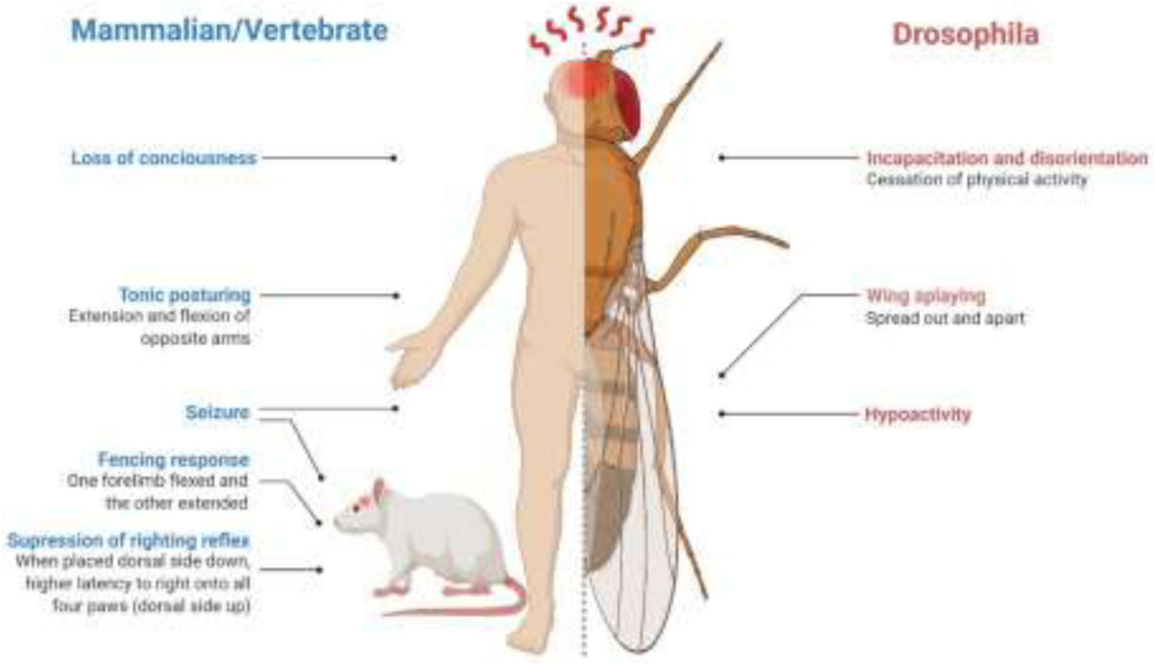

Figure 4.

Phenotypic similarity of mammalian/vertebrate and *Drosophila* acute response to diffuse TBI. Created with BioRender.com

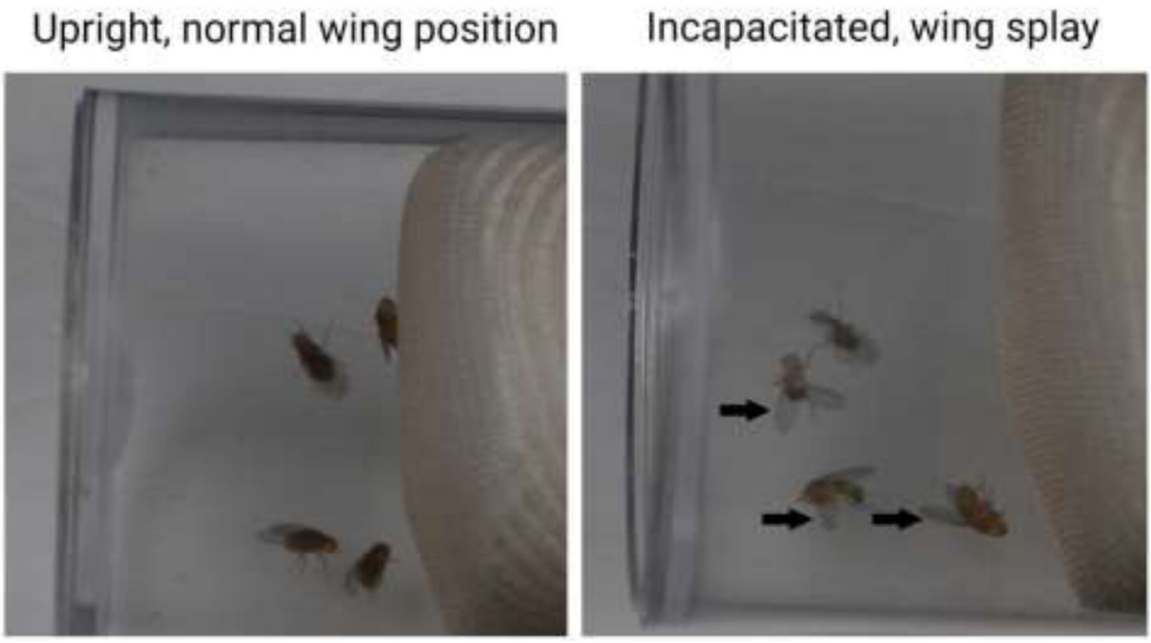

Figure 5.

TBI-induced *Drosophila* phenotypes. Sham and injured flies are shown trapped in the end of a standard food vial as part of a HIT device. Normal upright positioning and wing placement are depicted in uninjured flies (left). After TBI, Drosophila can become incapacitated, inverted, and their wings splayed (right, black arrows).

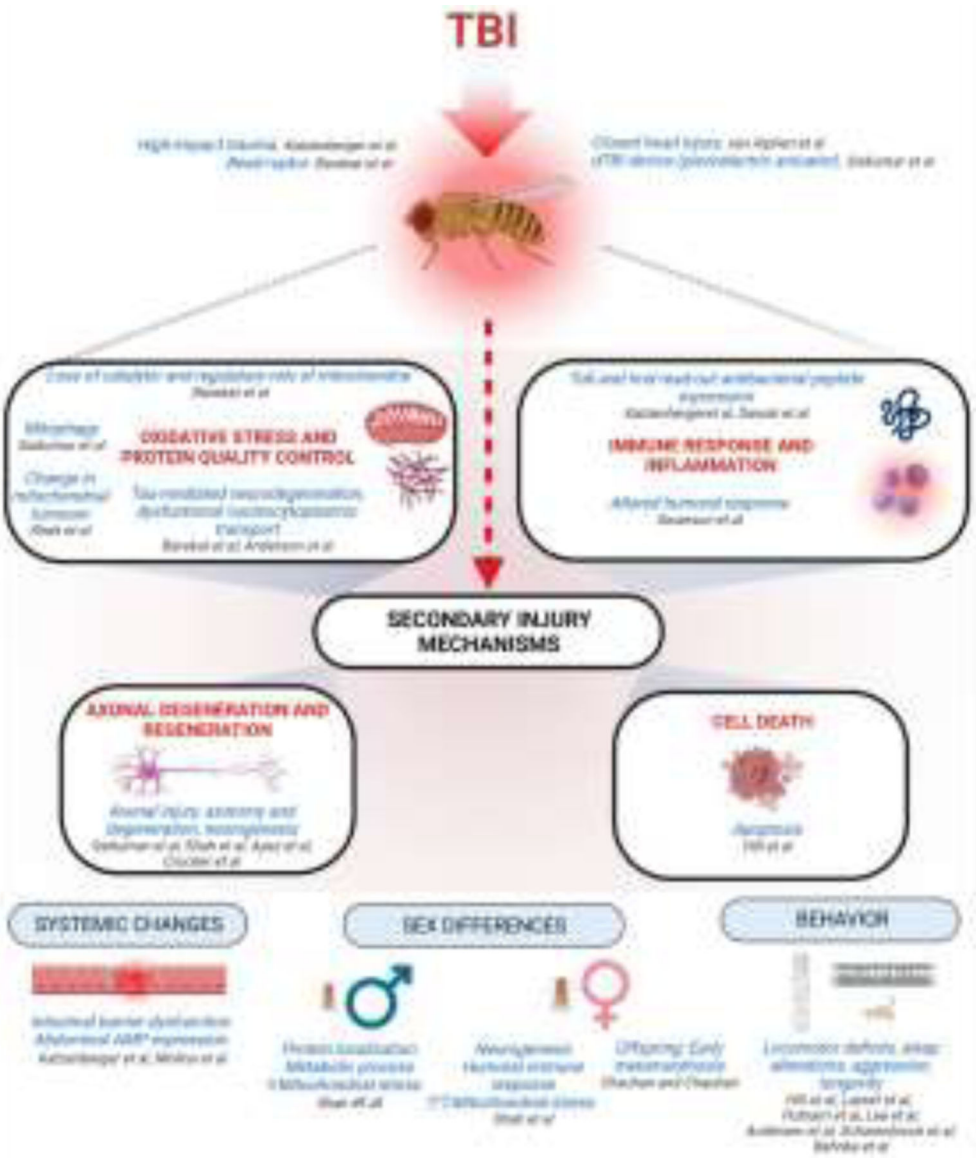

Figure 6.

Summary of findings from fly TBI studies to elucidate injury models, secondary injury mechanisms, systemic changes, sex differences, and behaviors (220-223). Created with BioRender.com

Table 1.

Vertebrate/mammalian homologs of *Drosophila* immune signaling molecules. Homologs listed were obtained from FlyBase, unless otherwise noted in the main text of the manuscript. Table subheadings indicate the relevant section of the manuscript in which they are discussed.

HOMOLOGS	
Drosophila melanogaster	**Vertebrate/mammalian**
4.2 Immune Response and Inflammation *Imd signaling*	
PGRP-LC (Peptidoglycan recognition protein LC)	*PGLYRP1* (peptidoglycan recognition protein 1)
Fadd (Fas-associated death domain-containing protein)	*FADD*
Diap2 (Death-associated inhibitor of apoptosis 2)	*BIRC2* (Baculoviral IAP repeat-containing 2)
Tak1 (Transforming growth factor-b activated kinase 1)	*MAP3K7* (mitogen-activated protein kinase kinase kinase 7)
IKKb	*IKBKB* (inhibitor of nuclear factor-kappa B kinase subunit beta)
key (kenny)	*IKBKG* (inhibitor of nuclear factor-kappa B kinase regulatory subunit gamma)
Dredd (Death related ced-3/Nedd2-like caspase)	*CASP10/CASP8* (Caspase-10/Caspase-8)
casp (caspar)	*FAF1* (human Fas-associated factor 1)
Rel (Relish)	*NF-kB1* (p105)/NF-kB 2 (p100)
Toll signaling	
T1 (Toll)	*TLRs* (Toll-like receptors)
MyD88	*MYD88* (MYD88 innate immune signal transduction adaptor)
pll (pelle)	*IRAK4* (IL-1 Receptor-associated kinase 4)
cact (cactus)	*NFKBIA* (NF-kB inhibitor alpha)
Dif (Dorsal-related immunity factor)	*RELA* (RELA proto-oncogene, NF-kB subunit/p65); *RELB*
dl (dorsal)	*REL* (REL proto-oncogene, NF-kB subunit); *RELA*
JNK signaling	
hep (hemipterous)	*MAP2K7* (mitogen-activated protein kinase kinase 7)
Mkk4 (MAP kinase kinase 4)	*MAP2K4* (mitogen-activated protein kinase kinase 4)
bsk (basket)	*MAPK10/MAPK8* (mitogen-activated protein kinase 10/8)
Jra (Jun-related antigen)	JUN (Jun proto-oncogene, AP-1 transcription factor subunit)
kay (kayak)	FOS (Fos proto-oncogene, AP-1 transcription factor subunit)
JAK/STAT signaling	
dome (domeless)	*PTPRQ* (Protein tyrosine phosphatase receptor type Q)
hop (hopscotch)	*JAK1/JAK2* (Janus kinase 1/2)
Stat92E (Signal-transducer and activator of transcription protein at 92E)	*STAT* (Signal transducer and activator of transcription)
Socs36E (Suppressor of cytokine signaling at 36E)	*SOCS* (Suppressor of cytokine signaling)
Tep1/Tep2 (Thioester-containing protein 1, 2)	*CD109* (a2-macroglobulin/C3 family)
HDAC1 (Histone deacetylase 1)	*HDAC1* (Histone deacetylase 1)
Dsp1 (Dorsal switch protein 1)	*HMGB3* (high mobility group box 3)
4.3 Axonal Degeneration and Regeneration	

HOMOLOGS	
Drosophila melanogaster	**Vertebrate/mammalian**
drpr (draper)	*MEGF10* (multiple EGF like domains 10)
Shark (SH2 ankyrin repeat kinase)	*ZAP70* (zeta chain of T cell receptor-associated protein kinase 70)
Src42a (Src oncogene at 42A)	*FRK* (Fyn related Src family tyrosine kinase)
ced-6	*GULP1* (GULP PTB domain-containing engulfment adaptor 1)
egr (eiger)	*TNFSF* (tumor necrosis factor superfamily)
wgn (wengen)	*NGFR* (nerve growth factor receptor); *TNFRSF* (TNF receptor superfamily)
Traf6 (TNF receptor-associated factor 6)	*TRAF6* (TNF receptor-associated factor 6)
4.4 Cell Death	
AIF (Apoptosis-inducing factor)	*AIFM1* (Apoptosis-inducing factor 1, mitochondrial)
Buffy	*BOK* (Bcl-2-related ovarian killer protein)
Cyt-c-d (Cytochrome c distal)	*CYSCS* (cytochrome c)
Cyt-c-p (Cytochrome c proximal)	*CYSCS* (cytochrome c)
Dark (Death-associated APAF1-related killer)	*APAF-1* (Apoptotic protease-activating factor 1)
DrICE (Death related ICE-like caspase)	*CASP3/6/7* (Caspase 3/6/7)
Dronc (Death regulator Nedd2-like caspase)	*CASP2/9* (Caspase 2/9)
Diap1 (Death-associated inhibitor of apoptosis 1)	*XIAP* (X-linked inhibitor of apoptosis)
Dcp-1 (Death caspase-1)	*CASP3/6/7* (Caspase 3/6/7)
Debcl (Death executioner Bcl-2)	*BOK* (Bcl-2-related ovarian killer protein)
HTRA2 (HTRA2-related serine protease)	*HTRA2*
Tor (Target of rapamycin)	*MTOR* (Mammalian target of rapamycin)