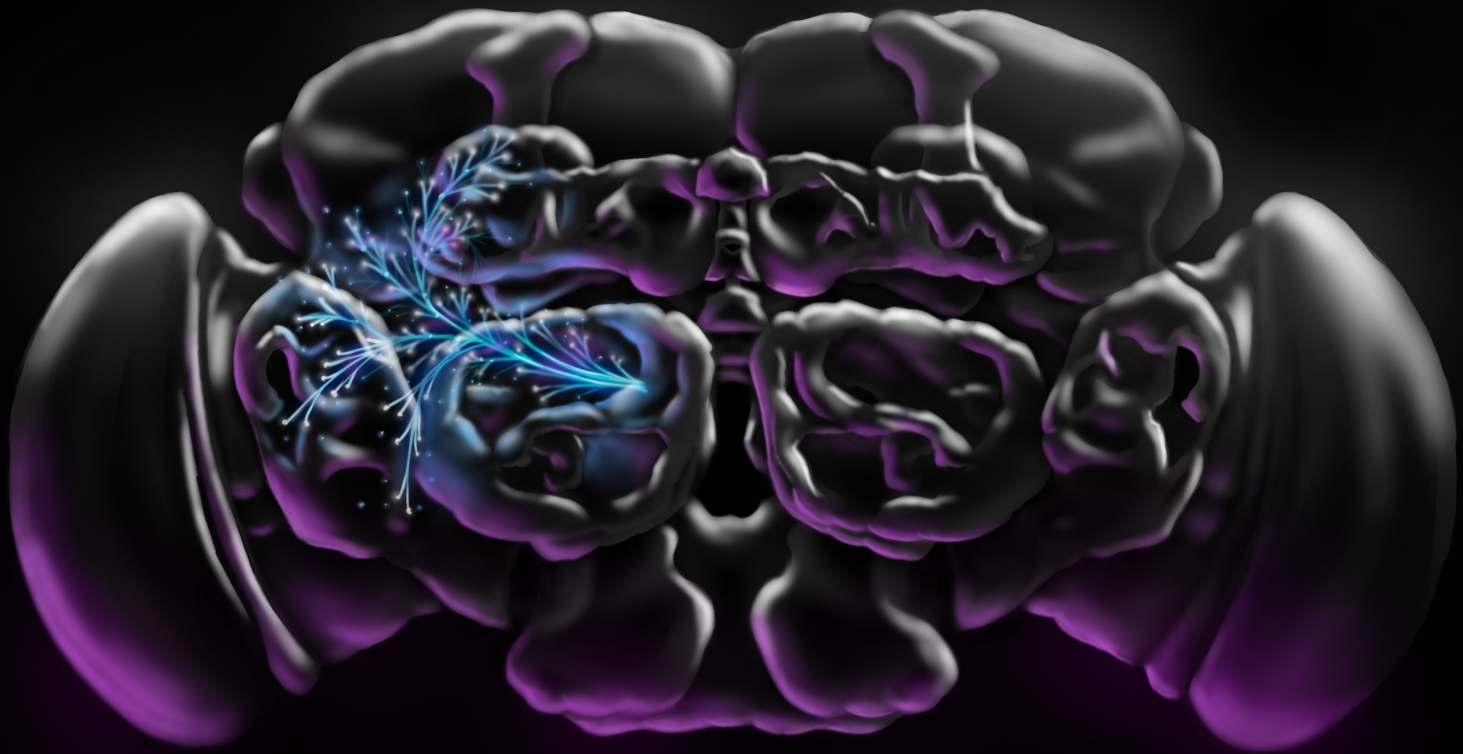
USJ Undergraduate Science Journal



VOLUME I

The Role of Ketone Bodies in Delaying Neurodegeneration Caused by Traumatic Brain Injuries in a *Drosophila melanogaster* Model

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Letter from the Editor-in-Chief

Sofya Levitina, UConn '24

Ideas are not born in solitude. Ideas spring out in lively conversation, they shape up in inspired collaboration, and they come true only as crimes of passion. The conception of the idea of the Undergraduate Science Journal I will always credit to a quick-witted discussion that my best friend, Nidhi, and I had in her dorm room way past waking hours. We were too caught up brainstorming the possibilities UConn students could have if it were easier to publish undergraduate research papers to notice time passing. Inspired by the amount of scientific discovery and curiosity the UConn community has, the recognition of how often it went unnoticed made the solution seem so simple. We were to be the ones to notice it and bring it to light through a publication dedicated exclusively to student research. Without a name and an understanding of how this would ever work, I searched for people who would share my devotion to the idea, as the idea itself was all I had in that moment, of starting a STEM-focused journal on campus.

Eventually, after weeks of elevator pitches and rejections, a group of five students came together to become the first executive board of what was to become known as USJ. It was a whiteboard in a windowless room that first saw the mapped out structure of the organization: there will be teams with separate areas of responsibility, there will be team leaders who meet weekly, and there will be work that needs to get done. And work there was. Recruiting, searching for papers, editing, promoting, putting up events and fundraisers, reaching out to organizations on campus, speaking to professors, writing and erasing, making mistakes and correcting them, disagreeing, apologizing, becoming a better team, and finally even publishing… But let me not get ahead of myself. The first order of business was not fantasizing about what such a group could accomplish, it was ensuring that our process was fair. We wanted a real journal, therefore we wanted a committee of dedicated faculty members who could ensure a proper peer-review process. With pure luck, we were able to convince six distinguished UConn professors across all STEM disciplines to help our cause. The Faculty Review Committee came together as volunteers out of their good will and as a gesture of their support of undergraduate students. Their faith in our vision and in us served as a catalyst, the final push for the creation of this journal. Retrospectively it is clear to me that it wasn't us they believed in - it was you. And this issue, the very first issue of the Undergraduate Science Journal, serves as concrete proof that they were right.

Starting this organization is something I know I will always be proud of. Seeing USJ, originally a budding idea being tossed around by two girls on the floor of a dorm room, fill with students impassioned by our goals and willing to put in hours of hard work, established a new conviction within me - a conviction that UConn students get it done. It was a newfound confidence that UConn graduates will make the world a better place.

Success is not achieved in solitude. I am so grateful to all the people who made this issue possible. Thank you to everybody who submitted their papers to us. Thank you to everybody who came to our events, supported our mission, and helped us achieve our goals. Thank you to everybody who believed in us. And a special thank you to the USJ team. They worked tirelessly, they overcame every challenge we faced, and they are the only reason you are able to read this. I hope in my life I will always be among such dedicated, passionate, talented, and humorous people, although I doubt it. This team is one in a million.

As any story told for the first time, this letter is overlong and fervent. If I dare to say - in short, I am beyond excited to present to you the very first issue of the Undergraduate Science Journal, and I am looking forward to seeing what USJ will accomplish next.

A Brief Overview

Introduction

Traumatic brain injuries (TBIs) are the leading global cause of death and injury, accounting for more than 50 million people suffering every year. In traumatic brain injury, a process known as neurodegeneration causes the death of neuronal cells, which can lead to diseases such as Alzheimer's Disease and Chronic Traumatic Encephalopathy. There are not many existing treatments to traumatic brain injury other than rehab to carry out basic, everyday tasks. But, research has shown that a ketogenic diet—a high-fat, low-carbohydrate diet designed to mimic fasting—can elevate brain energy and may be a potential treatment option. Directly injecting the molecules that result from a ketogenic diet, known as ketone bodies, has shown to delay neurodegeneration in animals. There are three ketone bodies: acetoacetate, 3-beta-hydroxybutyrate (BHB), and acetone. BHB is shown to have neuroprotective properties, and prior experiments on common fruit flies have shown that ketone bodies have shown benefits that arise from a ketogenic diet such as an extended lifespan. Thus, ketone bodies may be a sustainable alternative to the ketogenic diet. This paper hypothesizes that treating flies under a control diet and a ketone body infused diet will show a delay in neurodegeneration progression in the latter group.

Background and Description

TBI is classified in three categories: closed head, penetrating, and explosive blast penetration TBI, which is a more recently-established category in light of modern-day technology and its uses. Two different time-related classifications also play a role in TBI's effects and treatments approach. Primary injuries happen immediately and instantaneously while secondary injuries are delayed due to the initial issues being heightened or complex over time due to other processes. Many other symptoms can coincide with TBI other than the typical TBI events that

help to categorize them from the aforementioned classifications. The most common symptoms are among those like fatigue, blurred vision, sleeplessness, and noise and light sensitivity and can also be broken up into physical symptoms and cognitive symptoms. These symptoms can lead to many other post-TBI advancements that affect the brain on both cellular and tissue levels, later affecting the quality of life due to cell death and inflammation. Atg8 proteins regulate autophagy, a process that eliminates excess and unnecessary macromolecules, damaged organelles, and intracellular pathogens via fusion with lysosomes. Thus, Atg8 proteins can be used to determine the presence and severity of disease states such as neurodegeneration. The more these proteins are detected the more upregulation of autophagic processes, less neuronal damage should be present and vice versa. Namely, the development of chronic traumatic encephalopathy (CTE) is strongly associated with TBI. CTE is a progressive and fatal brain disease characterized by memory loss, aggression, impulsivity, depression and more. These symptoms tend to appear anywhere around 8-10 years after repetitive brain injury. There remain very limited treatment options for TBI victims, particularly due to the broad range of clinical symptoms that present with varying severity. The common fruit fly, known as Drosophila melanogaster, was employed as a genetic and physiological model for this research. Flies were provided a ketogenic diet to observe the neuroprotective properties of ketone bodies, which alleviate cognitive and physical damages. A ketogenic diet is high in fats and low in carbs, allowing for the metabolism to resort to gluconeogenesis that then make ketones the dominant source of fuel for the body in a process called ketogenesis.

Methods

There are three main components to the methodology of this paper: Fly reproduction and feeding, fly brain traumatization, and brain tissue extraction & analysis. There were 8 conditions for the flies: sex (male/female), diet (control/ketone supplemented), and TBI induced (banged/unbanged). The researchers used 100 flies for each condition, hence set out for 1000 flies in the f2 generation to make sure there were enough at the time of eclosion. This was achieved with 11 propagation vials. The new generation was sorted into sex segregated vials, each with 10 flies, with half having the control diet and half having the ketone supplemented diet. The researchers use a HIT (high impact device) to give the flies TBI on the 5th day of their adult life cycle.

After TBI is induced, the flies are placed into fresh vials with food of their respective types. On day 8 of the adult life cycle, the flies are placed into a bucket with ~1 lb of dry ice for 5 minutes, then removed to begin tissue harvesting. The brain matter is removed carefully by forceps, then blended into a liquid. The blended tissue is analyzed for protein concentration through a process known as a BCA Assay, after which the samples are incorporated into gel and are analyzed for their proteins through a process called Electrophoresis, where different types of proteins are segregated based on their reaction to an electrical current passing through the medium. The gel is imaged for analysis, and is finally transferred onto another membrane and imaged again.

Results, Discussion & Conclusion

Bicinchoninic acid (BCA) assay, western blot electrophoresis, and membrane transfer methods were used to study the movement of proteins in fly brains. When measuring these proteins in BCA, no significant differences could be identified in the tissues. This analysis was used for technical practice in preparation for the next analyses, so these results were expected. In addition to this, Western blot analysis was used to separate and identify proteins. It was found that lanes with MKBB, MCuB, FKBuB, and FCB diets had the strongest protein signal, revealing a high protein concentration in these samples. Zooming in further to observe lowly expressed proteins, these strong lanes were pushed to the surface of the gel using membrane transfer, which upon photographing, indicated a prominence of tubulin staining, along with faint markings of Atg8a, a protein that upregulates the management of damaged cells as a result of traumatic brain injuries. Initially, there was little travel of proteins through the gel. To combat this, beta mercaptoethanol was used on the gel in order to dissolve and break apart bonds of proteins in the brain tissue allowing them to flow easier. After seeing not much difference, the researchers centrifuged the samples at 4^{o}C to allow the proteins to travel smoothly through the gel and staggered the voltage to coax out the proteins from the gel walls. In this study there were six brains used per sample. However, if the concentration of brains used per sample increases, there would also be a higher concentration of proteins leading to a signal on the Western blot. The study did not find any significant results. However, this project has many potential leads and spurs knowledge on the interactions between ketone bodies and Atg8a in respect to their neuroprotective properties.

About the Author

Francine Cai is a recent UConn alumni who studied Physiology & Neurobiology with minors in Political Science and Business Analytics. As a UConn undergraduate researcher, she studied metabolism and neurodegeneration in the Tanner Lab and was a part of UConn Model UN. In her free time she loves trying new restaurants and listening to Renaissance.

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The Role of Ketone Bodies in Delaying Neurodegeneration Caused by Traumatic Brain Injuries in a *Drosophila melanogaster* Model

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ABSTRACT

The role ketone bodies possess as neuroprotective agents are examined in traumatic brain injury (TBI) models of D. melanogaster. In particular, the interactions they potentially have with Atg8 proteins, a family of proteins that upregulates autophagy, is studied. Autophagy is a metabolic process through which cellular waste and damaged organelles are managed, including neurons damaged from TBI as well as any neurofibrillary tangles created as a result of TBI (Wesch et al., 2020). Western blots are used to visualize and quantify Atg8 protein in 8 different conditions: non-TBI males on a control diet, TBI males on a control diet, non-TBI males on a ketone body-supplemented diet, TBI males on a ketone body-supplemented diet, non-TBI females on a control diet, TBI females on a control diet, non-TBI females on a ketone body-supplemented diet, and TBI females on a ketone body-supplemented diet. It is hypothesized that flies on the ketone body supplemented diet will see larger volumes of Atg8 protein compared to those not on the ketone body-supplemented diet, suggesting the mechanism by which ketone bodies enact their neuroprotective properties.

Keywords: Traumatic brain injuries (TBI), *Drosophila Melanogaster*, Ketone bodies, Tau protein, Atg8 protein

Introduction

Traumatic brain injury (TBI) has become an increasingly alarming health concern, with about 10 million TBI's resulting in death or hospitalization annually (Langlois et al., 2006). TBI can have debilitating long-term or even lifelong effects on one's physical abilities, cognition, behavior, and emotions that can interfere with daily productivity (McKee et al., 2013). Due to these adverse outcomes, TBI has been described as "one of the most disabling injuries" (Langlois et al., 2006). The link between TBI and neurodegeneration is increasingly evident with TBI possibly triggering multiple pathways that can lead to abnormal protein aggregation in the brain (Gavett et al., 2010).

One of the dominant markers of neurodegeneration is elevated intracellular tau protein; high levels of phosphorylated tau can indicate that certain neural processes are compromised (Orr, Sullivan, Frost, 2017). Under normal physiological conditions, tau is a protein that acts as a support structure and stabilizes the microtubules of the internal cytoskeleton, which can carry nutrients, metabolites, and essential substances, enabling axonal transport (Colodner and Feany, 2010; Alzheimer's Association, 2021). However when tau proteins misfold and aggregate, they can produce neurofibrillary tangles (NFTs), which are highly associated with decreased mental acuity and impairment of higher cognitive functioning (Edwards III et al., 2019); this aggregation of misfolded tau protein is termed "tauopathy." Typically, these tangles are lysed through autophagy, a metabolic process that regulates cellular waste (Nakatogawa, 2020). Autophagy is regulated by Atg8 proteins, but when NFTs are made at a faster rate than they are broken down, net neuronal death may occur (Wesch et al., 2020).

TBI can lead to tauopathy that can catalyze the onset of other neurodegenerative diseases, such as Alzheimer's Disease (AD) and Chronic Traumatic Encephalopathy (CTE; McKee et al., 2013; Edwards III et al., 2019). Those afflicted with TBI have been shown to exhibit increased phosphorylated tau levels compared with those who have not experienced TBI (Edwards III et al., 2019).

Despite its varied and widespread effects, there are not many effective treatments for TBI. One of the main treatments is rehabilitation, with a focus on restoring the patient's ability to carry out everyday tasks such as walking, eating, using the bathroom, and speaking; these exercises require maintenance, and may need to be done after treatment in order to sustain progress (Orr et al., 2017; Brain injury rehabilitation, 2021). Most prescribed medications are given to reduce the symptoms of TBI but do not combat the neurological damage itself (Orr et al., 2017); anti-anxi-

ety drugs, anti-coagulants, antidepressants, and others are given to those who have more acute TBI to ameliorate the cognitive and physical effects, but currently there is a lack of effective treatment that can reduce or delay the neurodegeneration caused by TBI (Orr et al., 2017). However, one promising possibility is the ketogenic diet (KD), which elevates brain energy charge (Appleton and DeVivo, 1974); direct administration of the KD metabolites known as ketone bodies (KBs), has shown promising abilities to delay neurodegeneration in animal models (e.g., Mooney, 2022).

The KD is a high-fat, low-carbohydrate diet that is designed to mimic the fasting state as glucose reserves are depleted over time. The KD induces ketosis, the synthesis from fat stores of ketone bodies (KBs); KBs are then used as a source of energy for the body (Har-Even et al., 2021), especially by the heart and brain. When there is a period of KB utilization lasting about 5-6 weeks, KBs become a significant contributor to the brain's energy source, comprising approximately 60% of the fuel the brain uses (Jensen et al., 2020). There are three physiological KBs, with the two predominant circulating forms being acetoacetate and 3-beta-hydroxybutyrate (BHB) (Laffel, 2000). The third KB, acetone, is exhaled during respiration and is thus irrelevant for consideration as an energy source (Laffel, 2000).

As seen in previous studies, BHB has neuroprotective properties (White and Venkatesh, 2011). It has been proposed that, after inducing a mild TBI (mTBI) in mice, KBs may alter SIRT1 expression and prevent its reduction, which plays a role in induced neuroprotection and hippocampal function (Har-Even et al., 2021). Caloric restriction (which can induce ketosis) and a KD have also been linked to reduced tau phosphorylation in the brain (Har-Even et al., 2021). Previous experiments with KB-treated D. *melanogaster* showed that KD-like effects, such as lengthened lifespan, could be achieved without the need for fasting (Lee et. al 2019). This direct KB treatment indicates a more sustainable alternative to a full KD, which is highly restrictive and not easily maintained (Pfeifer & Thiele, 2005). Male flies subjected to TBI exhibited increases in aggression (Lee et. al, 2019) and impaired learning (Vali & Tanner, unpublished result); flies put on a KB supplemented diet, specifically racemic BHB (a 50-50 mixture of the R- and S- enantiomers), showed a significantly decreased number of post-TBI aggressive episodes even when compared to the control standard diet (Lee et al., 2019; Mooney, 2022).

The induction of TBI in a *Drosophila* model results in apparent neuronal damage (Katzenberger and Wassarman, 2013) and likely results in higher levels of phosphorylated tau in the brain (Lucke-Wold et al., 2014). We hypothesize after treating flies under separate dietary conditions with a KB-free standard high carbohydrate (SHC) control diet (CD), and a SHC diet supplemented with a racemic mixture of BHB, measures of learning in these flies will also slightly improve when on the KB supplementation. It is expected that TBI-subjected flies treated with KBs will show a decrease or delay in neurodegeneration progression as measured by Western blot by levels of phospho-tau in TBI-subjected flies.

Background & Description

Traumatic Brain Injuries

Traumatic brain injury (TBI) has been a leading cause of disability and death globally, with more than 50 million individuals suffering from TBI annually (Ng and Lee, 2019). TBI has been defined as temporary or permanent physical damage sustained to brain tissue, and can lead to impaired cognitive function (Parikh et al., 2007). In many cases, TBI has been a cause of disability, or in worse cases death (Parikh et al., 2007). There are three main categories of TBI: closed head, penetrating, and explosive blast TBI (Ng and Lee, 2019). Closed head TBI events, or more commonly known as a concussion, are the most common amongst civilians, typically caused by blunt impact to the head in motor vehicle accidents, falls, and contact sports (Ng and Lee, 2019). Penetrating TBI, like the name suggests, occurs when an object or foreign body penetrates the skull into brain tissue. Due to the exposure to the environment, the probability of developing an infection can be quite high (Ng and Lee, 2019). Explosive blast TBI is a recently-developed category, where the brain is compromised due to rapid pressure shock waves generated by explosions (Ng and Lee, 2019), most commonly in improvised explosive device (IED) attacks in military contexts.

TBI events can be classified by two different types of injuries in terms of time course – primary and secondary. Primary injuries occur at the initial moment that the injury takes place and can manifest in the form of skull fractures, surface lacerations and contusions of the brain, and intracranial hemorrhage (Graham et al., 1995). Secondary injuries have a later onset, produced by complicating or amplifying processes initiated during impact such as inflammation. Neuronal axons, oligodendrocytes, and vasculature can all be affected by strong tensile forces, leading to a myriad of symptoms (Ng and Lee, 2019). These can present as brain swelling, augmented intracra-

nial pressure, ischaemia, and infection (Werner and Engelhard, 2007). Due to the nature of these injuries, primary or focal damage can only be lessened by preventative measures, while secondary traumas can be treated through therapeutic efforts (Werner and Engelhard, 2007). Depending on the severity of the injury, secondary tissue damage can lead to cognitive deficits, behavioral changes, and muscle weakness or hemiparesis (Ng and Lee, 2019).

Along with the different subclasses of injuries, TBI events can also see another host of symptoms manifest. Some of the most common symptoms that follow a TBI event include headaches, fatigue, dizziness, blurred vision, impaired attention span, noise and light sensitivity, irritability, memory displacement, anxiety, and trouble sleeping (Dikmen et al., 2010). Physical symptoms such as lightheadedness, fatigue, and dizziness typically decrease around 1 month after the TBI event, while cognitive symptoms such as memory issues, trouble sleeping, and irritability can persist up to a year or more after a TBI event (Dikmen et al., 2010). Furthermore, TBI events can lead to the development of many other pathologies later on in the brain on a tissue and cellular level, including excitotoxicity, apoptosis, inflammation, seizures, demyelination, white matter pathology, and decreased neurogenesis (Bramlett and Dietrich, 2015). These pathologies have been highly observed as post-TBI symptoms, which can greatly affect the quality of life for those affected.

One of the resulting pathologies of TBI, excitotoxicity, can lead to cell death through excess release of excitatory neurotransmitters, and is a common pathway to neuronal cell death upon induction of TBI (Baraclado-Santamaría et al., 2022). Excitotoxicity is upregulated when glutamate concentrations increase in the extracellular space. This is commonly due to cellular lysis (Baraclado-Santamaría et al., 2022). Apoptosis, or programmed cell death, another pathology connected to TBI, is thought to be a potential mechanism for the progressive nature of TBI events, specifically with the death of neurons or myelin-producing cells (Bramlett and Dietrich, 2015). It has been observed that both intrinsic and extrinsic pathways of apoptosis have been activated in models of TBI, and more recently, protein markers of apoptosis have been shown to be upregulated in the plasma and cerebrospinal fluid of TBI patients (Bramlett and Dietrich, 2015). Tumor necrosis factor alpha (TNF-α) levels increase 1-4 hours after a TBI event and play a crucial role in activating the extrinsic pathway of apoptosis (Keane et al., 2001). For intrinsic pathways, caspases, a family of cysteine proteases, are responsible for executing apoptosis. Caspases, specifically caspase-8 and caspase-9, have been shown to have increased levels in the brain following TBI, activating the apoptosis intrinsic pathway (Keane et al., 2001).

Furthermore, inflammation can be found in injured brain tissue in TBI patients weeks to months after the TBI event, contributing to further secondary damage and possibly even leading to long-term neuronal disorders (Bramlett and Dietrich, 2015). This outcome can occur because inflammatory cells are associated with the synthesis of chemokines and cytokines, which, when upregulated, can be potentially neurotoxic (Bramlett and Dietrich, 2015). Markers such as interleukin (IL)-1β, TNF-α, and IL-6 were measured to have elevated levels in injured brain tissues after TBI events, suggesting that inflammation persists after TBI (Bramlett and Dietrich, 2015). A combination of, or even one of, these symptoms can make the affected patient more susceptible to neurodegenerative conditions, especially chronic traumatic encephalopathy (CTE).

There is a very strong relationship between traumatic brain injuries and the development of CTE. CTE is a progressive neurodegenerative disorder characterized by the presence of tau neurofibrillary tangles (NFTs) and, occasionally, transactive response DNA binding protein 43 oligomers (Lucke-Wold et al., 2014). Transactive response DNA binding protein 43 is primarily responsible for regulating transcription, and when mutated, becomes

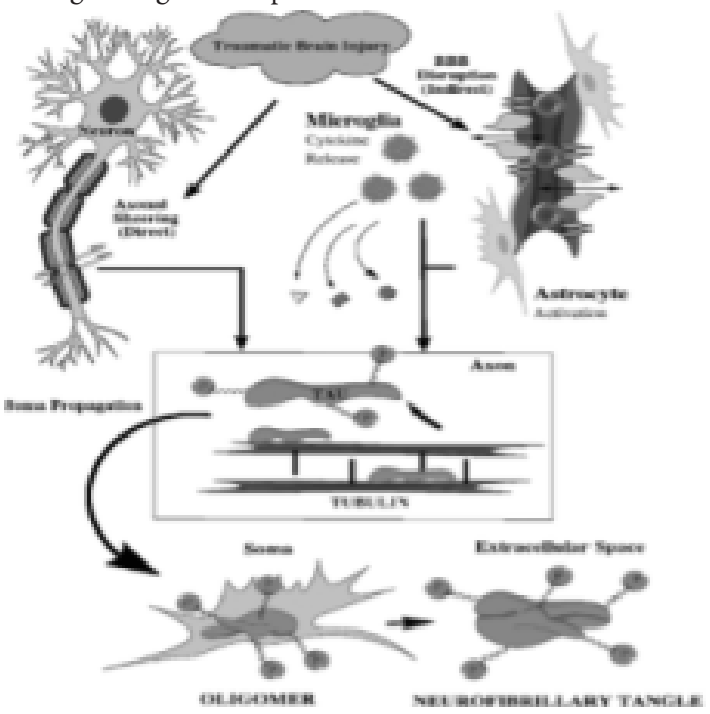

Figure 2.1 The formation of neurofibrillary tangles as a result of traumatic brain injury events
Lucke-World, et. al 2014

a component of ubiquitinated inclusions which contribute to neurodegenerative conditions like amyotrophic lateral sclerosis (ALS) (Gendron et al., 2010). CTE is clinically associated with symptoms of irritability, short-term memory loss, aggression, impulsivity, and depression, which usually appear 8-10 years after experiencing repetitive mild traumatic brain injury (McKee et al., 2013). The hyperphosphorylated tau abnormalities will initially form around the site of injury, then can later evolve to have an irregular perivascular distribution largely localized to the cortical sulci, or the convoluted regions between folds in the surface of the brain (Rubenstein et al., 2019). Neurofibrillary tangles form when tau proteins are dissociated from tubulin upon impact of TBI, exposing multiple phosphorylation sites (Lucke-Wold et al., 2014).

After phosphorylation of tau, it is no longer soluble in neurons, and thus aggregates in large forms producing tau oligomers. These precursors to neurofibrillary tangles can spread out to other neurons, grow, and harden, disrupting motor and cognitive functions (Lucke-Wold et al., 2014). This process is most likely due to NFTs causing neuronal denervation, or loss of nerve supply, ultimately leading to neural cell death (Lucke-Wold et al., 2014). With this established relationship between elevated levels of phosphorylated tau protein (p-tau) and those with CTE, p-tau has become a marker of CTE. This way, increased levels of p-tau can be further investigated into being utilized as a diagnostic marker of CTE, which is usually only confirmed post-mortem (Stein et al., 2014). Due to its aggressive nature, TBI and CTE can exacerbate loss of function in motor, physiological, and cognitive capabilities. The long-lasting damages of TBI emphasize the need for efficient recovery and treatment methods, of which currently there are not many.

The minimal medical or rehabilitation options for those who have endured TBI exclude validated pharmacological treatments for those who have been impacted by TBI. Instead, psychoeducation and providing support in symptom management are two common ways patients of TBI have been rehabilitated (Nelson et al., 2019). The challenges with treating TBI come from the range in which TBI symptoms can present. Due to the wide range of clinical symptoms and their severity, there is no standardized approach to treating those with TBI (Chung and Khan, 2014). Most treatment options are to prevent further damage from TBI and stabilize pre existing issues, but not much has been done with regards to reducing presenting symptoms. Cognitive remediative therapies have proved to be an effective method of treatment; however, due to the wide range of symptoms in TBI patients, treatment is typically assessed and given on a case-by-case basis, tailored specifically to the needs of the individual (Chung and Khan, 2014). With the current limited supply of treatments for TBI, the demand for new treatments and medication is high.

ATG8 Proteins and Neurodegeneration

Atg proteins are a family of autophagy modifiers and participate in multiple pathways, including membrane trafficking, neuronal signaling, and autophagy, one of its most prominent physiological functions (Wesch et al., 2020). Autophagy is a cellular mechanism that, in order to maintain homeostasis, degrades and recycles cellular material in compartments termed autophagosomes (Nakatogawa, 2020). A specific class of Atg proteins, Atg8 proteins, are synthesized as precursors, then processed by Atg4 proteases (Wesch et al., 2020). When autophagosomes mature, Atg8 proteins are ever present as they are conjugated to the inner membrane of autophagosomes when they fuse with lysosomes for degradation (Wesch et al., 2020). Autophagosomes are double-membraned vesicles formed during autophagy which engulf a wide variety of intracellular material to transport to lysosomes to be broken down (Nakatogawa, 2020). This consistent association between Atg8 proteins and autophagosomes illustrates the use Atg8 proteins have as a marker for autophagosome formation, and thus cellular breakdown (Wesch et al., 2020).

Atg8 proteins are a family characterized by a four-stranded central β sheet – with two parallel central strands and two outer strands antiparallel to the central ones. In between the two outer strands lie two α helices (Shpilka et al., 2011). During the autophagic process, Atg8 proteins bind to numerous factors, undergoing conformational changes to support the mechanism (Shpilka et al., 2011). The amino terminal region of Atg8 has been shown to hold a vital role in differentiating functions of the Atg8 protein family, undergoing flexible conformation changes to uphold protein function (Shpilka et al., 2011). There are three subfamilies of animal Atg8 proteins: microtubule-associated protein 1 light chain 3, or LC3, γ-aminobutyric acid receptor-associated protein (GABARAP), and Golgi-associated ATPase enhancer of 16 kDa (GATE-16) (Shpilka et al., 2011). These subcategories of Atg8 proteins all have distinct functions in the autophagy process.

As previously mentioned, Atg8 proteins play a crucial role in regulating autophagy – a process made to eliminate excess and unnecessary macromolecules, damaged organelles, and intracellular pathogens via fusion with lysosomes

(Lee and Lee, 2016). The first connection made between Atg8 proteins and autophagy was established in Saccharomyces cerevisiae, where a single Atg8 protein was marked as an autophagy and cytoplasm to vacuole targeting factor (Shpilka et al., 2011). This discovery has since evolved to include the entire Atg8 family, which is the only class of Atg proteins to be associated with mature autophagosomes (Shpilka et al., 2011). LC3 and GABARAP proteins partake in all steps in the autophagosome biogenesis process, including phagophore initiation and elongation all the way up to autophagosome maturation and lysosome fusion (Wesch et al., 2020). They also possess an extensive role in cargo recognition and selective targeting to autophagosomes, as autophagy receptors have a LIR motif that mediate association between ubiquitin-like domains of Atg8/LC3 proteins. This allows threatening structures such as protein aggregates, damaged mitochondria, and peroxisomes to be flagged and taken in by autophagosomes (Lee and Lee, 2016). Since Atg8 proteins hold a prominent role in autophagy, it can also be used to determine the presence and severity of disease states such as neurodegeneration.

Due to its role in maintaining homeostasis, compromising autophagic processes can lead to the development of various diseases, such as metabolic disorders, cancer, and neurodegeneration (Shpilka et al., 2011). Genetic mutations in autophagy receptors and adaptors can be linked to neurodegenerative disorders as they can alter the affinity that autophagosomes will have for specific cargo. This can lead to buildup of cellular waste, including misfolded proteins, which are a hallmark of neural cell death (Deng et al., 2017). Neurons are particularly sensitive to residual cellular waste as they hold relatively large amounts of cytoplasm and spatial heterogeneous endosomal populations and do not divide, so the waste does not get diluted (Park et al., 2020). In mouse models with Atg genes knocked out, the results led to embryonic or neonatal lethality, indicating that autophagy is absolutely critical for maintaining neuronal homeostasis (Park et al., 2020). In *Drosophila melanogaster*, Atg8 genes have two homologs, Atg8a and Atg8b. Through loss-of-function mutations, Atg8a has been linked to autophagy in various tissues, and complete loss of the gene can ultimately result in death at the late pupal stage (Jipa et al., 2020). Lipidation of Atg8a, the act of attaching Atg8a to the head group of phosphatidylethanolamine, is a hallmark of autophagy as it contributes to autophagosome formation. When Atg8a is lipidated, it takes on the form of Atg8ii and contributes to autophagy. Non-lipidated Atg8a, or Atg8ai, is not active and not used in the autophagy process (Jipa et al., 2020). Atg8b, on the other hand, is known to be present in the male germline and has a prominent role in ensuring male fertility in *Drosophila*, but the bulk of its function remains unknown (Jipa et al., 2020). These established relationships between the Atg8 family, autophagy, and neurodegeneration emphasize the importance Atg8 proteins can have as a marker of neurodegeneration. The more Atg8 proteins detected, the more regulated the autophagic process, and thus the less neuronal damage should be seen. With less Atg8 proteins, the autophagy mechanisms would be disrupted, leading to protein aggregate buildup and greater neuronal cell death.

Ketogenic Diet and Ketogenesis

The ketogenic diet (KD) consists of a high fat, moderate protein, and very low carbohydrate intake (Masood et al., 2022). It was originally used to treat epilepsy and has since amassed a large following due to its weight loss effects (Masood et al., 2022). The idea behind the ketogenic diet is to deprive the body of glucose, or carbohydrates, the body's main energy source. This way, metabolic efforts will thus be directed towards two processes - gluconeogenesis and ketogenesis (Masood et al., 2022). Gluconeogenesis is the mechanism by which new molecules of glucose are synthesized, primarily from lactic acid, glycerol, alanine, and glutamine in the liver. However, when blood glucose levels drop even further, ketone bodies become the dominant source of fuel in the body. Through ketogenesis, ketone bodies are produced by breaking down fatty acids, and three ketone bodies are produced – acetone, acetoacetate, and beta-hydroxybutyrate (Dhillon and Gupta, 2023). Once made, they can then be used as energy by various tissues across the body such as the heart, muscle tissue, and kidneys (Masood et al., 2022). Furthermore, ketone bodies

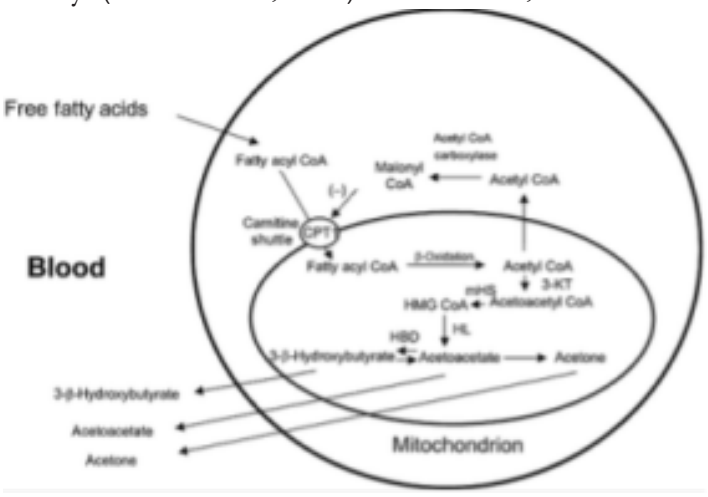

Figure 2.2 The production of ketone bodies via ketogenesis
Laffel, 2000

are the sole alternative source of fuel for the brain as they are able to cross the blood-brain barrier. Compared to glucose, ketone bodies are able to produce more ATP molecules, with 100 grams of acetoacetate yielding 9400 grams of ATP, 10,500 grams from beta-hydroxybutyrate, and only 8700 grams from 100 grams of glucose (Masood, et al, 2022).

Ketogenesis typically occurs in the mitochondria of the liver cells where fatty acids are brought in and broken down into acetyl CoA through beta-oxidation (Dhillon and Gupta, 2023). Two acetyl CoA molecules are then turned into acetoacetyl CoA, which then become HMG-CoA via HMG-CoA synthase. HMG-CoA lyase then comes to turn HMG-CoA into acetoacetate. From here, acetoacetate can be further converted into acetone through non-enzymatic decarboxylation or beta-hydroxybutyrate through beta-hydroxybutyrate dehydrogenase (Dhillon and Gupta, 2023). The two ketone bodies that are used for energy are acetoacetate and beta-hydroxybutyrate; upon exiting the liver tissue and entering other cell types, beta-hydroxybutyrate is converted back to acetoacetate. Then, acetoacetate is broken down into acetyl CoA, which enters the citric acid cycle to produce ATP (Dhillon and Gupta, 2023). This is done as a process known as ketolysis, where energy is produced as a result of ketone bodies going through the citric acid cycle (Dhillon and Gupta, 2023).

Neuroprotective Properties of Ketone Bodies

Ketone bodies have been shown to have a myriad of health benefits, with one of the most promising being its neuroprotective properties. The neuroprotective benefits of high levels of circulating ketone bodies are that they may save, recover, or regenerate parts of the nervous system (White and Venkatesh, 2011). The ketogenic diet started out as a treatment for those afflicted with epilepsy as fasting had great effects on reducing the frequency of seizures (Maalouf et al., 2009). However, long-term fasting is untenable, so R.M. Wilder developed the ketogenic diet to mimic the biochemical and metabolic profile of fasting without the negative side effects of zero caloric intake (Wilder, 1921). The KD is also associated with improving cognitive function, especially within children and adolescents (Maalouf et al., 2009). In both human and animal studies, it has been shown that the ketogenic diet both increases the threshold for seizures as well as delays their development. Outside of epilepsy, some patients with Parksinon's Disease, on average, improved their scores on the Unified Parkinson's Disease Rating Scale by 43% after following the ketogenic diet for about one month (Maalouf et al., 2009).

In mouse models, it has been noticed that ketone bodies have also had positive effects in recovering cognitive and physical damages in mice induced with mild TBI. Mice who were inflicted with mTBI performed worse than mTBI mice supplemented with ketone bodies on cognitive function tests measuring aptitude for visual memory and spatial awareness (Har-Even et al., 2021). mTBI ketone body supplemented mice also displayed less neuronal loss than mTBI mice, with the total number of neurons in the dentate gyrus of the hippocampus being significantly lower in mTBI mice not treated with ketone bodies (Har-Even et al., 2021). Cortical contusion volume, or the bruising of brain tissue, was reduced in a rat model of TBI after treatment of ketone bodies (White and Venkatesh, 2011).

One of the most promising mechanisms that suggests how this could happen is through alteration of SIRT1 gene expression when ketone body levels rise (Har-Even et al., 2021). The SIRT1 gene is responsible for developing the hippocampus and is involved in different physiological processes such as oxidative stress response, genetic silencing, genome stability, and cell line extension (Har-Even et al., 2021). Following caloric restriction, there is a correlation between an increase in neuroprotection and SIRT1 activation (Har-Even et al., 2021). Upon activation, SIRT1 can reduce the number of amyloid aggregates in the brain, a hallmark signal of Alzheimer's Disease (Maalouf et al., 2009). The suggested mechanism by which SIRT1 holds its neuroprotective properties is that SIRT1 exhibits deacetylase activity, acting on targets like the tumor suppressor protein p53, forkhead transcription factors, and the DNA repair protein Ku70 (Maalouf et al., 2009). The deacetylation of the p65 subunit of nuclear factor-kappa B (NF-KB) regulated by SIRT1 expression also contributes to the inhibition of neuroinflammation. NF-KB activity upregulates reactive oxygen species production, which accelerates cellular breakdown (Mishra et al., 2021). Therefore, by repressing NF-KB activity, SIRT1 has the ability to prevent further cellular deterioration (Mishra et al., 2021). When mouse models were subjected to mTBI, it was seen that their SIRT1 levels were significantly reduced compared to mice who were subjected to mTBI and fed a ketone body supplemented diet and mice who did not get subjected to any TBI at all (Har-Even et al., 2021).

Drosophila melanogaster as a Model Organism for Observing TBI

Drosophila melanogaster, the common fruit fly, has many versatile uses as a model organism and comes with many ad-

vantages for studying TBI. One of the main advantages is that D. *melanogaster* has a much shorter lifespan than other model organisms with a lifespan of 60-90 days compared to the 1.5-3 years of the *Mus musculus*, or the common laboratory mouse (Jennings, 2011). This way, researchers can study the effects of TBI and other conditions over the span of a few months rather than a few years. In addition, it only takes about 10-14 days to propagate hundreds of offspring, and D. *melanogaster* will reach sexual maturity one week into their life, making it accessible and efficient to cross certain genetic lines and study offspring (Jennings, 2011). With regard to human biology, 60% of the D. *melanogaster* genome is homologous to humans, and 75% of genes contributing to human disease are also homologous to D. *melanogaster*, making it a great genetic model to study various genes and conditions (Staats et al., 2018; Pandey and Nichols, 2011). Due to the multiple parallels between D. *melanogaster* and human biology, D. *melanogaster* serves as an efficient model for studying different pathological conditions, such as traumatic brain injuries.

In previous studies, neurodegeneration, memory, and sleep have been investigated using the D. *melanogaster* model, which are all affected by TBI events (Katzenberger et al., 2013). Fly and human brains also have similar structural, cellular, and molecular features that make for a seamless translation of effects from one model to another. For instance, the fly brain is also bilaterally symmetrical and is joined to a ventral ganglion that innervates the rest of the fly body – analogous to the human spinal cord (Katzenberger et al., 2013). In the fly brain, it is composed of three parts: the protocerebrum, deutocerebrum, and tritocerebrum, which mirror the forebrain, midbrain, and hindbrain, respectively, in humans. On the outside, the brain is encapsulated by an exoskeleton or cuticle, which acts as a skull for the fly. It is relatively rigid, and provides structural protection from the outer environment (Katzenberger et al., 2013). These structural and functional parallels between fly and human brains are further reasons for why D. *melanogaster* makes a good model for studying traumatic brain injuries.

In previous studies investigating TBI in D. *melanogaster*, it has been found that TBI is a contributor to reducing lifespan, and neurodegeneration has been observed in flies treated with TBI versus those who have not (Katzenberger et al., 2013). In flies, neurodegeneration manifests as vacuolar lesions in the neuropil, an area of the brain containing interwoven fibers, axons, synaptic terminals, and glial cells, making it a communication hotspot. Flies subjected to TBI have displayed small vacuolar lesions in the neuropil while non-TBI flies' brains have a smooth and uniform appearance (Katzenberger et al., 2013). These former studies can potentially contribute to a deeper understanding of the underlying mechanisms that lead to the progress of neurodegeneration and its associated pathologies, such as CTE.

Preliminary Studies

Based upon the previously-established work led by Dr. Geoffrey Tanner at the University of Connecticut suggesting the potentially-neuroprotective properties of ketone bodies, specifically beta-hydroxybutyrate, researchers like Lee et al. (2019) and Mooney et al. (2022) have pursued the idea that exogenously-supplemented ketone bodies – in place of a full KD – could alone act as a neuroprotective agent. In order to do so, they supplemented racemic BHB in the traditional standard high-carbohydrate (SHC) diet for D. *melanogaster* to examine its effects on behavior and longevity following a TBI event. The racemic BHB was added to fly food at concentrations of 2 mM to mimic the typical serum levels of BHB in those who are in ketosis (Mooney et al., 2022). To induce TBI in the D. *melanogaster* model, the High Impact Trauma, or HIT, device was used. The HIT device is a mechanical contraption that consists of a fly vial inserted into a metal spring, attached at one end onto a wooden board, and with the free end able to be pulled back up to 180 degrees and released over a rubber polyurethane pad to deliver high impact (Katzenberger et al., 2013; Lee et al. 2019). The open end of the spring also has a stopper attached to it, allowing the flies to be kept at the bottom eighth of the vial. Each fly makes contact with the pad in varying regions of their head and body, so the resulting primary injuries can differ. To ensure even distribution or consistent contact with the pad, the vial is retracted and released multiple times to inflict TBI multiple times. The HIT device has been successful in producing behavioral effects in fruit flies similar to humans who have undergone TBI events; its after-effects are highly reproducible (Mooney et al., 2022).

With regards to aggression, Lee et al. observed significant reduction in aggressive acts in male flies who were supplemented the ketone bodies, compared to those who were not, after a TBI event. This result indicates the effectiveness of BHB in reducing common behaviors seen in those who have suffered from TBI (Lee et al., 2019). Mooney et al. also had similar observations – a significant reduction in aggressive events and significant increase in latency to the first aggressive event following a TBI event in D. *melanogaster* administered BHB were noted com-

pared to those who were on the traditional control diet.

Examining longevity or lifespan of D. *melanogaster* following TBI indicated that even a single strike to the head can reduce lifespan (Katzenberger et al., 2013). A median lifespan of 38.3 days was measured in flies who received one TBI event, compared to a median lifespan of 48.3 days in flies who did not suffer from any TBI (Katzenberger et al., 2013). Investigating whether or not ketone bodies can aid in reducing this decrease in longevity, Lee et al. found that TBI flies not supplemented with BHB had the shortest lifespan, averaging to about 41.7 days, and KB flies not treated with TBI had the longest lifespan, of about 52.2 days (Lee et al., 2019). However, TBI flies on the control diet did not see a significant decrease in lifespan compared to non-TBI control diet flies. This suggests that ketone bodies extend lifespan in flies without head injury, but not in those who have TBI.

These findings have laid the foundation for further investigation of the role ketone bodies have in treating TBI and delaying neurodegeneration caused by it. Both Lee et al. and Mooney et al. have demonstrated that increased ketone body levels are associated with increasing longevity and decreasing aggression, both impacted by traumatic brain injuries. Understanding this can allow for a deeper dive into the role ketone bodies play in potentially reducing neuronal damage caused by TBI.

Methods

Fly Propagation

With 8 conditions to consider – male control diet banged (MCB), male control diet unbanged (MCuB), male ketone body diet banged (MKBB), male ketone body unbanged (MKBuB), female control diet banged (FCB), female control diet unbanged (FCDuB), female ketone body diet banged (FKBB), and female ketone body diet unbanged (FKBuB) – and an *n* of 100 per condition, 800 total flies were used. To ensure there were enough flies on Day 0, or eclosion day, a target of 1,000 flies in the F2 generation was made. Each propagation bottle can yield about 80 to 100 flies, so in order to reach the target number of 1,000 flies, 11 bottles were used. Canton S wild type flies were used.

Each bottle was filled with about 25 mL of D. *melanogaster* food. 20 females and 15 males were put into each bottle and capped with a gas-permeable cotton plug, or "flug." The flies were added into the bottles by knocking

Table 3.1 The below table organizes all conditions in this project for clarity and cohesion.

Sex	Diet	TBI Condition	*n*
Male	Control	TBI Induced	100
Female	Control	TBI Induced	100
Male	BHB Supplemented	Sham TBI	100
Female	BHB Supplemented	Sham TBI	100
Male	Control	Sham TBI	100
Female	Control	Sham TBI	100
Male	BHB Supplemented	TBI Induced	100
Female	BHB Supplemented	TBI Induced	100

them out using a CO_2 Anesthetizing Apparatus ("CO2 pad") and a CO_2 gun with a stainless-steel needle attached. The needle is inserted into the edge of the top of the bottle, and flies are knocked out in the vial. They are then gently tapped out onto the CO_2 pad, and separated using a paintbrush. Males are differentiated primarily by a black tip at the end of their abdomen, smaller size, and having less segments in their abdomen. Female flies are larger, with a wider abdomen. Their abdomen also comes to a more pointed end compared to males, who have a more rounded abdomen. After the proper number of flies are sorted into each propagation bottle, the flugs are put back on top of the bottle and the flies are put into an incubator. The flies were incubated at 25 degrees Celsius and at ambient humidity, creating optimal conditions for reproduction and development (Mooney et al., 2022).

After the flies were sorted and put into the bottles, they were given 24 hours to mate and lay eggs. This was to ensure that enough eggs were laid and first instar larvae were visible. After the 24 hours ended, the flies were gassed out and emptied from the bottles, allowing only the larvae to develop in the absence of the adult F1 generation. These larvae eclosed in 10-14 days, and on the day of maximum eclosion (the highest number of larvae eclosed in a 24 hr period), typically 11 or 12 days after gassing out the F1 generation, the newly born flies were collected, sorted by sex, and put into narrow vials. 10 flies were put in each vial, separated by sex. Each condition had 10 vials, totaling up to 100 flies per condition. Half of the vials had control diet D. *melanogaster* food, while the other half had the control diet supplemented with ketone bodies (2 mM racemic BHB).

Food Preparation

All the food used to feed the flies is traditional Nu-

tri-Fly Bloomington Food Formulation, and consists of yeast, soy flour, yellow cornmeal, agar, and light corn syrup. The food comes pre-mixed, and one packet makes about a liter of food (Mooney et al., 2022). The packet is emptied into a 2000 mL beaker, and 1 liter of distilled H2O is added to the beaker. The food is then brought up to a boil, stirred constantly with a metal spatula to avoid food burning at the bottom of the beaker. Once the food is brought up to a boil, it cooks for a minute at a boil, then is taken off the heat. The food is then brought down to around 65 to 70 degrees Celsius, which takes about 7 minutes. Once the food has cooled, it is treated with propanoic acid and Tegosept as antifungal agents. 4.9 mL of propanoic acid is added to every liter of food made, and 10 mL of Tegosept is added to every liter of food.

After treatment, racemic (+/-) BHB, was added to half of the food. An aliquot of +/- BHB, containing 0.5 mL of 1M +/- BHB, is needed and mixed thoroughly with a metal spatula. Since 40 vials needed BHB-supplemented food, and 10 mL of food is given per vial, 500 mL of food was treated with BHB since the BHB treatment requires 250 mL of food. The concentration of ketone bodies in each serving is 2 mM, to be consistent with the typical serum levels of an individual currently in ketosis, and with BHB concentrations used for *in vitro* experiments. The food is then poured into fly vials, filling about 10 mL per vial. The food is then covered with cheesecloths to prevent flies from entering the vials, and left to sit at room temperature for about 8-12 hours to set and dry. If food is poured in the propagation bottles, 12 hours is needed to let the food settle. If vials are used, it typically takes 8 hours for the food to set. Once the food has set, it should be more stiff but not dry. The vials are then topped with the flugs and ready to be used.

TBI Induction

TBI induction in *Drosophila* is done using a High Impact Trauma (HIT) device, which was designed in David Wasserman's lab (Katzenberger et al., 2013). The HIT device has the capability to inflict TBI in flies and has a reproducible effect. The device has a metal spring screwed into a wooden board on one end, and another open end where a fly vial can be attached. The open end also has a rubber stopper attached to it, so once a vial is inserted, the flies can be confined to a smaller area within the vial - typically the bottom ⅛ of the vial. Each vial, holding 10 flies, is then pulled back 120 degrees and released. The vial then makes contact with a polyurethane pad and this contact inflicts primary injuries of TBI onto the flies. This process is then repeated for a total of 7 strikes, in order to ensure even distribution of injury amongst the flies due to position variability within the vial. 7 strikes was determined by Lee et al. (2019) to be the maximum strikes that could induce injury without causing significant levels of death. This device can be adjusted by angle and strike frequency to either increase or decrease the severity of closed-head TBI delivered to the flies.

To add D. *melanogaster* to the device, the flies are gently tapped down to the base of the vial, and flipped into a new, empty, food-free vial. The new vial is then placed into the free end of the spring, with the stopper acting as the cap to the vial. Once the vial is properly attached to the HIT device, the flies are pulled back to 120 degrees, and released to bang against the pad 7 consecutive times, with each release occurring 1-2 seconds after the previous one. Flies are subjected to TBI on Day 5 of the adult phase of the fly life cycle. 10 vials of male on the control diet, 10 vials of males on the ketone body-supplemented diet, 10 vials of females on the control diet, and 10 vials of females on the ketone body-supplemented diet were subjected to TBI. After the flies are subjected to the TBI, they are flipped onto new vials with fresh food, containing their respective diets.

Fly Brain Dissections

On Day 8 of the adult phase of the fly life cycle, the flies are all gassed out and transferred to microcentrifuge tubes. Each condition, comprising 10 vials, is combined into one microcentrifuge tube where they are properly labeled. Once transferred, the flies are put into a bucket of dry ice holding about 0.8-1.2 pounds of dry ice. After 5 minutes have passed, the flies are pulled out to begin tissue harvesting. The flies that currently are not harvested are kept on dry ice to prevent further tissue degeneration. The brain tissue is dissected out in a RIPA/HALT cocktail, with a 100:1:1 ratio of RIPA buffer to HALT to EDTA. This buffer is meant to break down and suspend proteins in solution. However, after a few attempts to dissect with the RIPA buffer, it proved to be difficult to extract the brain as a whole piece. To prevent further breakdown, brain tissue was dissected in phosphate-buffered saline, or PBS. This way, the tissue does not break down while harvesting. The PBS is kept on regular ice while dissecting to ensure that it is cold. All extracted brains were stored in microcentrifuge tubes in the RIPA/HALT buffer, with 15 μL of the buffer used per brain. Each aliquot contained 6 brains, so 90 μL of RIPA/HALT total was added.

To dissect the brains, fine forceps were used, and a fly was placed in 10 μL of ice-cold PBS on a microscope slide.

Using forceps, the head was first separated from the rest of the body. Once the head is isolated, one pair of forceps is used to hold down the head at the nose or proboscis while the other pair of forceps removes the eyes and exoskeleton, revealing the brain. Once the brain is exposed, with minimal eye pigment and exoskeleton, the proboscis of the fly is detached and the brain is picked up and placed into the proper microcentrifuge tube. The microcentrifuge tube is then tapped against a hard surface to coax the brain down into the RIPA/HALT solution, and this process is repeated until six brains are in a microcentrifuge tube, and each condition has 3 aliquots filled to run 3 trials.

Homogenization

Once all the brain tissue was collected, the brains were homogenized to release cellular proteins and liquify all brain matter. This way, the liquid can be easily transferred into a gel during Western Blotting and into wells to run a BCA Assay. To homogenize the tissue, the aliquots are kept on ice, and, using a homogenizing tool, the tissue is blended in a pulsatile manner. It is important to not blend the tissue for more than a few seconds at a time so as to avoid the tissue mixture getting frothy or too hot. After 5 pulses, the tool is cleansed by pulsing the blender in a beaker or flask of distilled water. The homogenate is then examined for visible pieces, and if there are still some visible chunks, it is continuously blended until the solution is more or less combined well. Once no chunks are visible, the homogenate is spun in a centrifuge at 15,000 rounds per minute for 10 minutes at 4 degrees Celsius to ensure all unblended matter has separated. The liquid that is yielded after the centrifuge process, the supernatant, is then pipetted out to isolate it from the sediment at the bottom of the aliquot. This process is then repeated until all tissue has been properly homogenized and blended. Between each aliquot, the tool is cleaned and dried with a Kimwipe. The homogenate that is not used is stored in a -20 degree Celsius freezer.

BCA Assay

After homogenization, a BCA assay is used to determine the amount of protein in each sample and the volume of sample needed to input the wells of the gel used for the Western Blot. The BCA assay is conducted by combining a working reagent, made of reagent A and B, in a 50:1 ratio. To fill 12 wells with 200 µL of working reagent in the BCA assay plate, 2 aliquots of 1200 µL of reagent A and 24 µL of reagent B are combined, making 2 aliquots of working reagent totaling 1,224 µL each. Once the working reagent was made, 200 µL of it was pipetted into each well. The first nine wells were filled with an albumin standard, diluted at different concentrations to create a standard curve so the sample protein concentrations can be compared to the standard curve and their protein levels can be calculated. The albumin standards had concentrations of 2000, 1400, 1000, 700, 500, 250, 125, 25, and 0 µg/mL. 25 µL of premade standard were pipetted into the BCA assay as well in descending concentration order, and the last three wells were filled with 25 µL of a different condition's homogenate. The rest of the samples were added into a new row in the BCA assay plate after adding in 200 µL of working reagent first. The process for combining the working reagent mentioned above was repeated to ensure enough reagent was available. After adding in all the respective standards and samples, the BCA assay plate is then incubated for 30 minutes at 36 degrees Celsius and then put into the BioGen BCA Assay reading software to read the concentrations of protein in all the wells.

Once the reading is complete, a standard curve is made by plotting the standard concentrations. Then, the sample protein concentrations are calculated. The calculations are done based on where the sample absorbances are found on the curve, then using the equation of the curve the protein concentration is solved for, and the proper volume to add 20 µg of protein in the gel is calculated. This amount may differ from condition to condition due to varying protein concentrations, so calculating the proper volume to meet a set amount of protein would standardize the Western Blot for each condition. This assay is repeated two more times, once for each set of remaining aliquots. Once the BCA assay is finished, and the known volume of sample is calculated, it is then measured out from the samples and pipetted into new microcentrifuge tubes to be treated and added into a gel to start the Western Blot.

Western Blot Gel Electrophoresis

Once the sample protein volumes are known, the proper amounts are pipetted out into a new set of 1.5 mL microcentrifuge tubes and treated with 4x Laemmli buffer, a dye to visualize the sample in the agarose gel and denature proteins, and beta mercaptoethanol, which acts to break down disulfide bonds. The beta mercaptoethanol chemically breaks down the samples, allowing for further travel down the gel. 0.4 µL of beta mercaptoethanol was added for every 30 µL of the sample, including

the 4x Laemmli buffer. The gel used for Western blotting is a Mini-Protean TGX Stain-Free Precast Gel from Bio-Rad. It consists of 15 wells, with each well holding up to 15 μL. Once the samples have all been treated with 4x Laemmli buffer and beta mercaptoethanol, they are incubated at 90 degrees Celsius for 10 minutes.

After incubation, the samples are loaded into the gel along with 2 μL of Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards in a separate well. These standards, or protein ladders, allow for comparisons to be made with known sizes and proteins, so proteins from the sample can be identified. To set up the gel, the bottom strip of tape is removed to allow for current to flow through the gel. The gel is then locked into a cassette, with a buffer dam – a piece of plastic mimicking the gel case to create an enclosed container to fill with 1x Tris/Glycine buffer. Once the enclosure is made with the gel, buffer dam, and cassette, the comb in the gel is removed gently and the container is filled with 1x Tris/Glycine buffer, ensuring that the wells are covered. The samples are loaded into the gel, using the volumes calculated from the BCA assay which would hold 20 μg of protein each. The cassette with the gel is then placed into a buffer tank with two electrodes, placing black to black and red to red. Once the cassette is in place, the rest of the buffer tank is filled with 1x Tris/Glycine buffer, the lid is placed on top of the tank, and the power supply is started up. The gel is run for 20 minutes at 50 volts, 20 minutes at 100 volts, then 40 minutes at 150 volts. This is to coax the sample out of the well gently, then ensure it runs all the way to the edge of the gel.

After the gel has finished running, the power supply is turned off, and the lid taken off. The buffer is drained from the tank and the cassette and opened to remove the gel. The gel has two parts: a stacking gel and a separating gel. These two gels are placed between two pieces of plastic, which are pried open gently to expose the gel and remove it off the plastic. Once the plastic is removed and the gel is exposed, the separating gel with the dye is transferred using a scraper to the Azure imager to capture and image the gel. If the gel is stuck to the plastic, wetting the scraper with 1x Tris/Glycine buffer can help to lift it from the corners and onto the tray of the imager. Once on the imager tray, the gel is subjected to 5 minutes of UV exposure and then imaged. The image is captured with the nucleic acid with dye setting, using the gray color scheme and UV 320 nm, the image of the gel is then taken, adjusted to allow for clear distinction of protein travel and bands, and then saved.

Membrane Transfer

Once the gel has been imaged and that image saved, the gel is then prepped for a wash and membrane transfer. The gel is washed for 15 minutes with 1x Tris Glycine to remove excess salt, then moved to be stacked between filter paper and a membrane. The membrane used for the transfer is the Bio-Rad Trans-Blot Turbo Transfer Pack, which consists of a bottom layer of filter paper pre-soaked with buffer and the membrane on top of that layer, and a top layer with more filter paper. The gel is sandwiched between the two layers, with the gel laid on top of the membrane and rolling it over gently with a roller to ensure proper contact has been made between the gel and membrane. Once stacked, the membrane sandwich is loaded into the Bio-Rad Trans-Blot Turbo Transfer system and runs for 7 minutes to transfer the protein from the gel onto the membrane. Once the transfer is complete, the gel is removed from the membrane and imaged, to ensure all protein has been transferred. The gel should be void of protein and yield a blank image in the UV-activated channel.

After imaging, the gel is discarded, and the membrane is rinsed in blocking buffer consisting of 1x TBS with 0.05% Tween and 3% Milk (1x TBST with 3% Milk) solution for an hour at room temperature, or 20-22 degrees Celsius. 1x TBST with 3% Milk is made by adding enough Tween to TBS to be 0.05% by weight, and shaking it to combine. Nonfat milk powder is then measured out so it is 3% by weight, and added to a new container. 1x TBST is then added to the milk powder and combined. This membrane blocking prevents non-specific binding of antibodies, so when primary and secondary antibody incubation occurs the membrane is more sensitive to those antibodies and the signal is much clearer. Once blocking has been completed, the membrane is then placed and sealed in a plastic sleeve with 12 mL of primary antibody solution for Atg8a and tubulin (rabbit primary antibody). The primary antibody is placed in a 1:5000 solution with 1x TBST with 3% Milk and incubated overnight at 4 degrees Celsius.

After 12 hours have elapsed, the membrane is pulled out of the incubation and removed from the sleeve. It is then washed three times in about 10 mL of 1x TBST for 10 minutes each. After the washes, the membrane is then incubated with a secondary antibody solution for three hours at room temperature. The secondary antibody solution is 10 μL Alexafluor 488-conjugated goat anti-rabbit added to 50 mL of 3% milk in 1x TBST. Due to the fluorescent marker, the membrane is placed in a container and covered with aluminum foil to avoid bleaching by light. Once the incubation is finished the membrane is washed in 1x TBST

for 10 minutes 3 times, it is now ready to image. The membrane is transferred to the Azure imager and imaged using the fluorescent blot feature, with the green dye at UV 520 nm setting to capture the image. Once the image is taken, it is adjusted to display defined bands and lanes, the image is saved, and the membrane is sealed back up in a plastic sleeve in 10 mL of 1x TBST should further examination be needed.

Results

BCA Assay

With the first three BCA assays conducted, experimental tissue was not used. These BCA assays were run for technical practice, to sharpen laboratory skills prior to using experimental tissue. The first assay measured protein samples of 6 homogenized male Canton S wild type fly heads, not subjected to TBI, and on the control diet. The first BCA assay (Figure 4.1) yielded a proper standard curve, although there was slight variation with the standards concentration from the initial protocol. The protocol had standard B to be at 1500 µg/mL but it was made to be at 1400 µL, and standard D to be 750 µg/mL, but it was made at 700 µg/mL. Despite these differences, a standard curve was still made, and protein concentrations could still be calculated. This BCA assay was used as a trial run for technical practice, so sample protein concentrations were not calculated. Instead, 20 µL of each sample was used to load the Western Blot gel.

The second BCA assay did not have any samples, and was done for more technical practice. A standard curve was made, but there were no sample protein values to make comparisons to. The same pre-made standards were also used from the first BCA assay, so the same differences in concentration from the initial protocol was also present. For the third BCA assay

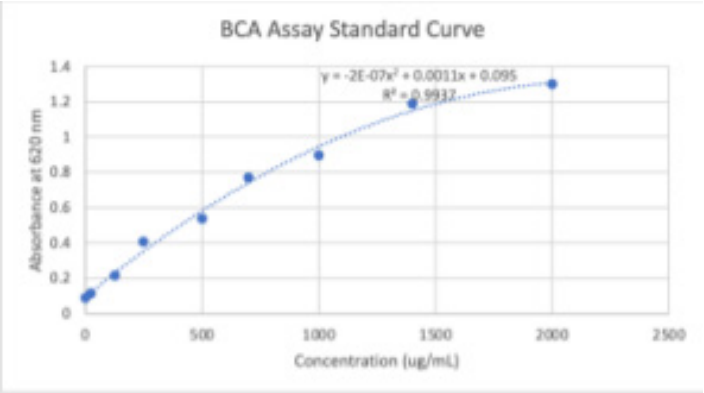

Figure 4.1 The standard curve for the first BCA assay, using male Canton S wild type, nonTBI, CD brains

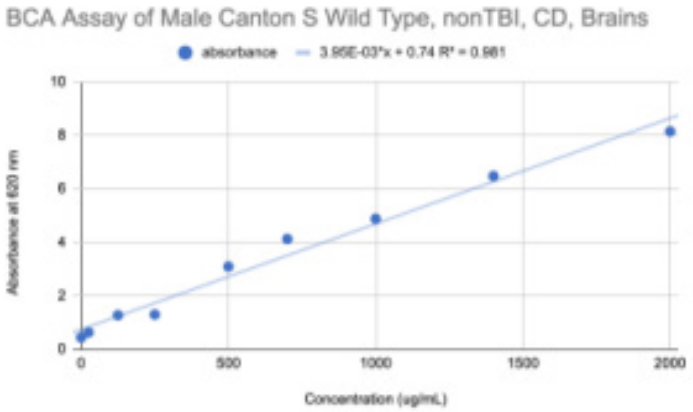

Figure 4.2 The third BCA assay standard curve using two samples of male Canton S Wild Type, nonTBI, CD brains

(Figure 4.2), homogenized brains were used, harvested from Canton S wild type flies not subjected to TBI, and fed the control diet. Before loading the samples into the BCA assay plate, one of the samples was treated with 4x Laemmli buffer and beta mercaptoethanol, which caused the BCA assay software to not be able to read at the absorbance level, resulting in an OVERFLOW value. This was because the 4x Laemmli buffer dyed the sample, and stained it beyond the threshold to be read at the wavelength of 620 nm. The other sample used had a calculated concentration of 375.7 µg/mL, using the equation of the standard curve. The sample had an absorbance of 2.224, and using the equation of the curve y = 3.95E-03x + 0.74, the concentration (x) was calculated. With this concentration, 53 µL of sample would be needed in order to load 20 µg of protein in the Western Blot gel (20 µg * (1 mL/375.7 µg) * 1000 µg/mL). Since this was not possible as the gel could only hold up to 15 µL of sample, the volume of sample needed to load 5 µg of protein was calculated, which came out to be 13.31 µL of sample. Since the other sample could not have been measured with the BCA assay, 15 µL was loaded into the gel. With the final BCA assay run (Figure 4.3), experimental tissue was used – measuring the protein concentrations of the first trial. This BCA assay was also done with the same concentrations of protein standards as the first three assays, and two rows of standards were filled to graph average absorbance versus average concentration. After calculations, it was found that the required volumes from each sample needed to load 20 µg of protein in the gel was significantly greater than 15 µL, indicating low protein concentrations, and so the volumes needed from each sample were calculated to fit 5 µg in the gel. These volumes were much more manageable after these calculations, ranging from 4.74 µL to 20.5 µL.

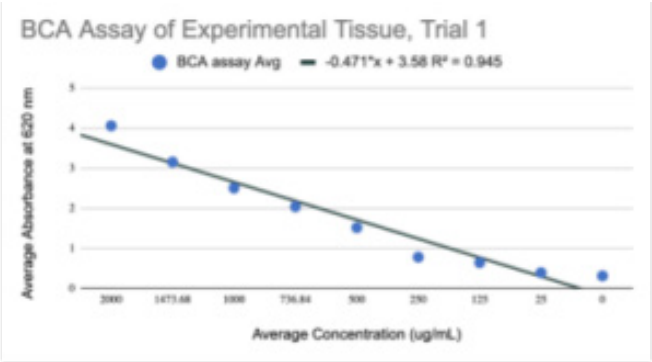

Figure 4.3 The standard curve of average absorbances and concentrations for this BCA assay. Used to calculate protein concentrations of experimental tissue

Western Blot Gel Electrophoresis

5 gels in total were run, with the first four using non experimental tissue. All collected samples run in the first four gels were brain tissue harvested from male Canton S wild type, nonTBI, CD flies. The first gel (Figure 4.4) yielded a signal, displaying proper protein movement, and a dye front that traveled all the way down the gel. Being the first gel, two samples, an older and more recently harvested sample, were treated with 4x Laemmli buffer and loaded into the gel. In lanes 5, 7, and 9, the old homogenate treated with just 4x Laemmli buffer was loaded. In lanes 2, 6, and 8, the newer homogenate, treated with 4x Laemmli buffer, was loaded. This was done primarily for technical practice loading the gel. The fourth well was loaded with 5 μL of protein ladder, and the rest of the wells were filled with the protein standards used in the BCA assay.

Lanes 10-15 had standards A-F, lane 1 had standard G,

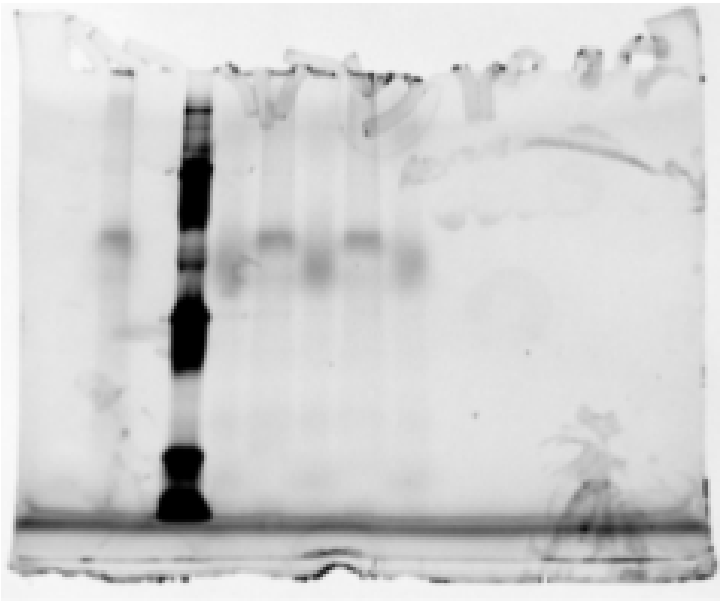

Figure 4.4 The first Western Blot gel, using a mix of old and new samples and BCA assay standards

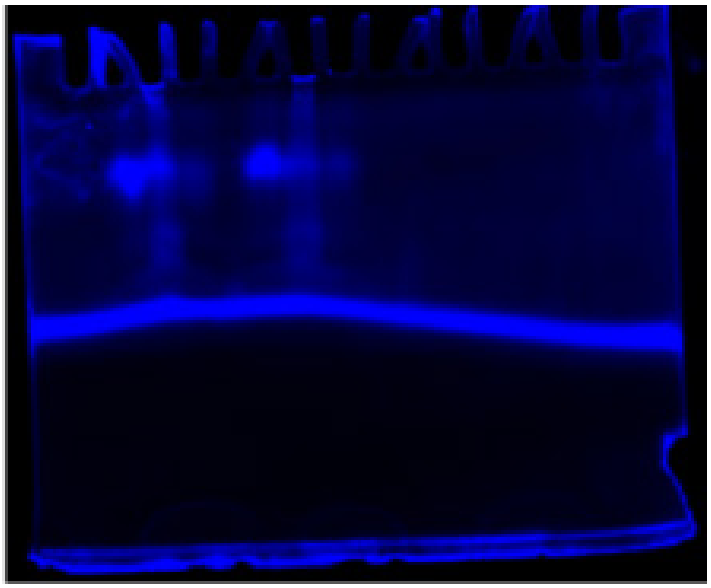

Figure 4.5 The fourth gel of samples taken from male Canton S wild type, nonTBI, CD flies. These samples were not treated with beta mercaptoethanol, but were spun at 4 degrees Celsius at 15000 RPM for 10 minutes

and lane 3 had standard H. With this gel, it was determined that 5 μL of protein ladder was too much as the lanes were enlarged and not distinct, so to prevent this from occurring in next gels, 2 μL of protein ladder was used instead. Also, although a protein signal was detected, much of the protein was still lodged in the wells of the gel, as seen from the darkened bands that appeared at the top of the gel and at the bottom of each well. This gel was also run for 90 minutes at 150 volts. The second gel used beta mercaptoethanol to treat the samples prior to incubation at 90 degrees Celsius. This way, the sample can be further broken down chemically and allow for more travel down the gel. They were also run at 150 volts for 90 minutes. This gel did not yield a strong protein signal despite these efforts to break down the protein. The third gel was treated with beta mercaptoethanol, centrifuged at 15,000 rpm at 4 degrees Celsius for 10 minutes, and used staggered voltage of 50 volts for 20 minutes, 100 volts for 20 minutes, and 150 volts for 40 minutes. It did yield anticipated results, with protein staining detected as well as a well-traveled dye front to the edge of the gel. The fourth gel (Figure 4.5) did not use beta mercaptoethanol due to experimental oversight, but included spinning down the samples prior to treatment at 15,000 rounds per minute for 10 minutes at 4 degrees Celsius. This did lead to a relatively strong protein signal in the gel; however, the dye front of the gel stopped midway through the gel. The fifth gel (Figure 4.6) did have experimental tissue used, and all techniques were used in terms of treating the samples and spinning it at 4 degrees Celsius

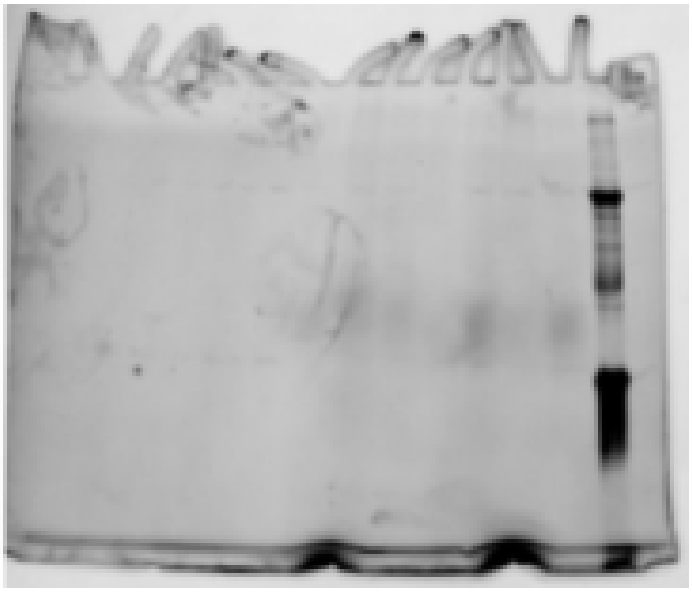

Figure 4.6 The fifth gel run, using all experimental tissue with one sample from each condition. All treatments were used, including beta mercaptoethanol and spinning the samples at 4 degrees Celsius for 10 minutes at 15000 RPM.

at 15000 RPM for 10 minutes. This gel did have a protein signal, and the wells at the top of the gel had significantly lower amounts of protein in them, suggesting that most of the protein was able to travel through the gel. Lanes 9, 10, 12, and 14 had the strongest signal, as the protein bands that were imaged came out the strongest. These lanes held the conditions of MKBB, MCuB, FKBuB, and FCB. These samples, with the exception of MKBB, also had the lowest volume of sample loaded into the gel, indicating a higher concentration of protein in these samples. This could explain why some of these samples were better detected than the others, who had between 20-25 μL of sample loaded into the well which was only designed to hold 15 μL.

Membrane Transfer

A total of three membrane transfers were performed from the five gels as the gels without a strong protein signal did not get transferred, to conserve resources. The first membrane transfer was done with the first gel, treated with primary tubulin antibody, and did not yield any tubulin results. This lack of signal led to the use of other treatments such as beta mercaptoethanol and spinning the sample at 4 degrees Celsius, as well as the staggered voltage when running the gel. The second membrane transfer (Figure 4.7) yielded promising signal, as both tubulin and Atg8a were captured on the membrane, although the Atg8a signal was quite faint. This transfer was not done on experimental tissue, as it was protein transferred from the third gel. The second gel was not transferred onto a membrane due to poor protein signal, and the fourth gel was not transferred as it used non-experimental tissue, so to conserve resources a membrane transfer was not performed. The fifth gel had the final protein transfer, which did contain experimental tissue. It had minimal signal despite all treatments and efforts utilized to ensure proper protein breakdown and travel in the gel.

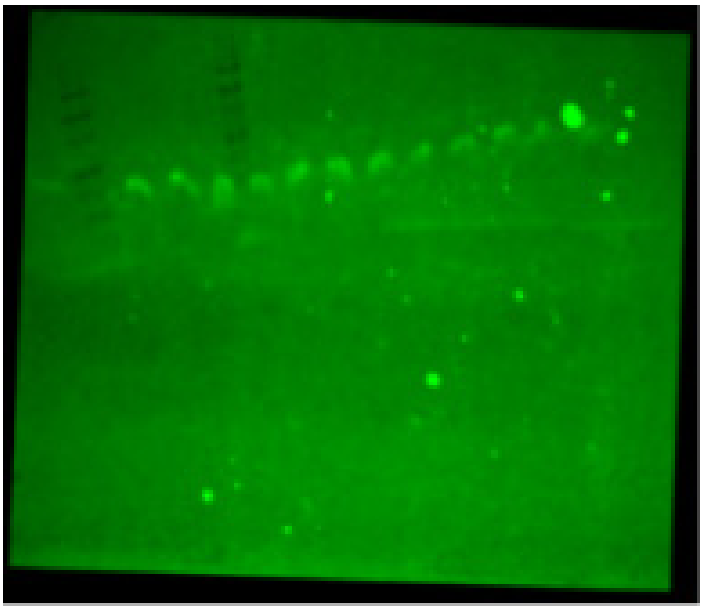

Figure 4.7 The membrane transfer from the third gel. Tubulin staining is seen as the most prominent lower row of markings, and Atg8 is quite faint but placed above.

Discussion

Whether or not experimental tissue was used, producing a protein signal with the Western Blot and membrane transfer was a series of trial and error. Although a signal was detected with the gel on the first run, there was minimal signal on the membrane. Furthermore, there was still protein left in the wells of the gel that had not been able to travel through the gel. This was what led to the use of beta mercaptoethanol – as it was most likely with the poor signal in the membrane and protein imaged still stuck in the wells of the gel that the protein in the sample was not broken down enough. Since beta mercaptoethanol functions to dissolve and break apart disulfide bonds, adding it to the homogenized brain tissue would chemically break down the proteins and allow them to flow through the gel easier.

After treating samples with beta mercaptoethanol and not seeing much difference in protein travel in the gel, it was also decided to centrifuge the samples at 4 degrees Celsius for 10 minutes at 15,000 rounds per minute. This

way, the unblended matter can be separated further from the homogenate and allow for smoother travel through the gel. It was also decided to stagger the voltage when running the gel in order to gently coax the sample out of the wells of the gel. Starting at 50 volts for 20 minutes would allow for the protein to set in the well and be ready to run through the gel. A higher voltage in a gel would create a greater electrical pressure, allowing the gel to run faster. Allowing the proteins to be able to travel the gel at a slower pace would increase chances of yielding bands of higher resolution. That way, the proteins are not pushed through the gel and can settle in properly. These different techniques that were adopted through this process helped to gain a better understanding of the technical and theoretical process that make up the Western Blotting protocol.

Beyond the technical obstacles of the Western Blot, there were other potential sources of error during this process. One major source was the lack of protein detected in each sample. Only 6 brains were used in each sample, but more brains per sample could have provided the higher protein concentration needed in order to detect a signal in the Western Blot. The first signal that points to this being a great source of error is the BCA assay, which calculated that upwards of 70 μL was needed in order to load the gel with 20 μg of protein. This sparse protein concentration could be a great contributor as to why protein signals were weak in the Western Blots. Since the amount of sample needed to input 20 μg of protein was not possible for the gel to hold, only 5 μg were loaded. This drastic reduction in protein concentration could and is most likely a large factor for the lack of signal that was detected in the gels and membrane transfer.

Understanding the mistakes made in this process would make way for future procedures, which would also be using these techniques. Practicing and perfecting the Western Blot protocol can lead to its future use in pursuing this project or others examining different gene functions in relation to the use of ketone bodies for neuronal damage recovery. Examining the role that KBs may play in preventing or delaying neurodegeneration can allow for further investigations into the possibility of KBs being used for therapeutic purposes. TBI's affect about 1.5 million people in the United States annually, and of those, 230,000 Americans are hospitalized, and 50,000 die from their sustained injuries (Centers for Disease Control and Prevention, 2016). If a positive relationship between KBs and reducing neurodegeneration is found, potential therapies can be developed in treating those with TBI.

Conclusion

While this project did not yield any expected or significant results, it was still a valuable learning experience. The Western Blot protocol and techniques were refined and laid a stronger foundation for those in the future looking to use similar techniques for their projects.

Further research following this would be to carry out another set of trials, and collect a higher volume of data for deeper analysis. Learning more about the potential interactions between ketone bodies and Atg8a could lead to further knowledge about the mechanisms by which ketone bodies produce their neuroprotective properties. If the hypothesized results that flies on the ketone body supplemented diet would see more Atg8a activity than flies not on the ketone body diet is observed, then further experiments can be done with Atg8a mutations to ensure that is the mechanism ketone bodies operate on to produce their neuroprotective properties. It would also further solidify the association between ketone bodies and TBI recovery. Overall, this project has many potential leads and can stimulate the investigation into the specific mechanisms ketone bodies operate by that contribute to their neuroprotective benefits.

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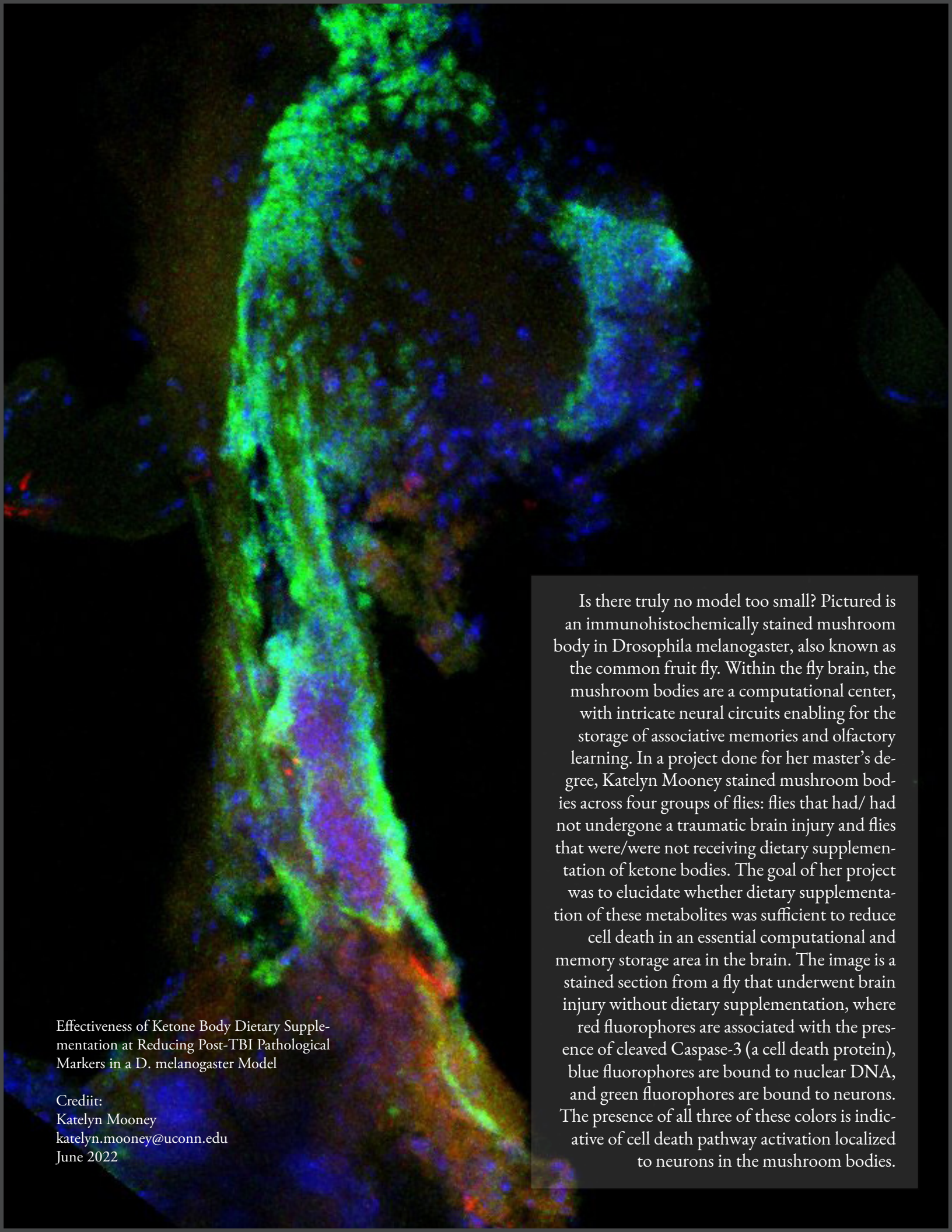

Is there truly no model too small? Pictured is an immunohistochemically stained mushroom body in Drosophila melanogaster, also known as the common fruit fly. Within the fly brain, the mushroom bodies are a computational center, with intricate neural circuits enabling for the storage of associative memories and olfactory learning. In a project done for her master's degree, Katelyn Mooney stained mushroom bodies across four groups of flies: flies that had/ had not undergone a traumatic brain injury and flies that were/were not receiving dietary supplementation of ketone bodies. The goal of her project was to elucidate whether dietary supplementation of these metabolites was sufficient to reduce cell death in an essential computational and memory storage area in the brain. The image is a stained section from a fly that underwent brain injury without dietary supplementation, where red fluorophores are associated with the presence of cleaved Caspase-3 (a cell death protein), blue fluorophores are bound to nuclear DNA, and green fluorophores are bound to neurons. The presence of all three of these colors is indicative of cell death pathway activation localized to neurons in the mushroom bodies.

Effectiveness of Ketone Body Dietary Supplementation at Reducing Post-TBI Pathological Markers in a D. melanogaster Model

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