The Effect of Ketone Body Supplementation of Autophagy Post-Traumatic Brain Injury in *Drosophila melanogaster*

by
Andreana (Andie) Hardin

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South Hadley, MA 01075

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This paper was prepared
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for eight credits.

To my wonderful and supportive Mom and Dad, Kim and Ken Hardin, for always supporting me. And to all of my siblings, Alex, Kenny, and Nic Hardin, for giving me enough TBI for a lifetime.

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ABSTRACT

Today, there are very few treatment measures for long-term brain injury, leaving it to be one of the leading causes of death. Autophagy is a natural process that degrades old cellular components to recycle amino acids and proteins to support the synthesis of new cellular structures in a turnover of cytoplasm. In autophagy, cytosolic vesicles fuse with a lysosome to turn over cytoplasmic contents for reuse. Autophagy has been linked to increased immune defense, tumor suppression, apoptosis, and the prevention of neuronal degeneration, leading many to explore the psychopharmacological benefits it may have for brain injury. In *Drosophila,* autophagy is regulated in part by the Atg8a protein. The ketogenic diet and ketone body supplementation have been known to increase rates of autophagy.

For this experiment, I inflicted traumatic brain injury on *Drosophila* fed on both a ketone-supplemented diet and a control diet. I hypothesized that *Drosophila* raised on a diet with ketone body supplementation will exhibit a higher rate of autophagy than *Drosophila* raised on control food. To achieve traumatic brain injury, the flies are put in a “High Impact Trauma” (HIT) device. *Drosophila* were then returned to control or ketone-supplemented food for 72, 24, or 6 hours. Then, their CNSwas dissected for analysis in two ways. First, I used a Western Blot approach to quantify autophagy via the ratio of lipidated to unlipidated Atg8a protein. Confocal microscopy was also used for analyzation. Images from the confocal microscope were analyzed quantitatively with Image J.

After analyses, I saw that there was a suggested difference between the sample groups, and the there was a lower rate of autophagy in flies raised on a BHB supplemented diet and remained on a BHB supplemented diet post TBI than flies raised on a control diet and remained on a control diet post TBI.

INTRODUCTION

Traumatic Brain Injury

According to the American Association of Neurological Surgeons, there are an estimated 1.8-3.8 million traumatic brain injuries (TBI) in the US every year, with a large percentage occurring in athletes (*Traumatic Brain Injury – Causes, Symptoms and Treatment*) However, TBI does not discriminate and can happen to anybody in many circumstances, such as a simple traffic accident. Traumatic brain injuries can range from something as seemingly simple as a concussion, to conditions much more severe, like second impact syndrome or chronic traumatic encephalopathy. The lasting trauma conditions from head impact can have long-term effects on both cell damage and degeneration, ultimately leaving the affected individual with long-term health deficits in both the physical body and the psyche (*Traumatic Brain Injury – Causes, Symptoms and Treatment*). To date, the primary way to combat long-term damage to the brain has been through prevention. Methods include alterations of helmets and safety rules being added to sports rules, safety features being added to motor vehicles, and the addition of safety laws. However, even with prevention, traumatic brain injury can be inevitable. Accidents will always occur in sports leading to injury, but modern-day society has also affected the rates of injury. American society has seen a large increase in firearm use that has increased rates of TBI, as well as a global increase in motor vehicle usage that has led to an increase in traffic accidents (Zeng et al., 2020). Unfortunately, today there isn't an effective therapy or clinical intervention method that exists (Zeng et al., 2020). With the increase in rates of traumatic brain injuries and the resulting premature

deaths, it is crucial to find a reliable method that will decrease the likelihood of long-term damage.

Psychological and Physiological Damage of TBI

High-impact trauma to the brain causes substantial physiological and psychological changes. Traumatic brain injury is categorized into two different phases: primary and secondary brain injury, with the first having the most impact on the prognosis (Zeng et al., 2020). Primary brain injury is the initial trauma caused by the impact of external forces that jeopardize the brain such as blunt force trauma or rapid movement (Maas et al., 2008). Secondary head injury, however, is the damage that occurs as a result of or post-primary head injury that typically leads to premature cell death (Maas et al., 2008). From the macro perspective, primary brain trauma can visibly appear as swelling, facial fractures, and ruptured eardrums, with symptoms such as hypoxia, hypotension, pyrexia, or coagulopathy (the body's ability to clot blood), among other symptoms that one would typically equate with brain damage. On the microscopic level, TBI damage can lead to detrimental injury that can compact to secondary damage. Possibilities include microporation of membranes, leaky ion channels, unwanted conformational changes in proteins, and higher rates of micro hemorrhaging (Maas et al., 2008). It is the extent of these injuries that determines the extent of secondary brain damage. Secondary brain damage develops over minutes to hours and days following primary brain injury. On the physiopathological level, secondary brain injury damage can include neurotransmitter release, free-radical

generation, calcium-mediated damage, unwanted gene activation, mitochondrial dysfunction, organelle death, and inflammatory responses (Maas et al., 2008). Although many symptoms and injuries can be easily traced back to TBI, large amounts of damage may go undetected for years, causing serious brain damage down the road.

Traumatic brain injury, once or multiple exposures, increases one's risk of developing a neurodegenerative disease, more specifically Chronic Traumatic Encephalopathy, Parkinson's, Alzheimer's, or Amyotrophic Lateral Sclerosis (sALS). CTE is a neurodegenerative disease caused by multiple closed-head traumas (TBI) over an extended period. Most commonly associated with football players. A study consisting of deceased NFL players found that 110 out of 111 included in the study were neuropathologically diagnosed with CTE post-mortem (VanItallie, 2019) A high school study provided that many student-athletes who participated in contract sports displayed many symptoms of traumatic brain injury such as brain atrophy, astrogliosis, myelinated axonopathy, microvascular injury, perivascular neuroinflammation, and phosphorylated Tau protein pathology (VanItallie, 2019).

Autophagy

Autophagy in most simple terms is our body's recycling system. In states of long-term fasting, autophagy degrades old cellular components to recycle amino acids and proteins to support the synthesis of new cellular structures in a turnover of cytoplasm. Autophagy plays a critical role and has been linked to

increased immune defense, tumor suppression, apoptosis, and the prevention of neuron degeneration. Typically, however, autophagy acts as a way to energize the body, as well as as a means of protection (Xie & Klionsky, 2007). There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. but for the sake of this paper, we will be investigating macroautophagy (referred to as autophagy). In short, autophagy can be summed as a degradation process in which cytosolic vesicles fuse with a lysosome to turn over cytoplasmic contents and for the reuse of protein.

The cellular mechanism of autophagy begins when the phagophore engulfs portions of cytoplasm to create a double membrane-bound vesicle around organelles and proteins, commonly called the autophagosome (Xie & Klionsky, 2007). ATG13 anchors ULk to the pre-autophagosomal structure (PAS). From there, Atg proteins are recruited and localized to the PAS. ATG proteins then create a cleation at the PAS (X. Li et al., 2020). These proteins carry the KFERQ amino acid sequence which unfold and translocate across the membrane of the lysosome through a channel with LAMP2A proteins (Mulakkal et al., 2014). This allows for the creation of the phagophore. The autophagosome is then formed when ubiquitin-like ATG conjugation pathways, Atg12-Atg5 and Atg8/LC3 conjugation systems enclose to form a lipid bilayer. This bilayer yields the creation of the autophagosome (X. Li et al., 2020). The autophagosome then fuses with the lysosome, allowing lysosomal hydrolysis to fuse the lysosome to the autophagosome to create the autolysosome which can then begin the process of breaking down the organelles and proteins (Xie & Klionsky, 2007).

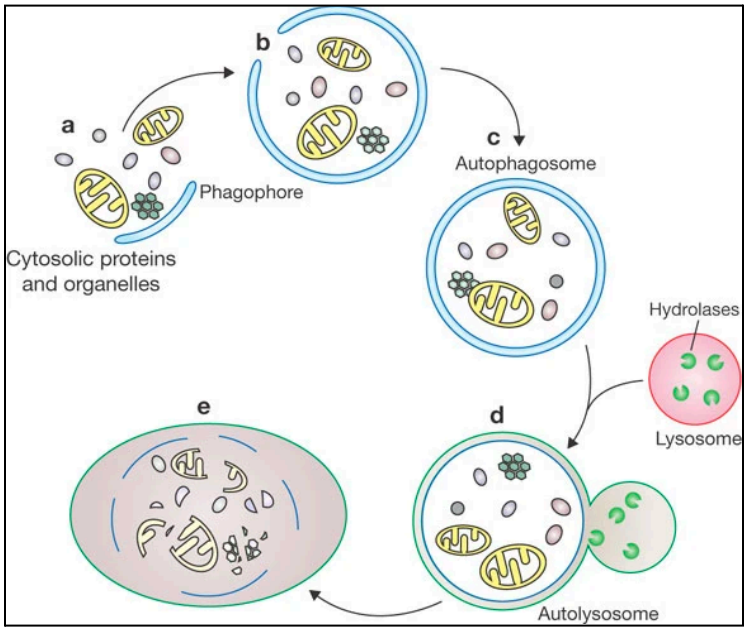

Figure 1: The cycle of the autophagosome, starting with the phagophore encapsulating the cytoplasm with the desired organic materials inside. The membrane then closes to form the autophagosome, which then fuses with the autolysosome to break down the materials inside the autophagosome so they can be recycled (Xie & Klionsky, 2007).

TOR regulation of autophagy

Autophagy is regulated through many mechanisms, regulated by many autophagy-associated genes and hormones. One mechanism of inducing autophagy is via nutrition or starvation which is regulated by de-phosphorylation and kinase substrates. Autophagy is primarily regulated by *TOR, the* target of rapamycin, and in mammals is regulated by Atg1 protein complexes TOR activity increases in the presence of nutrients and promotes cell growth. Therefore, TOR suppresses autophagy In starvation conditions, TOR activity is suppressed, therefore increasing the overall rate of autophagy (Mulakkal et al.,

2014). Phosphates such as PP2a may play a role in the suppression and antagonization of TOR, as well as serine/threonine kinase ATG1 having a direct link to the phosphorylation of TOR (Mulakkal et al., 2014).

Starvation Mediated Autophagy

Atg proteins also play a crucial role in starvation-mediated autophagy. Starvation can lead to the activation of ULK1/Atg1 (including ULK1, ATG13, FIP200, and ATG101) proteins, which act as the autophagy initiation complex (X. Li et al., 2020). The activation leads to an increase in autophagy and autophagosome formation. AMPK (Adenosine monophosphate-activated protein kinase) influences the ULK1 pathway, and the recruitment of autophagy-related proteins, also participating in the increase of autophagy.

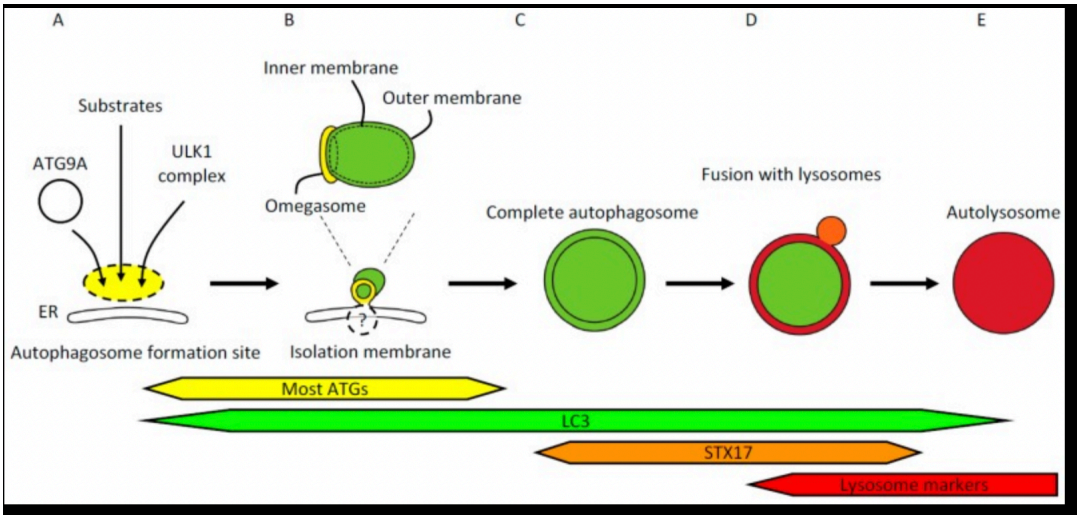

Figure 2: Overview of autophagy signaling pathways and proteins. Proteins involved in the induction of autophagy are shows to their perspective organelle and process (Yoshii & Mizushima, 2017).

As a result of post-traumatic brain injury, the pathways that autophagy takes are altered. While some impact studies have shown autophagy markers increasing, others have shown a decrease (Zeng et al., 2020). Although which direction is unclear, the inconsistencies in stimulation or decrease suggest a pathophysiological correlation between TBI and autophagy (Zeng et al., 2020). Autophagy can be influenced by oxidative stress, inflammation, and apoptosis, which are all increased post-TBI. Although post-TBI autophagy naturally goes either direction, there is a large benefit to increasing autophagy. An increase in autophagy has been linked to cognitive repair and a decrease in neuronal death (Zeng et al., 2020) Autophagy as a potential treatment was first introduced in 2007 (Mulakkal et al., 2014). After using rapamycin, a drug that increased autophagy, following traumatic brain injury. Their study showed a decrease in neuronal death and inflammation via signaling pathway modulation (Zhang & Wang, 2018). An increase in autophagy post-TBI also promotes hippocampal repair, which in turn, helps decreases cognitive deficits (Zhang & Wang, 2018). There has also been evidence that autophagy-regulating pathways, more specifically P13K, AKT, and mTOR can reduce apoptosis via autophagy regulation (Zhang & Wang, 2018). The turnover of cytosol and cellular components brought on by autophagy decreases mitochondrial dysfunction triggered by TBI (Maas et al., 2008). Mitochondrial dysfunction decreases ATP production, as well as depletes oxygen, in turn causing premature apoptosis and

necrosis (Maas et al., 2008). For these reasons, the induction of autophagy presents to be a very promising treatment option for TBI.

Induction of Autophagy

Certain conditions can either increase or decrease the rate of autophagy in cells. In the human CNS, autophagy will decrease with age and is commonly associated with cognitive decline. However, there are conditions in which autophagy has been shown to increase, such as engaging in strenuous, regular, exercise. Individuals who have engaged in exercise regularly actually exhibit a slower rate of cognitive decline and decreased autophagy with age (Andreotti et al., 2020). The natural state of ketosis is also linked to an increase in autophagy as well. In order to induce ketosis, certain lifestyle choices and diets may be used to reap the therapeutic benefits of autophagy.

Ketosis and the Ketogenic Diet

In the 1990's a high-fat low-carb diet called the "keto diet" became popular overnight in the dieting world. The keto diet comprises a 4:1 lipid-to-carbohydrate ratio to simulate starvation and send the body into a state of ketogenic metabolism (D. C. Lee et al., 2019)Many have attributed weight loss and a healthier overall lifestyle to the regime, and the diet has been promoted in many lifestyle magazines. Those on the diet for cosmetic and lifestyle purposes have claimed that they feel more energetic, aware, and overall healthier, as well as exhibiting a slimmer figure to boot (*Keto Diet*, 2020) Although gaining traction in

recent years, the origin of the keto diet is anything but new. The therapeutic effects have been documented for hundreds of years, and it has been used as a mechanism for seizure control since around 460 BCE (Hartman et al., 2007)What many fad dieters fail to realize when adding butter to their coffee for the name of health, is that the ketogenic diet is a treatment for pediatric epilepsy. The ketogenic diet has anticonvulsant effects, making it a reliable and standardized treatment option for seizures since the 1920s (Hartman et al., 2007; J. Li et al., 2017). Utilization of the diet's neuropharmacological properties is also still being considered, with trials used in the treatment of Parkinson's disease, Alzheimer's Disease, and traumatic brain injury such as CTE (chronic traumatic encephalopathy), as well as several psychiatric and mood disorders (Danan et al., 2022; Yang et al., 2019).

Mechanisms of Ketosis

To understand the Ketogenic diet, it is important to understand the underlying mechanisms of Ketosis. During starvation or low access to carbohydrates, the body must switch from the metabolic pathway of glycolysis to the alternative, fatty acid oxidation. This process occurs initially in the liver, where large amounts of acyl-CoA are made via acyl-CoA synthetases (metabolic intermediate), which are turned into ketone bodies in the liver (Hartman et al., 2007; Ruppert & Kersten, 2023). Ketone bodies are a metabolic intermediate used in lue of glucose when it is not readily available. The primary Ketone body being generated in the human brain is β-hydroxybutyrate, otherwise known as BHB.

When lipolysis occurs duing ketosis, mitochondria produce ATP and acetyl-CoA during beta-oxidation. Acetyl-CoA is then reduced to three ketone bodies, but most abundantly, β-hydroxybutyrate. The amount of BHB that is being produced is directly correlated with the starvation status of the body (Ruppert & Kersten, 2023). Ketone bodies can cross the blood-brain, whereas fatty acids are too large, making them a perfect replacement for glucose. After the ketone bodies are turned to Acyl-CoA, they are oxidized in the mitochondria when the CoA group is exchanged for carnitine via CPT1A and forms acyl-carnitines. The acyl-carnitines are then transported to the mitochondria by SLC25A20 (Carnitine-acylcarnitine translocase). Once in the mitochondria, the acyl-carnitines are then reversed back into acyl-CoAs via CPT2 and undergo beta-oxidation, yielding acetyl-CoA. The reformed Acetyl-CoA then is further oxidized in the TCA cycle, yielding NADH and FADH2, which after the electron transport chain produce ATP (Ruppert & Kersten, 2023).

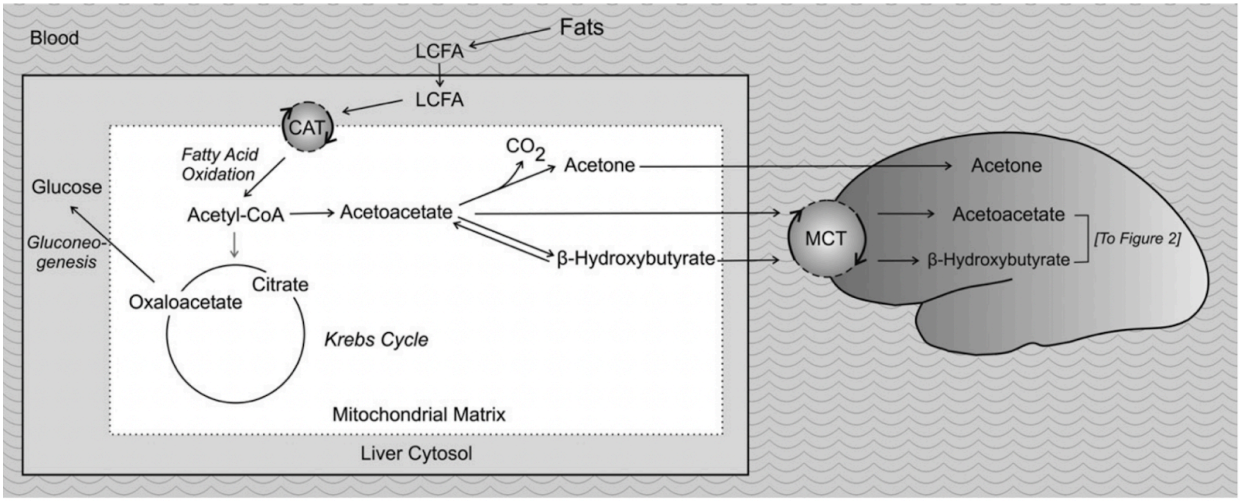

Figure 3: illustration depicting the mechanism of ketone bodies being used as an energy source originating from the liver cytosol, transferring to the mitochondrial matrix of hepatic cells, then entering the blood to cross the blood-brain barrier. The Acetyl-CoA yielded from fatty acid oxidation breaks down into acetoacetate, which then forms ketone bodies, the keone bodies are acetone and β-hydroxybuteryrate . The ketone bodies then enter the blood-brain barrier, in which they can be used to form ATP in the MCT cycle (Hartman et al., 2007).

B-hydroxybutyrate (BHB)

β-hydroxybutyrate, otherwise known as BHB is one of three ketone bodies that is produced from Aceyl-CoA during beta-oxidation. Studies have suggested that β-hydroxybutyrate is associated with histone de-acylation. Typically, histones are wrapped around DNA to inhibit gene expression. However, when de-acetylated, gene expression may commence (D. Lee, 2020)This de-acetylation may hold many therapeutic benefits when it comes to traumatic brain injury, including the expression of increased autophagy. For this reason,

β-hydroxybutyrate has been largely recognized as a ketone body of choice for TBI investigations (D. Lee, 2020).

Ketone bodies and anticonvulsant/ neuroprotective effects

The alternative source of energy provided by ketone bodies is under extensive investigation of how they may be applied in treatments for other neurological pathologies due to the observed therapeutic effects in epilepsy as well as other neurological and neurodegenerative diseases.

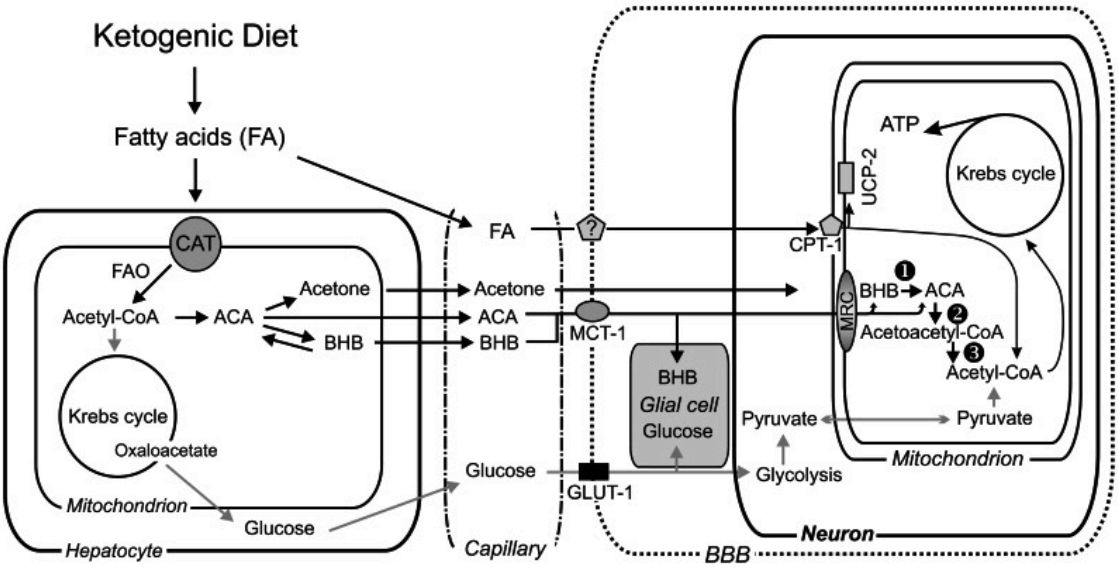

Figure 4: portrayal of the mechanism of the keto diet from consumption to ketone body production. Fatty acid oxidation yields acetyl-CoA, which then produces ketone bodies. These ketone bodies then enter the bloodstream and cross the blood-brain barrier where they can be converted into ATP (Masino & Rho, 2012).

The keto diet protects the brain from epilepsy by using ketone bodies as energy and producing energy stores which not only increase effective neurons but can lower neuronal excitability by increasing the synthesis of GABA. GABA

(-aminobutyric acid) is the major inhibitory neurotransmitter in mammals (Hartman et al., 2007). These effects have been noted to help the brain become more resistant to metabolic stress, lowering the risk of both seizures and overall damage as seen in epilepsy studies. The keto diet also mirrors the therapeutic effects of autophagy, responding to oxidative stress and high-impact trauma. Besides epilepsy, the keto diet is being considered as a possible treatment for a range of diseases. Patients with severe treatment-resistant refractory mental illnesses such as schizophrenia, major depression, and bipolar disorders exhibited a lower severity of symptoms while on the diet. However, it is important to note that due to the patient's diagnosis, long-term treatment was difficult as they did not want to remain on the diet and were largely only tolerated in an inpatient setting (Danan et al., 2022). The Keto diet has also more recently been applied as a possible treatment for Parkinson's Disease. Parkinson's disease is largely affected by inflammation that can lead to neuron degeneration. One pathway of neuron degeneration from the disease is the increased synthesis and reuptake of glutamate, an excitatory neurotransmitter in which large amounts can cause neurotoxicity. When applied in the Keto Diet it is theorized that the inhibitory effects can help slow this process (Yang et al., 2019) In addition to al the benefits listed above, the keto diet also has been shown to increase autophagy.

Studies have suggested that autophagy increases under the keto diet. In vivo and in vitro rat studies have suggested that autophagy-related proteins Atg5, Beclin-1 along with other autophagy-associated proteins, were expressed in higher rates in rats fed the KD than in rats fed control food (Wang et al., 2018).

Rats fed the KD also exhibited the therapeutic effects outlined previously. When applied in the case of traumatic brain injury, a keto diet that increases the overall rate of autophagy may help decrease the rate of secondary brain damage post-trauma.

Ketone body supplementation is a great alternative for those who may not be able to stay on the keto diet full-time or as an alternative to the strict rules of the diet as a whole. Ketone bodies act as signaling ligands, even at low concentrations, leading to the increase in ketogenesis and therefore autophagy (Lee, 2020). Studies have shown that feeding human subjects ketone body supplements elevated ketone body circulation as a whole (Stubbs et al., 2017). In mouse models, supplementation relieved neuropathological symptoms as well. In addition, when studied in *drosophila*, ketone body supplementation has shown seizure relief (Lee et al., 2019). Due to the pharmacological effects of ketone body supplementation, one may use supplementation of ketone bodies as an alternative to the keto diet altogether.

Drosophila as a model organism

Drosophila melanogaster, known as the fruit fly, is often used as a model organism for research. *Drosophila* make a great model organism as they reproduce quickly and have relatively simple needs. Their entire generation cycle is 10 days long from fertilization to pupal eclosion, making them a perfect candidate for generations of study and as a constant flow of experimental organisms to use. The flies themselves may live a relatively long time, of about 40

days. They follow a life cycle of embryonic development, to the larval stage, to the pupal stage, and into adulthood (Fernández-Moreno et al., 2007).

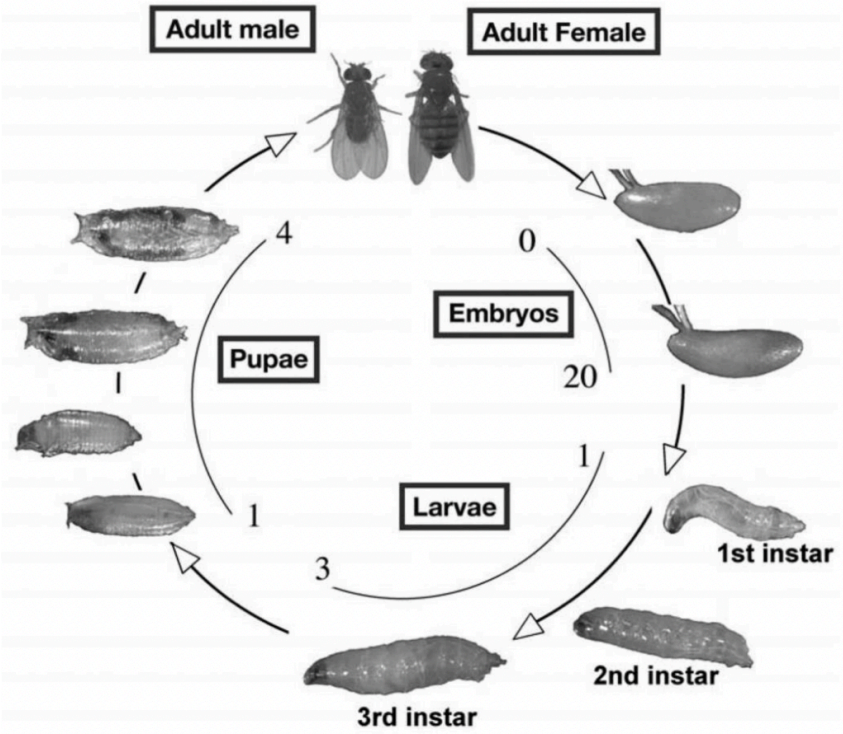

Figure 5: Lifecycle of *Drosophila melanogaster* from embryonic development, into the first through third instar larvae stages, followed by pupae development into post inclusion into adulthood. The arrows within the inner circle of the figure depict how many days of development that stage lasts. The embryonic stage duration is indicated in hours, while the larvae and pupalstages are in days. (Andreotti et al., 2020).

The impact of traumatic brain injury on *Drosophila* is also very apparent. In a study done at Uconn, *Drosophila* post-impact showed elevated levels of aggression as measured on a negative geotaxis assay (D. C. Lee et al., 2019). *Drosophila* also displays very similar behavior post-TBI to humans as well,

including seizure activity, temporary loss of consciousness, or even premature death (D. C. Lee et al., 2019). Also, *Drosophila* and *Homo sapiens* share many similarities in brain structures they are both bilateral and joined by a ventral chord, making them a go-to organism for neuroscientific research (Katzenberger et al., 2013)

Purpose of this study:

The keto diet and ketone body supplementation have shown great promise as a pharmacological treatment for TBI, neurodegenerative diseases, and seizure protection. The purpose of this study is to further understand how ketone body supplementation may be applied as a treatment for traumatic brain injury. Supplementation will be used as opposed to the keto diet as a whole, as this diet is not realistic for many people to consistently stick to to achieve ketosis.

I investigated the possibility that an increase in autophagy in the CNS of *Drosophila melanogaster* is caused by ketone body supplementation and that this will ameliorate post traumatic brain injury. The big picture of this study is to understand how this increase in autophagy may act as a neuroprotective factor against traumatic brain injury. This study could have translational implications to future research regarding the treatment of human traumatic brain injury, as well as the overall prevention of long-term damage.

RESEARCH QUESTION AND HYPOTHESIS

My research question for this study is whether or not flies raised on a diet supplemented with ß-hydroxybutyrate will express higher levels of autophagy post-traumatic brain injury than flies raised on a control diet. I hypothesize that flies raised on a BHB-supplemented diet that undergo traumatic brain injury will express higher rates of autophagy as compared to flies that were raised on a control diet that undergo traumatic brain injury. I also expect that flies raised on control food and switched to a BHB-supplemented diet post-traumatic brain injury will also exhibit higher rates of autophagy as opposed to flies raised solely on the control diet that remains on the control diet post-traumatic brain injury.

MATERIALS AND METHODS

Fly husbandry

Materials and methods were largely based on a 2021 study, "Genetic reduction of *tyramine β hydroxylase* suppresses Tau toxicity in a *Drosophila* model of tauopathy"(Nangia et. al 2021). *Canton-S* wild-type *Drosophila melanogaster* were used for all experiments, originating from the same stock. Flies were transferred into new vials approximately every 3 days. For this experiment, flies were used 6-24 hours post enclosure to eliminate age as a potential factor in autophagy.

Food Preparation

Canton Special Drosophila melanogaster (CS) were raised on Bloomington Formulation diet food prepared one bag at a time. 1 L of water was allowed to boil in a pot before adding 1 bag of Bloomington Formulation diet fly food. The water along with the BF formulation was then brought to a **vigorous** boil for about 15 minutes before being removed from heat and allowed to cool. The food was also stirred consistently to make sure nothing congealed at the bottom or burned. Once off the heat, the food cooled until the temperature reached 70°C. The mixture must be under 70°C to eliminate the possibility of decarboxylation due to temperature. Once cooled, 10 mL of 10 mg/mL tegosept

was added. Tegosept is a common antifungal agent added to preserve *drosophila* food. Tegosept was prepared by mixing 50 g -hydroxybenzoic acid methyl ester in 5000 mL ethanol. The standard Woodard Lab protocol includes the addition of both propionic acid and tegosept to *Drosophila* medium. However, it is important to note that food made with β-hydroxybutyrate can not contain any propionic acid, a common addition as an antifungal preservative to fly food, because propionic acid will degrade the β-hydroxybutyrate, rendering the food unusable. Therefore no propionic acid was added. Before adding the β-hydroxybutyrate supplement, the mixture was split in half to separate the control food from the BHB food, and separated into labeled beakers. DL-β-hydroxybutyrate acid sodium salt (Product number: H6501-5G) was then added to the experimental beaker which was stored at 4°C. 0.063mg β-hydroxybutyrate powder was added to the BHB beaker. This concentration was detailed in previous studies in vitro and in vivo. (Tanner et al 2011) Vials were then poured according to control and experiment, taking care to make sure that the food was not allowed to solidify. The food was then covered in a cheesecloth overnight before being sealed with flugs the next morning. Flugs are a stopper for the vials. Vials containing β-hydroxybutyrate were marked with a black dot on the top. All the food was then stored in the 4° until used.

Fly Preparation

Five *Canton Special Drosophila melanogaster* males and five females were used to originate the fly stocks. Flies were transferred every 24-48 hours.

For this experiment, only flies from 6-24 hours post-enclosed were used. This is to eliminate age as a factor affecting autophagy.

High Impact Trauma Device

To measure the ratio of autophagy post-TBI, adult flies were subjected to a High Impact Trauma (HIT) device. A High Impact Trauma device, also referred to as a HIT device is a simple but efficient way to inflict closed head injury on flies for experimental use (D. C. Lee et al., 2019).

Five flies from both the experimental at random and 10 flies from control Food food (10 flies overall) were anesthetized with CO_2 to be collected.
The sex of the fly was not considered in this study. Flies were allowed to regain full consciousness before beginning the HIT episode. Four HIT episodes were run on the *Drosophila* one minute apart (Nangia et al., 2021). Then plugged into the HIT machine with the stopper. The spring was pulled to a 90-degree angle and let go. From there, the flies were returned to the food for a prospective time before freezing them for preservation.

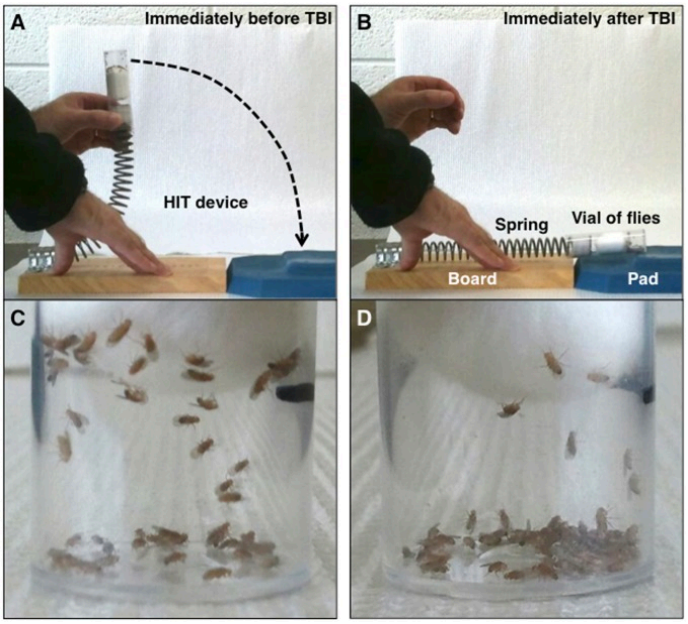

Figure VI: image of the High Impact Trauma (HIT) device as used in the Katzenberger studies. This image portrays a demonstration of the methodology of the device. Image A shows the spring being lifted to 90°. Image B portrays the immediate release of the spring to the impact on the pad (Katzenberger et al., 2013).

The flies were separated into three categories for this experiment. 5 flies raised on BHB were fed BHB post-HIT, 5 flies raised on control food were fed BHB food post-HIT, and 5 flies fed control food pre and post-HIT. The three groups of flies were returned to their food for 6 hours, 24 hours, or 72 hours before being anesthetized once again with CO_2 and placed back into the -20°C freezer. Theses time amounts were chosen to observe the effects of BHB supplementation over time as well. The flies were placed at -20°C to preserve the Atg8a proteins for Western Blot. Note that anesthetization for the second time is optional (Katzenberger et al., 2013). Flies used for confocal microscopy were

returned to their food for their perspective times, then immediately dissected and analyzed when ready.

Decapitation

One block of solid carbon dioxide was prepared. Dissection plates were placed in a styrofoam container with the CO2 until visually appearing frosty. *Drosophila* were then taken out of the freezer and 1 drosophila from each of the groups was taken for decapitation. Vials containing the HIT *Drosophila* taken from the -20°C freezer were also placed briefly in the solid CO2 container. Flies weren't allowed to develop frost on them. It is important that the flies are frozen, but if frost were to melt on them, dissection becomes more difficult. The more solid the fly, the easier the dissection.

Using Dumont #5 tweezers, both the body and head were held. Pulling the body away from the head, flies were dissected. One head was placed into their perspective 15 µl 2x Lammeli buffer solutions.

Sample Preparation

To prepare *Drosophila* for western blot analysis, Central Nervous Systems (CNSs) were dissected and homogenized. Using a 20 µl pipette, 15 µl of 2x Lammeli buffer was pipetted into three Eppendorf microtubes. Nine microtubes were used for days in which all heads were decapitated at once (Nangia et. al 2021)

Homogenization:

The microtubes containing 15µl Lammeli buffer containing one head (or 5 depending on the blot) were homogenized with a pestle until there was a slight color change within the buffer. The mixture of the eyes of the fly causes this color change. Then, the microtubes were vortexed and centrifuged, making sure all of the solution was together. Heads were then boiled on the hotplate safe for microtubes at 100 degrees for 10 minutes. (Nangia et. al 2021) After boiling, the heads were once again vortexed and centrifuged for 1 minute to ensure the solution was adequately blended. The solutions were placed in the -20°C freezer for storage, or used immediately.

The Western Blot

Western blot analysis was used as an assay to detect the ratio of lipidated to un-lipidated Atg8a proteins in *Drosophila* CNS. The Atg8a gene encodes ubiquitin-like proteins (family of proteins found in almost all eukaryotes), and uses similar E1, E2, and E3 activation steps. Atg8a-I is the target of the membrane lipid phosphatidylethanolamine and has core responsibilities for regulation of autophagosome trafficking, and lysosomal fusion (Jipa et al. 2020). Phosphatidylethanolamine attaches to the autophagic membrane. This converts Atg8a-I, the lipidated version of Atg8a, to the lipidated Atg8aa-II, making it distinct from Atg8a-I. The lipidation occurs to promote the elongation of the phagophore, allowing the encapsulation of the autophagophore. For this reason by

comparing the ratio of un-lipidated Atg8a-I to the lipidated Atg8a-II, the rate of autophagy can be determined (Jipa et al., n.d.)

Drosophila have two genes encoding Atg8a, Atg8ab. For this analysis, I will be using the Atg8a gene as a biomarker for autophagy. I used the recombinant Anti-GABARAP+GABARAPL1+GABARAPL2 antibody to detect the ratio of Atg8a-II to Atg8a-I proteins. GABARAP proteins are a subcategory of the Atg8a gene. A high quantity of autolysosomes is associated with Atg8a-II, allowing us to measure the ratio of lipidated to unlipidated Atg8a proteins.

To prepare the flies for SDS- PAGE the 2x Lamelli Buffer solutions containing homogenized fly heads were taken out of the freezer and placed on ice to thaw before usage. All samples were taken out to be analyzed on the same blot. First, the Mini-PROTEAN Tetra cell was assembled. Using a 20µl pipette with SDS-PAGE gel loading tips, 10µl of each sample was transferred into a Mini-PROTEAN® TGX™ Precast Protein Gel containing 10 wells, along with 5µl of protein ladder. The SDS-PAGE tank was filled halfway with running buffer and the inner chamber within the tank was filled to the top with running buffer (100mL 10x Tris/Glycine/SDS buffer, 900 mL distilled water). Electrophoresis was run for 1 hour at 120V. After the hour, the gel was removed from the cassette and transferred onto a Nitrocellulose membrane. Great care was taken to ensure that the gel did not break during the transfer. The running buffer in the electrophoresis chamber was replaced with transfer buffer (100 mL 10x transfer buffer, 200 mL Methanol, 700 mL ddH2O), and run at 30V for 1 hour.

The Nitrocellulose membrane was then removed from the cassette and placed in a 5% blocking solution (5% dry milk in 1x TBST solution) the membrane in the blocking solution was shaken for 1 hour. The membrane was blocked to prevent non-specific binding. After the hour concluded the blocking solution was replaced with 50 mL of 1:5000 primary antibodies (10 µLrabbit anti-GABARAP (Abcam#ab52866), in blocking solution and shaken overnight in the 4°C cold room.

The following morning the membrane was removed from the blocking solution containing the antibodies and stored. The membrane was then washed 3 times for 10 minutes each with 1 x TBST solution. After the third wash, the membrane was blocked with a secondary antibody 10 µL anti-Rabbit antibody in 50 mL 5% dry milk in 1xTBST) on the shaker for 2.5-4.00 hours. Once the membrane had finished blocking in the secondary antibody, the membrane was once again washed with 1xTBST solution 3 times for 10 minutes to prepare for imaging.

To prepare for imaging, ECL substrate was used to stain the membrane. While incubating, great care was taken to make sure that the membrane was not exposed to light. After incubating for 5 minutes, the Azure Biosystems c600 Imager was used for imaging.

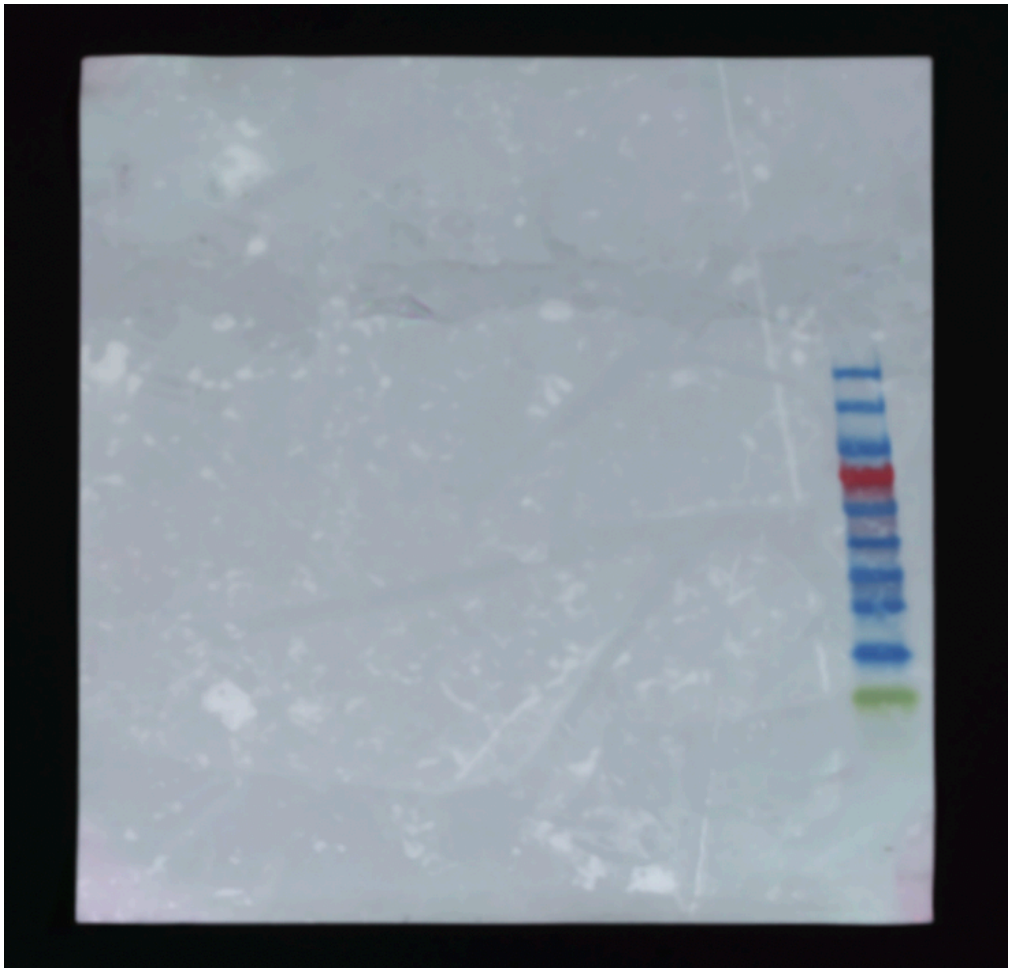

Figure 7: Nitrocellulose membrane imaged on Azure Biosystems c600 Imager, November 10th, 2024. The image shows a clear lack of bands on the blot.

Confocal microscopy

Another method that I chose to take for the measurement of autophagy was confocal microscopy to quantity autolysosomes. This approach includes the preparation of samples via dissection and staining in order to directly visualize the ratio of autolysosomes to nucleus for chosen samples. I chose this as another method for analyzation as a continuation of previous Woodard lab research (Becher, 2023). For this approach, I dissected the whole CNS (central nervous

system) from adult flies 6 hrs to 1 day post-enclosed and stained with mM LysoTracker Red DND-99.

Dissection:

Between 5-10 flies were anesthetized with CO2 to dissect. The amount of flies was dependent on how many samples were viable after dissection. Adult flies were placed on a depression slide with a drop of PBS solution. Heads were then removed from the body by using Dumont #5 tweezers and grasping both the head and the base of the head. The body was then pulled away from the head and the head was placed into the PBS solution. Next, to dissect the CNS, the head was turned to face the back. One pair of tweezers firmly grasped the proboscis, while the other created a clean tear along the back of the head. This should create an opening. In this opening, slide the tweezers into the slits and pull them apart, taking care to not destroy the eyes (as this can make dissection more difficult and cuticle may get stuck in the brain tissue). The brain should now be visible and removable. I then placed the brains into a PCR tube with a few µl of PBS solution.

Autolysosome Staining

1µl of LysoTracker Red DND-99 was mixed with 99µl PBS solution to stain brain tissue with 10 µM LysoTracker Red. It is important not to expose LysoTracker to light. To avoid this, the samples were wrapped in aluminum foil. The LysoTracker was placed in the foil-wrapped PCR tube and allowed to

incubate for 20 minutes. After the incubation period was complete, the samples were briefly rinsed in PBS solution before being mounted in VectaSheild + DAPI solution to be taken up to the confocal microscope as to stain nuclei and avoid photobleaching. At any given time post-stain, the samples were shielded from light. To move the dissected brains and to avoid losing brains, a 1000 µl pipette tip was cut, to create a mechanism for transport.

Confocal Microscopy:

The program used to analyze the samples was CLSM was Nikon Elements. Both DAPI and TRITC channels were used to identify the nucleus and the autolysosomes. The BHB control image was taken two days after the initial two images. Each image was initially focused on 20x using DAPI channels. Then, the image was focused on 40x before switching to the laser channels. To prevent bias, DAPI channels were primarily used for focusing on the samples. Unfortunately, due to time constraints, each sample only has one clear image to be analyzed.

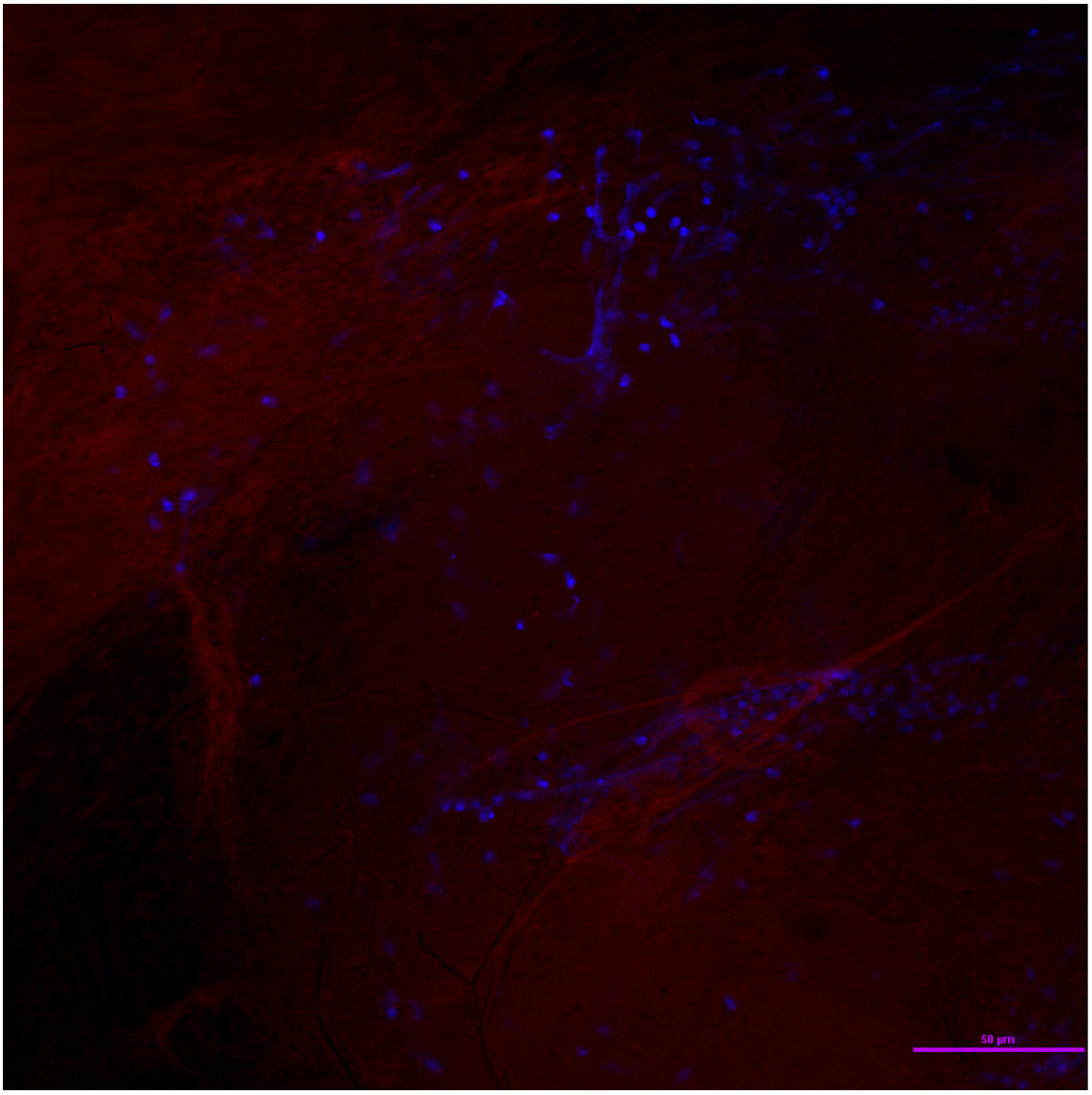

Figure 8: CNS of *Drosophila* visualized using Confocal Laser Scanning Microscopy from flies raised on a control diet and allotted control food for 24 hours post TBI. Cells were observed under 40x magnification. CLSM image obtained by exciting blue and red colors via DAPI and TRITC channels to show autolysosomes and nuclei. The scale bar indicates 50 µm.

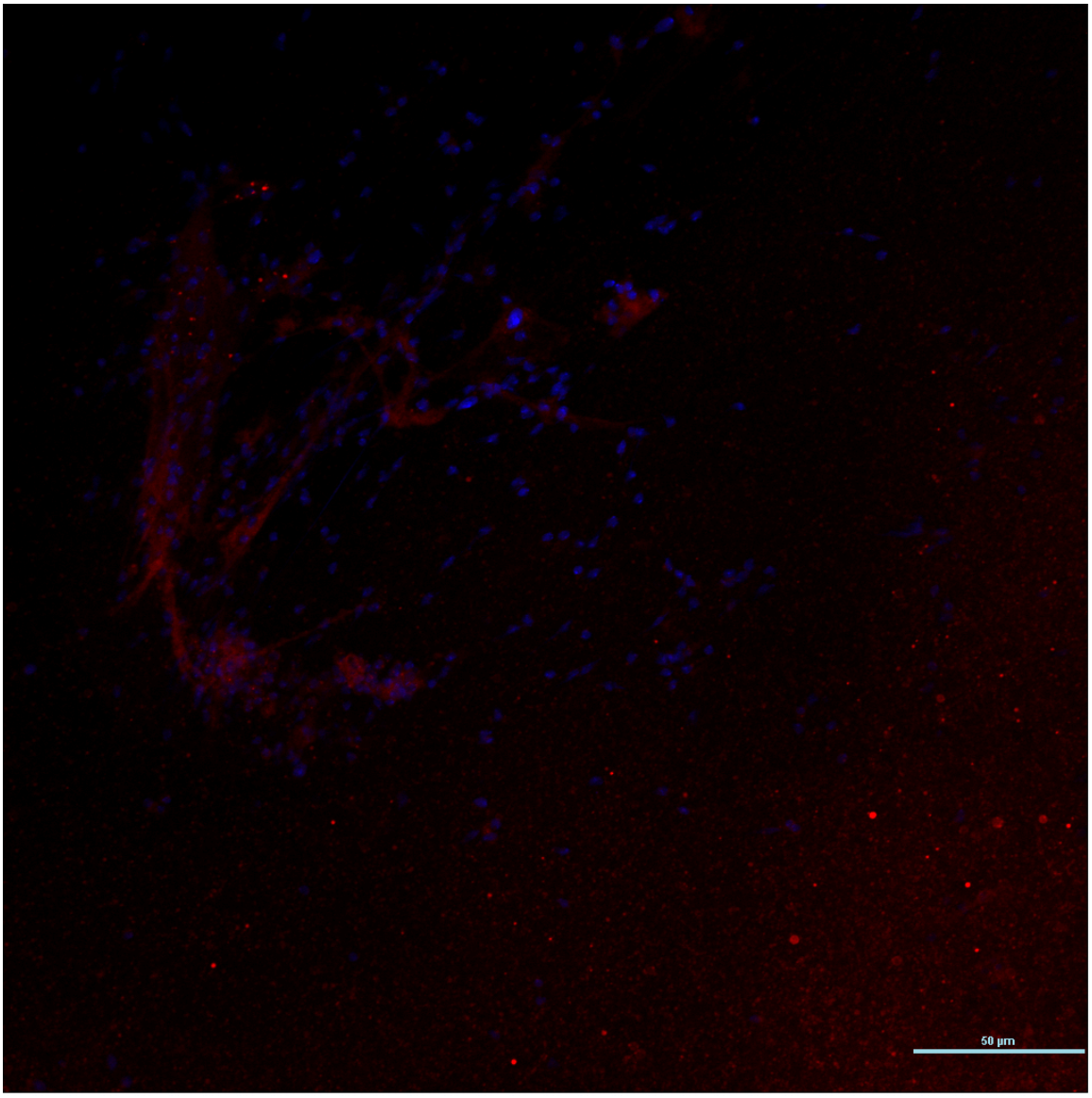

Figure 9: CNS of *Drosophila* visualized using Confocal Laser Scanning MicroscopyFrom flies raised on a BHB-supplemented diet and returned to a BHB-supplemented diet 24 hours post TBI. Cells were observed under 40x magnification. CLSM image obtained by exciting blue and red colors via DAPI and TRITC channels to show autolysosomes and nuclei. The scale bar indicates 50 µm.

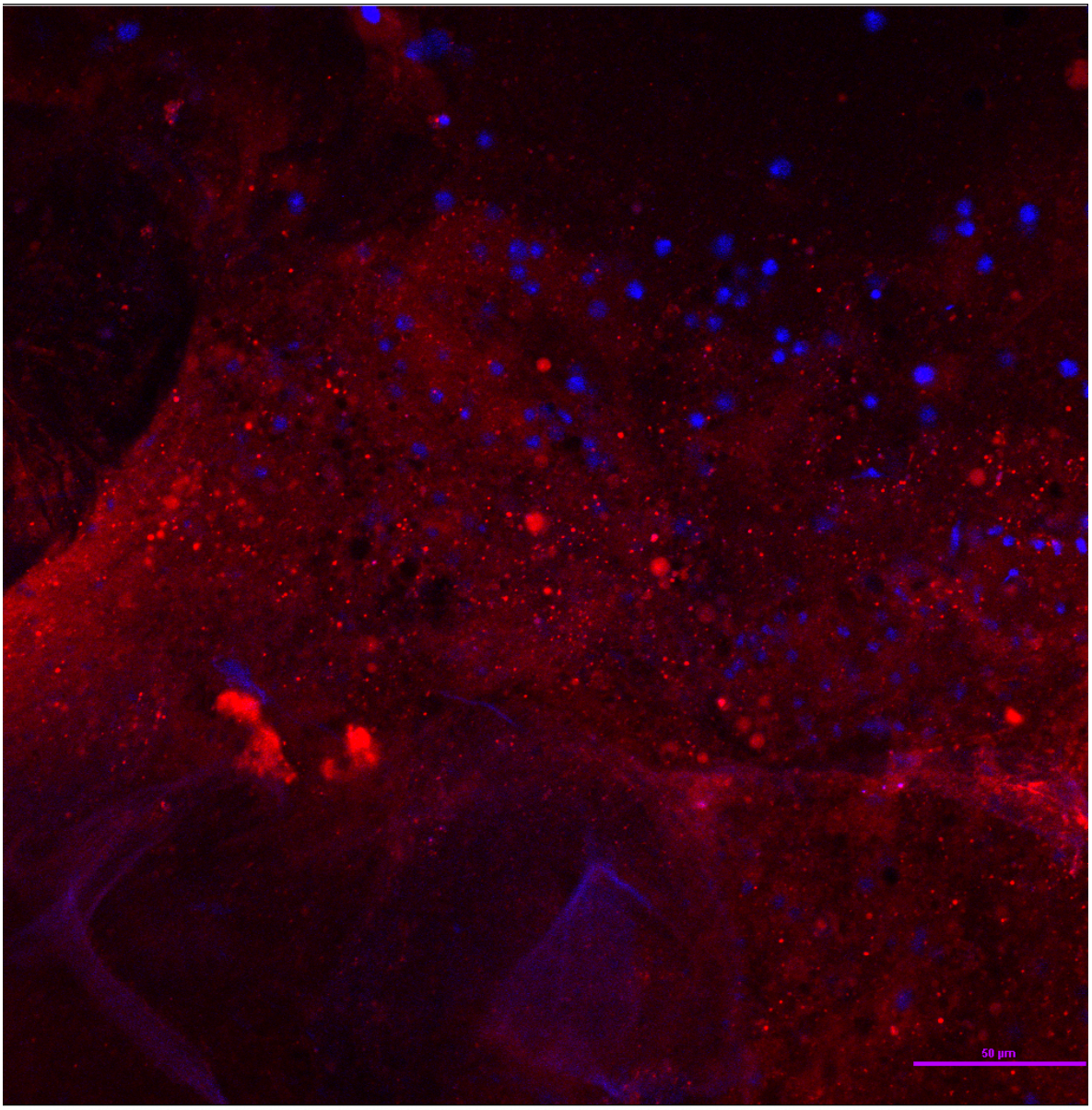

Figure 10: CNS of *Drosophila* visualized using Confocal Laser Scanning Microscopy from Drosophila fed initially with a control diet but switched to BHB-supplemented food post TBI for 24 hours. Cells were observed under 40x magnification. CLSM image obtained by exciting blue and red colors via DAPI and TRITC channels to show autolysosomes and nuclei. The scale bar indicates 50 µm.

Quantification of confocal microscopy:

The BHB-BHB and Control-Control images were taken two days after the Control-BHB image. To quantify the number of puncta per nucleus images were analyzed under the TRITC and DAPI channels to reveal the autolysosomes and nuclei under 40x magnification. TRITC stains autolysosomes, whereas the DAPI stains nuclei. In the images, autolysosomes appear as red puncta, whereas the nuclei fluoresce blue.

The image was analyzed using FIJI (otherwise known as Image J) to quantify the number of puncta per nucleus. Brightness and contrast were adjusted for visualization, as well as the threshold of the brightness of fluorescence that the program identified as an organelle. To avoid bias, adjustments were made as sparingly as possible. Next, I allowed FIJI to quantify both the number of autolysosomes and nuclei per image. Unfortunately, due to time constraints, only the CNS of flies removed from food 24 hours post-hit were analyzed and limited images were taken for a qualitative analysis.

RESULTS

For this project, I explored whether there would be a large induction of autophagy post-traumatic brain injury in *Drosophila* raised on control food or a BHB-supplemented diet. I also explored what the difference in autophagy would be if flies were raised on control food and switched to a BHB-supplemented diet post-traumatic brain injury. Flies raised on control, BHB, and raised on control and switched to BHB were examined.

The results of the western blot analysis were inconclusive as there were no bands that indicated the presence of Atg8a-I or Atg8a-II proteins. The procedure was followed directly as written, however, I was still unable to collect a proper image. Multiple adjustments were taken to the procedure post-imaging to attempt to retrieve a result. Both the amount of *Drosophila* heads and the allotted time for blocking the secondary antibody were adjusted. I also decided to use entirely new buffers and fresh blocking solutions and antibodies. Unfortunately, this did not affect the result of the blot.

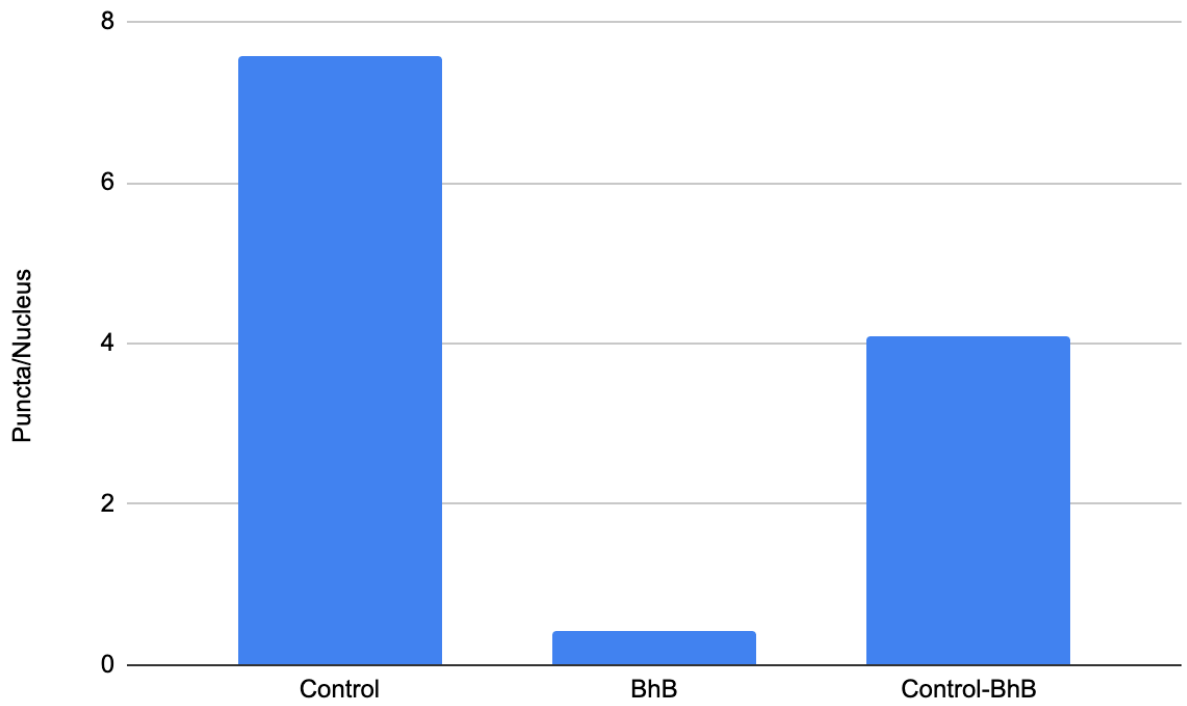

Figure 10: Number of puncta/nucleus for each experimental group. The number of puncta per nucleus for the control group was 7.57, while the number of puncta per nucleus for flies raised solely on BHB was 0.41. The number of puncta per nucleus for flies raised on control food and switched to BHB post-TBI was 4.10. (n=1 for each of the three groups)

Evidence suggests that *drosophila* raised on a control diet and remained on a control diet post-TBI induct a greater amount of flies raised on a control diet that remain on a control diet post-TBI. Evidence also suggests that flies raised on a control diet and switched to a BHB-supplemented diet post-TBI also exhibit less autophagy than flies raised on a control diet and kept on a control diet.

A qualitative analysis of the results of confocal microscopy suggests that there is a decrease in autophagy on flies fed on a BHB diet and remained on a

BHB diet post tbi induce less autophagy than flies raised on a control diet alone and kept on a control diet post-TBI. Statistical analysis was not possible for these samples as there was only one viable image taken for each of the sample groups. Due to the lack of data analysis is only observational and suggestive from the samples provided.

DISCUSSION

After qualitatively comparing the differences in means of average puncta/nucleus between control, BHB, and BHB switched to control groups, the result suggests that BHB fed flies induceless autophagy than flies fed on a control diet. Although my statistical analysis suggested that there is not a significant difference between the groups, due to time constraints I was unable to collect what I deem to be a sufficient amount of samples to draw a solid conclusion from this study. I was also unable to analyze more than one of the time frame groups (only the 24-hour group). For future study, I strongly recommend analyzing the ratio of puncta/ nucleus of the 6-hour and 72-hour time frame groups as well. I also believe it would be helpful to analyze the CNS of unhit flies raised on control food, BHB food, and control food moved to BHB food after a time period, to better understand how BHB affects autophagy without injury.

Due to the western blot not working, I reached out to Dr. Geoffrey Tanner Tanner Lab at The University of Connecticut (Uconn). I was informed that Dr. Tanner's lab was also unable to yield any result about the ratio of Atg8a-I to Atg8a-II proteins using western blot analysis (Tanner,G., Personal communication). This led me to the conclusion that the CNS of adult *Drosophila* does not contain enough protein to be detected via western blot analysis, even when the number of heads used in the analysis was increased in an attempt to increase the amount of protein present in a sample. For future direction, if this

method were to be replicated, there would need to be a significant increase in the number of heads used per sample to increase the amount of protein detected in the sample.

The results of microscopy suggest that flies raised on a BHB diet exhibit less autophagy post traumatic brain injury than flies kept on a control diet, or originally on a control diet and switched to a BHB diet. The results also show that flies raised on a control and switched to a BHB diet post traumatic brain injury also induce less autophagy than flies raised on a control diet alone. However, due to the limited amount of samples that were analyzed due to time constraints I can not say that this would have remained the same had there been other data. To gain a more accurate understanding, I would have liked to collect data from many more samples to find a more true mean than the means that I had found. Additionally, confocal microscopy is not a fool proof option. Although great measures were taken to prevent photobleaching, this effect could be a factor in the difference between the samples. A way to combat this could be by using an alternative stain.

In the Woodard lab, previous students have also tested the effects of BHB-supplemented diets vs control diets. In a 2023 honors thesis, Meredith Becher found no statistically significant increase in autophagy in control vs. supplemented diets in fat bodies of *Drosophila melanogaster* (Becher, 2023). However, in 2022 another student found increased autophagy in fat bodies of BHB fed *Drosophila* (Zhai, 2022). However, Becher quantified autophagy using microscopy, while Zhai used a western blotting approach. The discrepancy

between the two results led me to believe that there still isn't a full conclusion or understanding that be drawn about BHB supplementation autophagy as a whole.

To fully understand the effects of BHB supplementation on TBI it is important to study more than just the effect on autophagy. Both behavioral studies as well as using other biomarkers for indications of damage serve a strong purpose in fully understanding this concept. Studies have shown that there is a difference between ATP/ADP ratios in *Drosophila-fed* BHB-supplemented diets in comparison to control diets for post-traumatic brain injury. The results of this study showed that BHB-supplemented *Drosophila* had better utilization of ATP in the brain, suggesting that ketone bodies help with the utilization of energy post-injury. (Lee, 2020) Behavioral studies have also found that Ketone bodies act favorably with post-traumatic brain injury. The Tanner Lab at the University of Connecticut has found that flies raised on a BHB-supplemented diet exhibit less aggression post-traumatic brain injury (aggression being a distinct marker of brain damage in both *Drosophila* and mammals) as opposed to flies raised on a control diet (D. C. Lee et al., 2019). For this reason, the exploration of the therapeutic effects of ketone body supplementation and the keto diet is far from over.

It is also important to remember the true importance of this study and those like it, to prevent and treat traumatic brain injury. Traumatic brain injury is a leading cause of death in both developed and developing countries. Despite medical advancements, there is not much medicine has to offer for both prevention and treatment. About only 25-30% of those categorized with severe TBI have a positive outcome, about 33% will die, and the rest will be left

permanently disabled (*Traumatic Brain Injury – Causes, Symptoms and Treatments*). For these reasons it is vial to find a viable option for not only treatment, but prevention as well.

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Appendix

WHOLE					
CCDAPI24_RGE	95	1651	17.379	0.157	88.668
CCLYSO24_RG	719	2669	3.712	0.255	24.964
BhB24dapi(2).tif	518	13176	25.436	1.257	57.043
BhB24Lyso (2).ti	214	804	3.757	0.077	93.605
CBHB24DAPI_R	126	1803	14.31	0.172	91.746
cBHB24LYSO_R	516	1758	3.407	0.168	65.873

Table 1a: Image J quantification raw data