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Ketogenic Diet and Seizure Susceptibility in a Whole-Animal *Drosophila melanogaster* Model: Effects and Mechanisms

By

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

fed in either BF diet or BHB supplemental diet in the third-round experiment

ABSTRACT

Seizures are abnormal epochs of neuronal hyperexcitability. The low-carbohydrate, high-fat ketogenic diet (KD) is an effective treatment for epilepsy in juveniles especially for drug-resistant seizures. KD produces ketone bodies (KB, e.g. β- hydroxybutyrate, or βHB) that are thought to have anticonvulsant properties, however, their exact mechanism of action is unknown. In vitro, presumably anticonvulsant KB effects appear to be mediated by K_{ATP} channels and $GABA_B$ signaling. In order to study the role of $GABA_B$ signaling and K_{ATP} channels, the *eas* bang-sensitive mutant strain, which exhibits seizures upon mechanical stimulation, was used. This strain is expected to have reduced seizure like activity (SLA) in the presence of βHB after mechanical shock. Fly SLA was examined under various conditions of diet and pharmacological treatment. Using automated computer scoring of behavior, *eas* flies fed the βHB supplemented diet exhibited significantly fewer "jumps" (interpreted as SLA's) compared with the control diet. Application of CGP55485 (a $GABA_B$ blocker), or tolbutamide (a K_{ATP} blocker) partially reversed these KB effects in this whole-animal model, validating its use for further study of seizure disorders.

CHAPTER I: INTRODUCTION

Seizure Disorder Overview

Seizures are abnormal changes in the electrical activity of neurons and neural networks and circuits (Fisher et al., 2005). Recurrent seizures in the central nervous system are classified as a seizure disorder called epilepsy (Cunliffe et al., 2015). Epilepsy influences around 1% of the US population and at least 50 million people worldwide (Kobau et al., 2008, Cunliffe et al., 2015). Statistics have shown that although many patients respond positively to anti-epileptic drugs, one third of cases cannot manage their seizures with currently available anti-convulsant medications (Remy and Beck, 2006, Cunliffe et al., 2015). The causes of seizures vary from genetic defects to brain injuries (Cunliffe et al., 2015). The major conventional surgical intervention implements a cut into the corpus callosum connecting the two hemispheres of the brain in order to limit the spread of epilepsy (Glickstein&Berlucchi, 2008). Some surgical resections of abnormal tissues like tumors have also been a common practice (Glickstein&Berlucchi, 2008). However, treatment options remain limited because not all patients develop such lesions or abnormal tissue growths such as tumors (Glickstein&Berlucchi, 2008).

In addition, an alternative dietary treatment for epilepsy called the ketogenic diet (KD) was developed in the 1920's (Wilder 1921). This diet emphasizes a high-fat, low-glucose diet and has also been considered as an effective therapy to treat drug-resistant epilepsy, especially in children (Thiele,

2003, Hartman & Vining 2007, Neal et al. 2008). Evidence has shown that fasting could be applied as a treatment to epilepsy, and the KD was first designed to mimic these metabolic effects of fasting (Bailey et al., 2005). However, many patients could not tolerate a long-term KD since the diet is restrictive and specific. Therefore, one of the modifications for KD is a low glycemic index treatment (LIGT) that makes the diet more favorable. LIGT allows a more liberal daily intake of carbohydrates compared to the stringent KD (Pfeifer, 2008). The KD requires followers to restrict all kinds of carbohydrates equally regardless of sources (Pfeifer, 2008). However, the LIGT allows people to consume a more variety of carbohydrates, as long as they have a low glycemic index (Pfeifer, 2008). To develop a better understanding of the mechanistic underpinnings of the diet, researchers have been using animal models such as zebrafish, mice, and fruit fly *Drosophila melanogaster*to study epilepsy (Song et al., 2008)

Drosophila melanogaster **as an animal Model for Seizures**

Because of its simple nervous system, rapid life cycle, and genetic tractability, fruit flies Drosophila melanogaster are a useful and commonly-studied model for understanding numerous human diseases, including seizures and diabetes (Song et al. 2008, Parker et al. 2011). Although the fly's genome is only about 5% the size of that in humans, and is distributed on four chromosome pairs (Kemppainen, 2015) instead of 23, about 75% of human disease-causing genes are believed to have a functional homolog in the fly (Pandey and Nichols, 2011). Additionally, the short four-stage metamorphic developmental process, in

which normal flies develop in the larval stage for 7-10 days and typically eclose as reproductive adults about 4 to 5 days after the pupa stage (Kemppainen, 2015). This results in a quick life cycle, wherein multiple generations can be produced in a short amount of time. Therefore, another powerful feature of this model is the ability to measure seizure susceptibility accurately across genotypes over time (Kuebler and Tanouye, 2000; Kemppainen, 2015). Finally, many well-characterized mutants exist, including those featuring elevated seizure susceptibility, making fruit flies a highly suitable model to study seizure disorders (Pandey and Nichols, 2011).

Bang-Sensitive Mutants, Metabolism, and Seizure

Drosophila mutants called bang-sensitive (BS) paralytics are commonly used to study seizures (Song et al. 2008, Parker et al. 2011, Stone et al., 2013). BS paralytics are strains of flies featuring loss-of-function mutations in genes involved in regulating seizure activity resulting in seizure behavior and paralysis when activated by mechanical stimulation (Fergestad et al., 2006). The BS mutants flies are more susceptible to seizure-like activity (SLA): rapid, uncontrollable movement of the wings, abdomen and legs (Stone et al., 2013), violent bursts of uncontrollable shaking, paralysis-like static movement, and delayed contraction and recovery (Baraban 2007) compared to wild-type flies. These behavioral readouts are easy for people to observe and record in order to study seizures. Several identified BS mutations (e.g., technical knockout or *tko*, stress-sensitive or *sesB*, and easily shocked or *eas*) affect genes that encode

products intimately involved with metabolic processes (Fergestad et al., 2006). Those mutants with mitochondrial defects suggest that there is most likely a relationship between mitochondrial and metabolic alteration in human seizure, which was tested by Fergestad et al. (2006) using the *knockout* (*tko*) mutant encoding an essential metabolic protein, which is essential for citrate synthase. Significantly reduced ATP levels in *eas, tko*, and *sesB* mutants suggest that defects in cellular metabolism are related to BS mutations, although the exact mechanistic connection is still unclear (Fergestad et al., 2006).

The *tko* gene encodes the ribosomal protein S12 (mRpS12) in the mitochondria that is essential for translation of mitochondrial proteins (Toivonen et al., 2001). Mutation of this gene causes defects of mitochondrial ribosomes through reduced activity of mitochondrial redox enzymes containing mitochondrial translation products and decreased levels of ribosomal S12 (Toivonen et al., 2001). If rRNA does not bind to ribosomes, it becomes unstable; the low amounts of 12S rRNA result in either a ribosomal assembly or stability defect (Toivonen et al., 2001). These mitochondrial translation defects lead to limitations on mitochondrial protein synthesis; the resultant loss of metabolic proteins may lead to seizure disorders (Toivonen et al., 2001).

In addition of *tko*BS mutant strain, the*sesB* mutation encoding another mitochondria protein called the adenine nucleotide (ADP/ATP) translocator (ANT), which plays an important role in ATP export from mitochondria resulting in defects in energy metabolism (Zhang et al., 1999, Toivonen et al., 2001). ANT

is the transporter protein shuttling ADP and ATP across the inner mitochondrial membrane in order to release energy from catabolism to the rest of the cell. ANT is a key link between cytoplasmic energy usage and mitochondrial oxidative phosphorylation (OXPHOS), the metabolic pathway in which the mitochondria in cells use their structure, enzymes, and energy released by the oxidation of nutrients to reform ATP (Zhang et al., 1999, Fergestad et al., 2006, Vartiainen et al., 2014). *sesB* mutant fly adults exhibit a decreased respiratory pathway due to a reduced activity of cytochrome oxidase (the last enzyme in the ETC) in response to the limitation of ATP synthesis in mitochondria (Vartiainen et al., 2014).

Lastly, the *eas* mutant encodes genes for ethanolamine kinase, which is crucial for synthesis of phosphatidylethanolamine (PE) in the biosynthesis of phospholipids, the key component of the cell membrane (Pavlidis et al., 1994, Zhang et al., 1999). The *eas* mutation results in overproduction of lipids; the increased lipid metabolism in this mutant fly strain alters neuronal excitability (Fig. 1). The *eas* mutant flies have decreased synthesis of PE, which dysregulates phospholipid metabolism and induces constant compensatory overactivation of the transcription factor dSREBP (Drosophila sterol regulatory element-binding protein) signaling and excess lipid production (Swope et al 1992, Li et al., 1993). The effect of this mutation on lipid metabolism may increase *eas* behavioral defects by altering ion channel activity to change neuronal excitability (Swope et al 1992, Li et al., 1993).

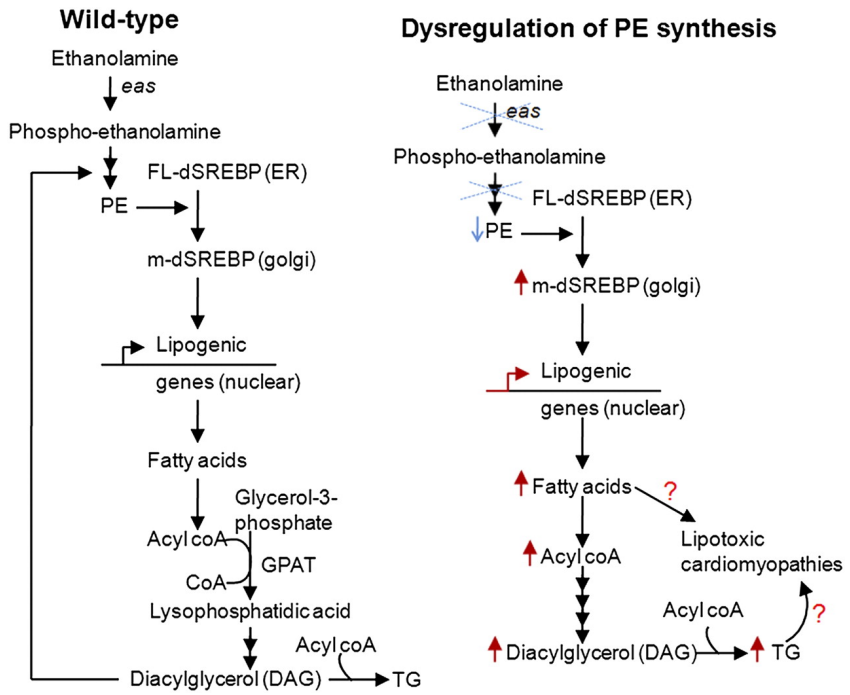


Figure 1. A model depicting how phospholipid homeostasis regulates lipid metabolism and cardiac function through SREBP signaling in Drosophila (Lim et al., 2011).

Ketogenic Diet and Ketone Bodies

Recent research has shown that bang sensitive flies fed with the ketogenic diet (KD) have reduced seizure like activities (SLA) (Stone et al., 2014). In addition, this dietary therapy is considered an effective treatment for patients with epilepsy, especially for pediatric cases with drug-resistant seizures (Thiele, 2003, Hartman & Vining 2007, Neal et al. 2008). The ketogenic diet is a high-fat and low-glucose dietary therapy with a 4:1 ratio of fats to proteins and carbohydrates (Thiele, 2003, Hartman & Vining 2007, Neal et al. 2008, Lutas and Yellen, 2013). The effect of the KD can be disrupted if additional sugar is supplied (Lutas and

Yellen, 2013). The KD produces ketone bodies (KB's; for example, β-hydroxybutyrate or acetoacetate) that are found in the blood and urine of patients consuming a KD (Hartman & Vining, 2007). Based on observation of patients treated on KD, KBs are considered to have anticonvulsant properties (Sokoloff, 1973, McNally & Hartman, 2012).

Under a KD in mammals, elevated fats generate high levels of acetyl-CoA and ketone bodies produced by the liver through exchanging excess acetyl-CoA to ketogenesis (Fig. 2) (McNally &Hartman, 2012). Ketogenesis is the process of breaking down fatty acids to generate ketone bodies. Very little acetyl-CoA enters the TCA cycle in the liver; instead, excess acetyl-CoA is converted to the ketone body acetoacetate, which is converted to beta-hydroxybutyrate (βHB) through enzymatic catalysis by 3-hyrdoxybutyrate dehydrogenase (Melo et al., 2007, McNally & Hartman, 2012). Ketone bodies are then released from the hepatocytes, enter the bloodstream, and cross the blood brain barrier (Melo et al., 2007, McNally & Hartman, 2012). Acetoacetate is reactivated to its CoA derivative in order to be converted to acetyl-CoA eventually. This acetyl-CoA will enter the TCA cycle for the production of energy and other metabolic intermediates (Melo et al., 2007, McNally & Hartman, 2012). Differently from the production of ketone bodies in mammals, ketone bodies are formed in the flies' fat body (Trinh &Boulianne, 2013). Additionally, cells called oenocytes in the fat body stores lipids/fats under starvation.

Because of the increased levels of ketone bodies observed in patients treated with the KD, coupled with the KD's clear anticonvulsant effects, ketone bodies are used to understand the KD's anticonvulsant mechanism. Although the mechanism is still unclear, there are various hypotheses that may explain the action of ketone bodies. These include: inhibition of vesicular glutamate loading, activation of ATP-sensitive K^+ channels (K_{ATP} channels), glycolytic inhibition, and increased GABA signaling (Ma et al., 2007, McNally & Hartman, 2012, Lutas&Yellen, 2012, Hartman &Stafstrom, 2013). In my project, we investigated the role of K_{ATP} channels using the K_{ATP} inhibitor tolbutamide, and we also investigated the role of GABA signaling using the $GABA_B$ blocker, CGP55845.

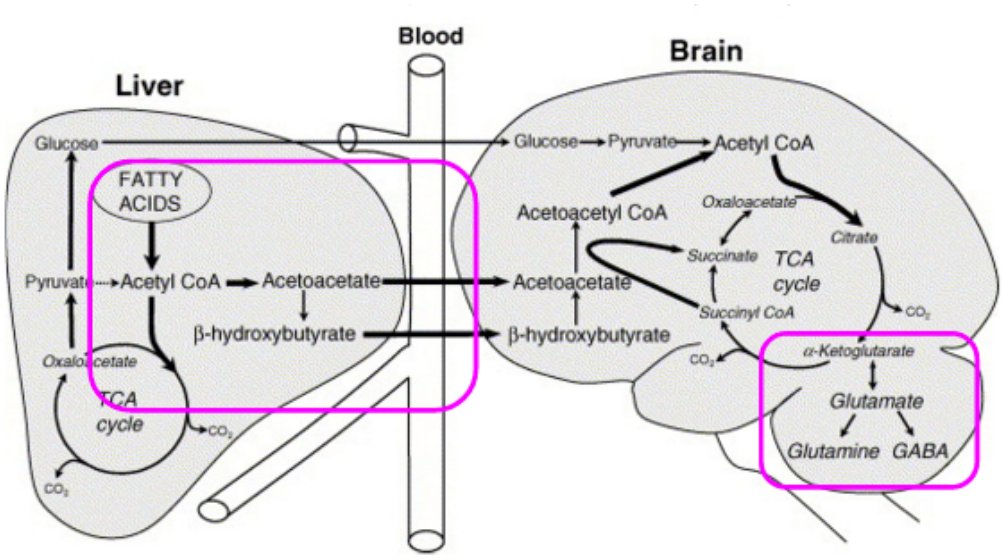


Figure 2. A high-fat, low-carbohydrate ketogenic diet causes a shift in metabolic activity (Melo et al., 2006).

Ketone Bodies and K_{ATP} Channels

One way that ketone bodies may reduce SLA is through an effect on neuronal membrane potential. Previous research demonstrated the role of ATP-sensitive K^+ (K_{ATP})channels as a clear link between cellular metabolism and neuronal excitability (Ma et al., 2007, Tanner et al., 2011, Gimenez-Cassina et al., 2012, Lutas and Yellen, 2013, McNally and Hartman, 2014). K_{ATP} channels, inhibited by intracellular ATP, are hetero-octamers, composed of four pore-forming potassium-channel subunits (Kir6.2) and four regulatory sulfonylurea-receptor subunits (SUR1) (Fig. 3) (Ashcroft et al. 1984, Ashfield et al., 1999, Glyon et al., 2004, Gimenez-Cassina et al., 2012). They are located throughout the central nervous system, and are generally found in the closed state because of high intracellular ATP production through glucose metabolism (Ashcroft et al. 1984, Proks et al., 2008, Gimenez-Cassina et al., 2012). K_{ATP} channels are also present in flies such as the insulin-like peptide-producing cells (IPCs) in the brain, and cardiac muscle (Fridell et al. 2009, Akasaka et al., 2006).

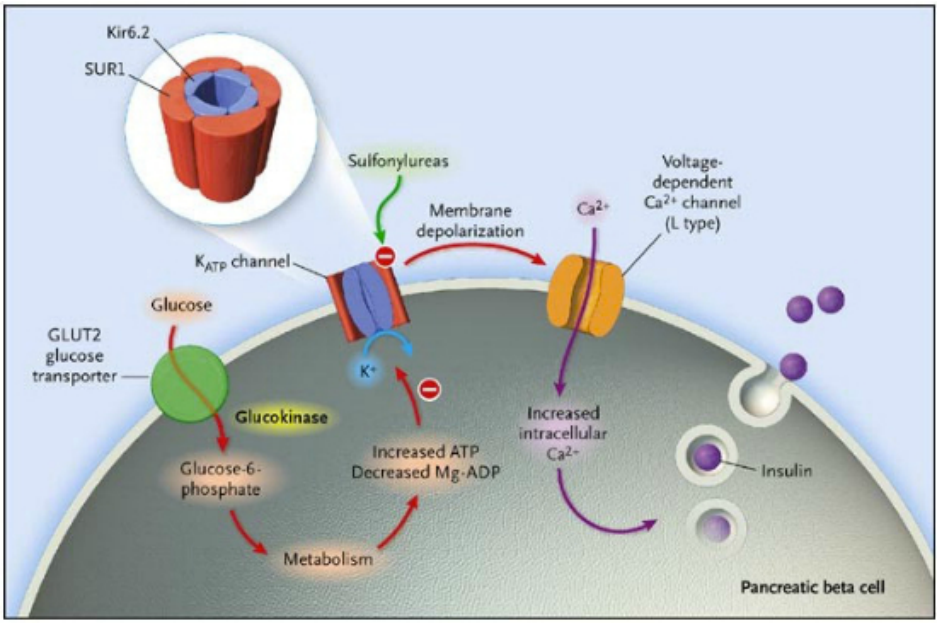


Figure 3. Schematic representation about the role of the ATP-sensitive potassium (K_{ATP}) channel in insulin secretion (Glyon et al., 2004).

A connection between ketone bodies and K_{ATP} activity has been shown in several mammalian brain regions thought to play a role in seizure. In neurons of the mouse hippocampal dentate gyrus, a region of the brain with high levels of K_{ATP} channels, an increased level of K_{ATP} channel opening was found in the presence of βHB; such K_{ATP} opening is thought to be one possible mechanism to limit the passage of hyper-excitation and thus, to temper seizure (Tanner et al., 2011). In the substantianigra pars reticulata (SNr), a key regulator of motor output and a brain region known to act as a "seizure gate" controlling seizure generalization, spontaneously-active neurons in mouse brain slices exhibited reduced firing rates in the presence of βHB or acetoacetate on top of normal glucose concentrations (Deransart et al., 2003, Ma et al., 2007). This effect was

eliminated or reduced with the either the application of tolbutamide (an inhibitor of K_{ATP} channel opening), or in K_{ATP} channel knockout animals, suggesting that K_{ATP} opening might, at least in part, mediate the KB effect (Ma et al., 2007).

Ketone bodies may enhance K_{ATP} channel activity through ATP compartmentation by decreasing glycolytic ATP levels (Fig. 4) (Lutas and Yellen, 2013). KBs can enter neurons and be metabolized in mitochondria by oxidation in the tricarboxylic acid (TCA) cycle, which results in increased mitochondrial ATP production and decreased glycolytic ATP production (Lutas and Yellen, 2013, McNally and Hartman, 2014). This metabolic change activates K_{ATP} channels, putting the neuron into a hyperpolarized state. Reduced seizure activity is an expected consequence of hyperpolarization. Since ketone bodies elevate the production of acetyl-CoA, acetyl-CoA enters the TCA cycle via citrate synthesis (acetyl-CoA + oxaloacetate → citrate) resulting in increased production of citrate, an early intermediate in TCA cycle, which inhibits phosphofructokinase (PFK) I, a key glycolytic enzyme (Berg et al. 2002, Yudkoff et al., 2007). Thus, glycolysis is inhibited. In addition, application of an alternative energy source (e.g., pyruvate, lactate instead of glucose) or the glycolytic inhibitor *in vitro* in rat brain slices, researchers found that epilepicform burst frequency was reduced (Stafstrom et al., 2009). Therefore, inhibition or reduction of glycolysis might suppress seizures (Figure 4b) (Appleton &DeVivo, 1974; DeVivo et al., 1978; Berg et al. 2002,Stafstrom et al., 2009). Furthermore, the KD would lead to an overall increase in cellular ATP while reducing ATP levels by the plasma

membrane, where most glycolysis is localized. Due to the low local concentration of ATP, plasma membrane K_{ATP} channels are activated (Tanner et al., 2011, Lutas and Yellen, 2013, Tantama et al. 2013). This activation of K_{ATP} channels would cause potassium ions to flow out of the cell, which would subsequently cause the membrane to hyperpolarize, thereby reducing the probability that an action potential will fire (Tanner et al., 2011, Lutas and Yellen, 2013).

K_{ATP} channels may not only be activated via metabolic inhibition, but also due to ATP consumption by the Na^+/K^+ pump (Fig. 4) (Tanner et al., 2011). The main consumer of ATP at the plasma membrane is the Na^+/K^+ pump, which maintains the electrochemical gradients of sodium and potassium using ATP (Tanner et al., 2011). Increased activity of Na^+/K^+ pumping activates nearby K_{ATP} channels because the intracellular ATP level is lowered and high intracellular ATP inhibits these channels (Tanner et al., 2011). In conclusion, activation of these channels through metabolic inhibition or ATP consumption by the pump is likely to result in reducing seizure activity. Therefore, it will be interesting to see whether K_{ATP} is the main factor that helps to reduce seizure in the presence of βHB using tolbutamide.

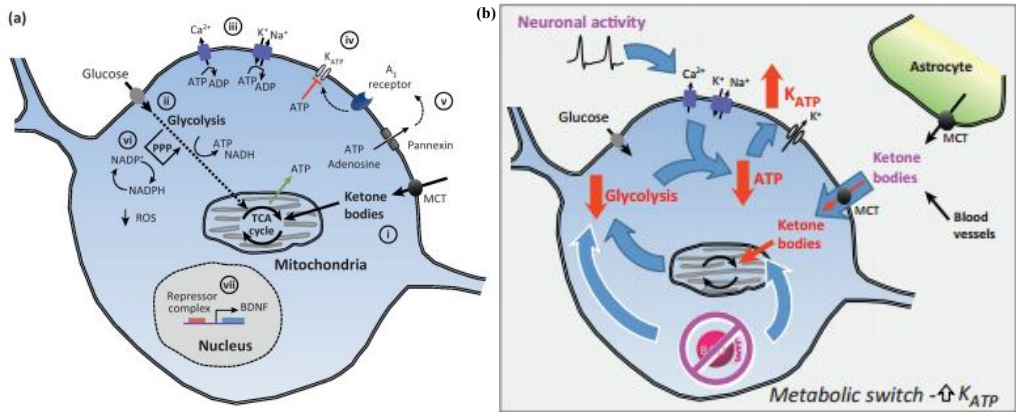


Figure 4. Schematic representation about the relationship between K_{ATP} channels and ketone body (Lutas and Yellen, 2013). (a) The diagram displays the key metabolic pathway and targets. (b) Metabolic switch increased K_{ATP} channels.

Ketone Bodies and GABASignaling

Application of ketone bodies reduced firing rate in spontaneously-active CNS neurons and this effect could be reversed by the $GABA_B$ receptor antagonist CGP55845 (Ma et al., 2007, McNally and Hartman, 2014, Hartman &Stafstrom, 2013, Lutas and Yellen, 2013). Ketone bodies are converted to acetyl-CoA, metabolized via citrate synthase (acetyl-CoA + oxaloacetate → citrate + CoA); this limits the production of oxaloacetate and its ability to convert glutamate to aspartate via transamination (Fig. 5) (Yudkoff et al., 2007, Melø et al., 2006). Thus, more glutamate is available, which yields increased production of gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter and a key anticonvulsant agent in order to slow the firing rate in SNrGABAergic neurons (Yudkoff et al., 2007, Melø et al., 2006, Ma et al., 2007).

Under exposure to βHB in cultured astrocytes or under a KD for mice, more glutamate was available (Yudkoff et al., 1997, Yudkoff et al., 2001). However, there are contradictory findings reporting no change in GABA signaling (Yudoff et al. 2001, Yudkoff et al., 2005, Melo et al., 2006, Appleton &DeVivo 1974, AlMudallal et al., 1996). Therefore, the role of GABA signaling functions in anti-seizure mechanisms is still unresolved.

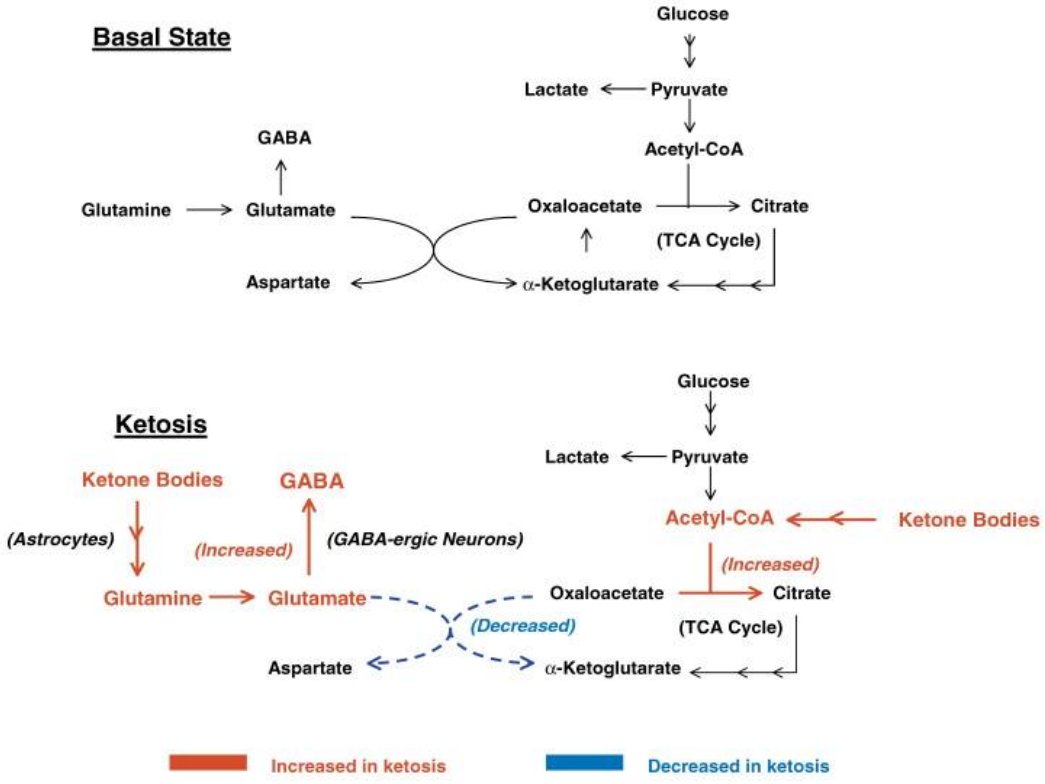


Figure 5. Diagram of the role of glutamate under the impact of ketone bodies (Yudkoff et al., 2007).

The Present Study

The present study is designed to understand and elucidate, in a whole animal model, what role, if any, K_{ATP} channels and $GABA_B$ signaling may play in

the effect of ketone body (βHB) application on SLA. Bang-sensitive (BS) mutant flies' seizure like activities (SLA) were examined under various dietary conditions (standard versus KB-supplemented diets) and pharmacological manipulations (using either the K_{ATP} inhibitor tolbutamide or the $GABA_B$ blocker CGP55485). Because BS mutant flies have different types of SLA behaviors and are identifiable, I hypothesized that *tko, sesB,* and *eas* mutant fly strain would exhibit greatly decreased SLA with the application of βHB over the entire course of development, a moderate decrease of SLA after transferring flies from a control diet to βHB diet at eclosion, and some decrease or no change of SLA in the presence of either βHB + Tol or βHB + CGP55485.

CHAPTER II: MATERIALS AND METHODS

Fly Husbandry Supplies and Videography Equipment

Drosophila vials and plugs for Drosophila vials were purchased from Genesee Scientific (San Diego, CA). Transfer chambers, enclosed recording chambers (3cm width x 3 cm length x 4 cm height), and the open recording arena (same dimensions as enclosed recording chamber) were designed and manufactured in-house (Figure 6). A Vortex Genie2 (Scientific Industry, Bohemia, NY) was set to its highest intensity (10) for shaking flies to induce seizures. After vortexing, flies in their recording arenas were placed under an Olympus SZ40 zoom stereo microscope (Micro-Tech Optical INC, Bloomfield, CT) connected to a PixeLink USB microscope camera (PixeLink, Ottawa, ON). Videos of fly behavior were recorded using Camtasia Studio software (TechSmith Corporation, Okemos, MI) at a framerate of 10 fps and a size of 640 x 480 pixels. Recorded videos were analyzed off-line using either manual scoring or automated scoring with Ethovision software (Noldus Information Technology, Leesburg, VA).

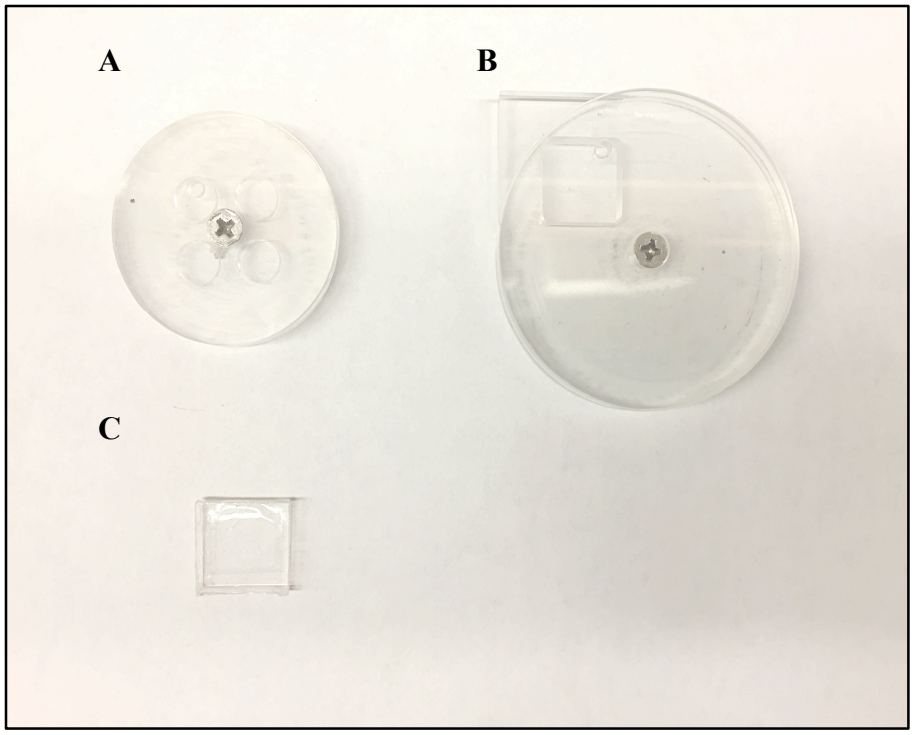


Figure 6. A representative equipment diagram involving transfer chambers (A), enclosed recording chambers (B), and the open recording arena.

Subjects and Materials

(I) Drosophila stocks and maintenance

tko^{3}, *sesB*^{1}, and *eas*^{alaE13} mutant flies and Canton-S (wild type) flies were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). All flies were cultured on a standard Bloomington Formulation food ("BF"; Genesee Scientific, San Diego, CA) with the anti-fungal agents Tegosept (15g/L food; Apex BioResearch Products, Whitmore Lake, MI) and propionic acid (4.9 mL/L food). All experimental flies were raised and maintained at room temperature.

All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted.

(II) Fly cultures

Nine different culture media were prepared for these experiments. In addition to the control diet (BF), and eight other diets or pharmacological conditions were used. Each diet included a dietary supplement or a drug, or drug vehicle, or a combination thereof, mixed with the standard BF diet, which included: for supplement, sodium 3-hydroxybutyrate (βHB); for drugs, tolbutamide (Tol) and CGP55845 hydrochloride (CGP); for vehicle, dimethyl sulfoxide (DMSO, to dissolve CGP) and ethanol (EtOH, to dissolve Tol). Tolbutamide was made as a 40 mM stock solution in 95% EtOH and diluted in BF food to a final concentration of 200 μM; CGP55845 was made as a 11.4 mM stock solution in DMSO and diluted in BF food to a final concentration of 2 μM. Further details are presented below in Table 1.

Table 1. Formula of each diet.

Diets	Formula
Control (BF)	Standard Bloomington food (1L) with Tegosept (15 g/L) and propionic acid (4.9 L/L)
βHB supplement	2 mM βHB dissolved in BF diet
A mixture of βHB supplement and tolbutamide (βHB + Tol)	200 μM Tol dissolved in 95% EtOH on top of βHB supplemented diet
Tolbutamide (Tol)	200 μM Tol dissolved in 95% EtOH on top of BF diet
Ethanol (EtOH)	BF diet with 95% EtOH

Diets	Formula
A mixture of βHB supplement and CGP 55485 (βHB + CGP)	2 μM CGP 55485 dissolved in 2.5 mM DMSO on top of βHB supplemented diet
CGP 55485 (CGP)	2 μM CGP 55485 dissolved in 2.5 mM DMSO on top of BF diet
A mixture of βHB supplement and DMSO (βHB + DMSO)	2.5 mM DMSO dissolved in βHB supplemented diet
DMSO	2.5 mM DMSO dissolved in BF diet

Procedure

Virgin female flies of a given BS mutant strain were paired with male flies of the same strain. Mating pairs were distributed one pair per BF vial; each such pair was classified as the original parents.

First-round experiments: So that all offspring in a given genotype would be siblings, the original parents were transferred to other diets subsequently in the following order: BF diet, βHB supplemented diet, Tol diet, a mixture of βHB supplement and tolbutamide (βHB + Tol), EtOH diet. For each transfer of original parents, wafers of BF food were placed to cover surfaces of diets to ensure parents were always only allowed to eat solely control diet (BF). To make wafers, cylinders of solid BF were pulled from pre-existing food vials and sliced into thin circles, which were placed on top of experimental diets.

Second-round experiments and third-round experiments: Alternatively, experimental offspring were produced by mating one pair of BS mutant parents per given diet vials. BF wafers were also used in this case so that parents' diets were unaffected.

During first-round experiments, offspring collected immediately after eclosion were anesthetized using CO_2, sexed, and separated into individual empty vials. Flies were allowed to recover from anesthesia for a minimum of 3 hours. Each individual fly was transferred to a transfer chamber and vortexed for 10 seconds to induce temporary seizure like activity (SLA). Immediately after vortexing, each fly was transferred to the open recording arena for behavioral recording their behaviors. Recordings began at the time of vortexing and continued for 3 minutes.

An alternative method was used for the second-round experiments and third-round experiments that each individual fly was transferred to an observation chamber one at a time without anesthesia, vortexed for 10 seconds and recorded for 3 minutes. Anesthesia and sex identification were performed after completion of recording. In the second-round experiments, fly offspring were collected immediately after eclosion instead of three days after eclosion in the third-round experiments.

(I) Selecting the ideal BS mutant fly strain for further study

Three BS mutant strains were compared with the Canton-S (wild-type) strain to determine whether there were the differences in seizure-like activities (SLAs). Regardless of genotypes, all flies were fed a control (BF) diet in trial recordings from the first- and second-round experiments to select the ideal BS mutant strain. Upon determination of the ideal strain (eas), further investigation

into the role of the K_{ATP} channel blocker or the effect of $GABA_B$ receptor blocker on top of βHB supplemented diet was investigated.

(II) Effect of K_{ATP} channel blocker

The BF diet and the BHB + Tol diet were compared in *eas* BS mutant flies to test the effect of K_{ATP} channels using K_{ATP} blocker, tolbutamide. Mating pairs were set up in six diets (BF, BHB, BHB + Tol, Tol, EtOH) in third-round experiments.

(III) Effect of $GABA_B$ blocker experiment

The BF diet and the BHB + CGP diet were compared in eas BS mutant flies to test the effect of $GABA_B$ signaling using $GABA_B$ blocker, CGP55485. Mating pairs were set up in six diets (BF, BHB, BHB + CGP, CGP, BHB + DMSO, DMSO) in third-round experiments.

Analysis of Video Data

Recordings were viewed in Windows Media Player. Each recording was 3 minutes long, including 10 seconds vortexing time. During first-round experiments, there was a 30-second delay for fly transfer time between vortexing and visualization; for second-round experiments, there was a 15-second delay for fly transfer time.

Two methods were employed to analyze behavior: Ethovision software and manual scoring. In Ethovision, initially, several different behaviors were quantified to assess the level of SLA: sudden jumps, and paralysis-like static movement. Sudden jumps were typically reported by Ethovision as “subject not

found," static behavior was reported as "immobile frequency or immobile cumulative duration." Parameters were set to generate data on: total distance (cm), mean velocity (cm/s), subject not found (%), immobile frequency, and cumulative immobile duration (s), defined as the state of activity lower than 5% of mean velocity. Optimization of the parameters was carried out by comparing the consistency of SLAs between manual screening and automatic screening under different parameterization in first-, second- and third-round experiments. "Subject not found" percentages appeared to most reliably report incidence of SLA; therefore, this parameter was chosen for all statistical analysis.

Another way to measure SLA was performing manual scoring on effect of $GABA_B$ blocker diet experiment. Four levels of SLA could be identifiedreliably: "jumps," "flips," "disappearance interpreted as high-frequency tremors," and "mixed" behaviors. A jump was classified as a movement of more than one body distance away from the original location; A flip was classified as body rotation and/ or body spinning; a disappearance is classified as a video frame with no detectable fly image while the fly was still at the same place before and after losing image; a mix is classified as the presence of more than one described SLA behaviors above. Frequency of each eas BS mutant fly's total SLA and duration of each SLA in each individual fly in both control diet (BF) and βHB supplemented diets were counted. Lastly, the average frequency of individual flies' total SLA and mean durations of each SLA in both diets were calculated.

Statistics

Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA) and SPSS (IBM) software. Univariate analysis of variance (ANOVA), Mann-Whitney U-tests, and Kruskal-Wallis tests were used to compare SLA between different diets and genotypes.

CHAPTER III: RESULTS

Seizure Disorder Overview

A total of 226 trials of first-round experiments were performed with all four genotypes over five diets (BF, βHB, βHB + Tol, Tol, and EtOH) (Table 2). A total of 187 trials of second-round experiments were performed with four genotypes collected over four diets (BF, βHB, βHB + Tol, and Tol) (Table 3). For first- and second-round experiments, not all diets were used with all genotypes. A total of 196 trials of third-round experiments were performed only in *eas*BS mutant flies over ten diets including one where offspring were transferred from BF immediately after eclosion to βHB diet for three days (dietary switch - BF → βHB) (Table 4). Table 2 and Table 3 show that the *eas*BS mutant strain had the greatest reproductive rates as well as the highest seizure-like activity (SLA) on the tested diets among all BS mutant strains.

The automatically detected "Subject not Found" parameter in Ethovision—interpreted as uncontrolled jumps, flips, or convulsions—was used as a proxy for SLA for all experiments. In third-round experiments, both automatic and manual SLA scoring were used.

Table 2**.** First-round experiments “Subject not Found” percentages.

Genotype	Diets	Mean percentage of “Subject not Found” (%)	Std. Error of the mean	Sample Size
Canton-S (Wild-type)	BF	0.178	0.089	18
	βHB	0.100	0.109	12
	βHB + Tol	0.325	0.188	4
	Tol	0.100	0.154	6
eas	BF	0.337	0.051	54
	βHB	0.366	0.057	44
	βHB + Tol	0.600	0.218	3
	Tol	0.393	0.073	27
	EtOH	0.317	0.109	12
sesB	BF	0.131	0.105	13
	βHB	0.331	0.094	16
	Tol	0.000	0.377	1
tko	BF	0.181	0.094	16

Table 3. Second-round experiments “Subject not Found” percentages.

Genotype	Diets	Mean percentage of “Subject not Found” (%)	Std. Error of the mean	Sample Size
Canton-S	BF	0.020	0.027	20
	βHB	0.025	0.027	20
	βHB + Tol	0.040	0.027	20
	Tol	0.022	0.029	18
eas	BF	0.050	0.027	20
	βHB	0.180	0.027	20
	βHB + Tol	0.090	0.027	20
	Tol	0.055	0.037	11
sesB	BF	0.060	0.039	10
tko	BF	0.177	0.034	13
	βHB + Tol	0.087	0.032	15

Table 4. Third-round experiments "Subject not Found" percentages. Differences in percentage of time of "subject not found" were compared between the control diet and each of the other nine diets using a Kruskal-Wallis test in *eas BS* mutant flies. In *eas*BS mutant flies, there was a significant difference in behavior detection in βH*B* diet ($p< 0.001$), or EtOH diet ($p< 0.01$), or CGP diet ($p< 0.05$) compared to the control diet.

Genotype	Diets	Sample size	Mean percentage of "Subject not Found" ± Standard error of the mean	*p*-value from control (BF) diet
eas	BF	20	0.800 ± 0.111	1.000
	BF→ βHB	20	0.350 ± 0.111	0.492
	βHB	20	0.065 ± 0.111	0.000
	βHB + Tol	19	0.337 ± 0.114	0.639
	Tol	20	0.200 ± 0.111	0.200
	EtOH	18	0.189 ± 0.117	0.005
	CGP	19	0.137 ± 0.114	0.032
	CGP + βHB	20	0.290 ± 0.111	1.000
	DMSO	20	0.170 ±0.111	0.131
	βHB + DMSO	20	0.225 ± 0.111	0.193

eas Bang-Sensitive Mutant Was Chosen as the Focal Strain for Further Experiments

To determine the ideal BS mutant strain for further experimentation, we compared the average percentage of "Subject not Found," an SLA, between Canton-S (wild-type) flies and each of the three BS mutant fly strains (*eas, sesB,* and*tko*) in the first-round (Figure 7, Table 5) and second-round experiments (Figure 8, Table 6). In both sets of experiments, all flies were fed the control diet. The average percentages of "Subject not Found" were not normally distributed; only one independent categorical variable (genotype) was compared across four different groups. Therefore, a Kruskal-Wallis One-Way ANOVA was used to test whether there was a significant difference between the average percentages of "Subject not Found" across genotypes. There was no significant difference in average percentage of "Subject not Found" in the first-round experiments (n = 243, $p = 0.243$). Therefore, I retained the null hypothesis that the distribution of average percentage of "Subject not Found" was the same across different genotypes on the control diet.

However, I observed the inconsistency of mean percentages of SLA in BS mutant flies namely this *eas*BS mutant and *sesB*BS mutant in the first-round experiment. Therefore, the second-round experiment was conducted to verify the reliability of the first-round experiment. The *p*value for the second-round experiments suggest a rejection of the null hypothesis (n = 62, $p< 0.05$) that all groups were the same. Based on this significant Kruskal-Wallis test, pairwise

comparisons between each genotype using multiple Mann-Whitney U's were evaluated, which helped protect against Type I error by correcting alpha (Table 7). Although Mann-Whitney U tests showed that there was no significant difference in average percentage of "Subject not Found" between each genotype, *eas*BS mutant had the greatest mean percentage of SLA (n = 20, mean = 0.180 %, SEM = 0.038 %) compared to the other strains in the second-round experiments (Figure 8). In addition, *sesB*BS mutant strain had the lowest mean percentage of SLA (n = 20, mean = 0.056, SEM = 0.057)compared to the other BS mutant strains, but was still greater than the Canton-S strain (n = 20, mean = 0.025 %, SEM = 0.038 %). The differences between these two experiments were likely due to the method of data collection, for instance, transferring time.

Average percentage of "Subject not Found" was calculated by Excel after extraction from Ethovision output (Table 5, Table 6) to see the difference of SLA among four different genotypes on the BF diet. Both tables show that *eas*mutant flies had highest mean percentages of SLA compared to other genotypes, therefore I selected *eas*BS strain for future studies.

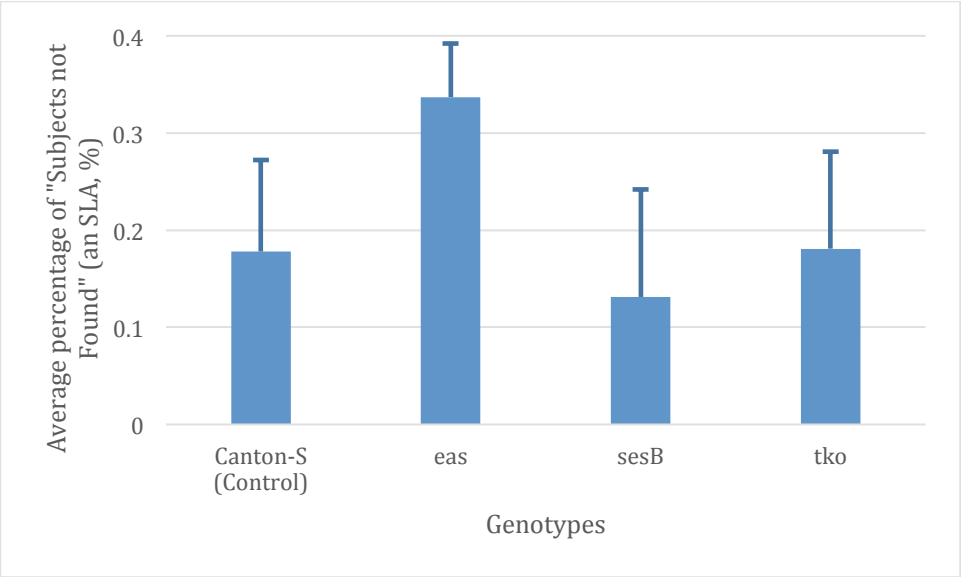


Figure 7. A comparison of average percentage of "Subject not Found," an SLA, between Canton-S flies and each of the three BS mutant flies in first-round experiments. The difference of SLA was not significantly different across genotypes on the BF diet (n = 243, p = 0.243, a Kruskal Wallis test). *eas*BS mutant flies (n = 54, mean = 0.337 %, SEM = 0.055 %) had the greatest average percentage of "Subject not Found" (mean ± SEM) compared to the other genotypes. Canton-S flies had a greater average percentage of SLA (n = 18, mean = 0.178 %, SEM = 0.094 %) compared to *sesB*BS mutant flies (n = 13, mean = 0.131 %, SEM = 0.111 %).

Table 5. Summary of mean "Subject not Found" percentages in first-round experiments with the control (BF) diet for all four genotypes.

Genotype	Diet	Sample size	Mean percentage of "Subject not Found" ± Standard error of the mean (%)
Canton-S	BF	18	0.178 ± 0.094
eas		54	0.337 ± 0.055
sesB		13	0.131 ± 0.111
tko		16	0.181 ± 0.100

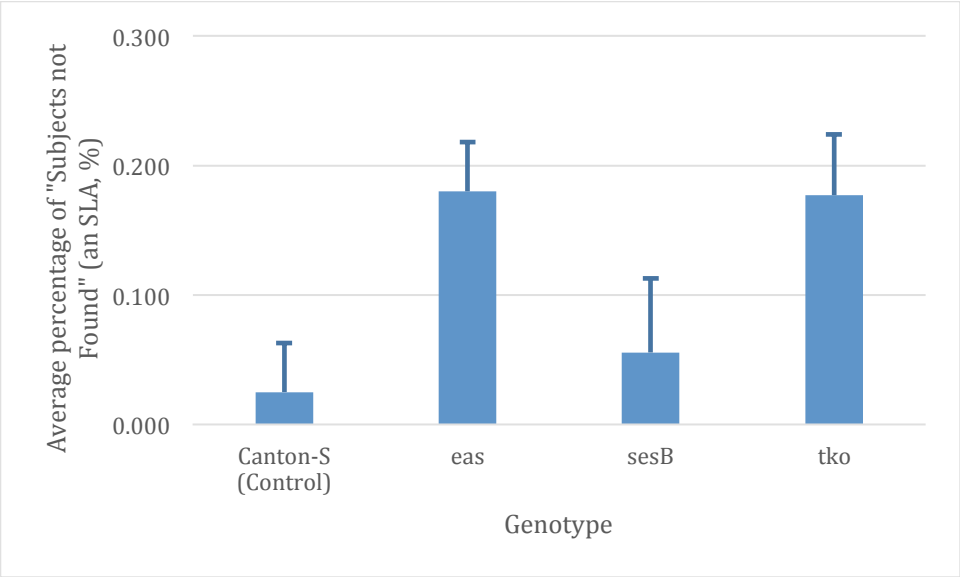


Figure 8. A comparison of average percentage of "Subject not Found," an SLA, between Canton-S flies and each of the three BS mutant flies in second-round experiments. There was a significant difference in mean percentage of SLA across genotypes on the BF diet (n = 62, $p < 0.05$, aKruskal Wallis test). *eas*BS mutant strain (n = 20, mean = 0.180 %, SEM = 0.038 %) had the greatest average percentage of SLA (mean ± SEM) compared to the other strains. The differences in average percentages of SLA between *eas*and *tko*BS mutant flies (n = 13, mean = 0.177 %, SEM = 0.047 %) were little. Furthermore, *sesB*BS mutant flies' average percentage of SLA (n = 20, mean = 0.056, SEM = 0.057), which was the lowest in BS mutant flies, was greater compared to the Canton-S flies, but the difference was not significant (n = 20, mean = 0.056, SEM = 0.057).

Table 6. Summary of mean "Subject not Found" percentages in second-round experiments with the control (BF) diet for all four genotypes.

Genotype	Diet	Sample size	Mean percentage of "Subject not Found" ± Standard error of the mean (%)
Canton-S	BF	20	0.025 ± 0.038
eas		20	0.180 ± 0.038
sesB		9	0.056 ± 0.057
tko		13	0.177 ± 0.047

Table 7. Pairwise comparisons between each genotype in BF diet in second-round experiments using multiple Mann-Whitney U-tests.

Diets	Genotypes	*p*-value
BF	Canton-S vs*sesB*	1.000
	Canton-S vs*eas*	0.055
	Canton-S vs*tko*	0.051
	*sesB*vs*eas*	1.000
	*sesB*vs*tko*	1.000
	*eas*vs*tko*	1.000

To further study whether *eas*BS mutant strain would yield interpretable results in the second-round experiments, the difference of SLA between wild-type flies and *eas*BS mutant flies across four diets (BF, βHB, Tol, and βHB + Tol) was tested (Table 8). A two-by-four factorial ANOVA was used to check the difference of SLA between four genotypes and four diets through assuming data were normally distributed (N=149). Pairwise comparison using the Bonferroni correction (Table 9, Table 10) shows that there was a significant difference in mean percentage of SLA between Canton-S flies (mean = 0.025%, SEM = 0.026 %) and *eas*BS mutant flies (mean = 0.180 %, SEM = 0.026 %) fed with βHB supplemented diet ($p< 0.001$), where *eas*flies had a greater mean of SLA compared to the Canton-S flies (Table 9). In addition, the differences in mean percentage of SLA between βHB supplemented diet and BF diet (mean = 0.05 %, SEM = 0.026 %) ($p< 0.01$), and the differences between the βHB supplemental diet and the Tol diet (mean = 0.055 %, SEM = 0.036 %) ($p< 0.05$) were significantly different in *eas*BS mutant flies (Table 10), where *eas*BS mutant flies had a greater mean percentage of SLA on the βHB supplemental diet compared to either the BF diet or the Tol diet.

Table 8. Summary of mean "Subject not Found" percentages in second-round experiments for two genotype and four diets.

Genotype	Diets	Sample size	Mean percentage of "Subject not Found ± Standard error of the mean (%)
Canton-S (Wild-type)	BF	20	0.020 ± 0.026
	βHB	20	0.025 ± 0.026
	βHB + Tol	20	0.040 ± 0.026
	Tol	18	0.022 ± 0.028
eas	BF	20	0.050 ± 0.026
	βHB	20	0.180 ± 0.026
	βHB + Tol	20	0.090 ± 0.026
	Tol	11	0.055 ± 0.036

Table 9. Within-diet comparisons of genotypes in second-round experiments.Differences in mean percentage of "subject not found" were compared within a given diet across each of two different genotypes using a two-by-four factorial ANOVA with a post hoc Bonferroni correction. On the βHB supplemented diet, there was a significant difference in behavior detection between *eas*BS mutant flies and Canton-S (wild-type) flies ($p < 0.001$).

Diets	Genotype	Genotype	*p*-value
BF	Canton-S	eas	0.424
	eas	Canton-S	0.424
βHB	Canton-S	eas	0.000
	eas	Canton-S	0.000
βHB + Tol	Canton-S	eas	0.184
	eas	Canton-S	0.184
Tol	Canton-S	eas	0.477
	eas	Canton-S	0.477

Table 10. Within-genotype comparisons of diet effects in second-round experiments. Differences in mean percentage of "subject not found" were compared within a given genotype across each of four different dietary conditions using a two-by-four factorial ANOVA with a Bonferroni correction. In *eas*BS mutant flies, there was a significant difference in behavior detection between the BHB supplemented diet and either the BF diet ($p < 0.01$) and the Tol diet ($p < 0.05$).

Genotype	Diets	Diets	*p*-value
Canton-S	BF	βHB	1.000
		βHB + Tol	1.000
		Tol	1.000
	βHB	BF	1.000
		βHB + Tol	1.000
		Tol	1.000
	βHB + Tol	BF	1.000
		βHB	1.000
		Tol	1.000
	Tol	BF	1.000
		βHB	1.000
		βHB + Tol	1.000
eas	BF	βHB	0.004
		βHB + Tol	1.000
		Tol	1.000
	βHB	BF	0.004
		βHB + Tol	0.105
		Tol	0.033
	βHB + Tol	BF	1.000
		βHB	0.105
		Tol	1.000
	Tol	BF	1.000

		βHB	0.033
		βHB + Tol	1.000

Different Diets Affect Seizure Like Activities (SLA) Ineas BS Mutant Flies

The *eas*BS mutant flies were chosen for investigation of the effect of ketone body (βHB) supplemented diet on SLA, and the impact of K_{ATP} channels and $GABA_B$ signaling on top of ketone body supplemented diet, in third-round experiments. Average percentages of SLA were not normally distributed and only one independent categorical variable, diets with ten groups, including the BF diet, the βHB supplemented diet, the βHB + Tol diet, the Tol diet, the EtOH diet, the βHB + CGP diet, the βHB + DMSO diet, the CGP diet, the DMSO diet, and a transferring diet which transferred BS mutant flies in BF diet immediately after eclosion to βHB supplemental diet for three days (or the dietary switch). A non-parametric Kruskal Wallis One-Way ANOVA test was used to determine whether there was a difference in mean percentage of SLA across diets in *eas*BS mutant flies. In accordance with the results of the Kruskal-Wallis test ($n = 196$, $p < 0.01$), the null hypothesis, in which the distribution of percentage of jumps was the same across diets, was rejected. Pairwise comparisons between each diet in *eas*BS mutant flies using multiple Mann-Whitney U-tests (Table 4) revealed that there was a significant difference in mean percentage of SLA between specifically selected diets: BF and βHB, BF and EtOH, and BF and CGP.

Ketone Bodies (βHB) Reduced SLA

To determine whether ketone body (βHB) supplemented diet reduces SLA in *eas* BS mutant strain, we compared the average percentage of SLA between the control diet and the βHB supplemented diet. Based on the result of the multiple Mann-Whitney U-test, there was a highly significant reduction ($p< 0.001$) in average percentage of SLA on the control diet (n = 20, mean = 0.800 %, SEM = 0.111 %) compared to the βHB supplemented diet (n = 20, mean = 0.065 %, SEM = 0.111 %) (Table 4, Figure 9). In addition, there was a reduction in mean percentage of SLA with the dietary switch (n = 20, mean = 0.350 %, SEM = 0.111 %) compared to the control diet. Although this difference in mean SLA was not significant ($p = 0.492$), it displays a trend that even if BS mutant flies were not fed on a ketone body supplemented diet during their entire life spans, their SLA could still be reduced after the application of ketone bodies.

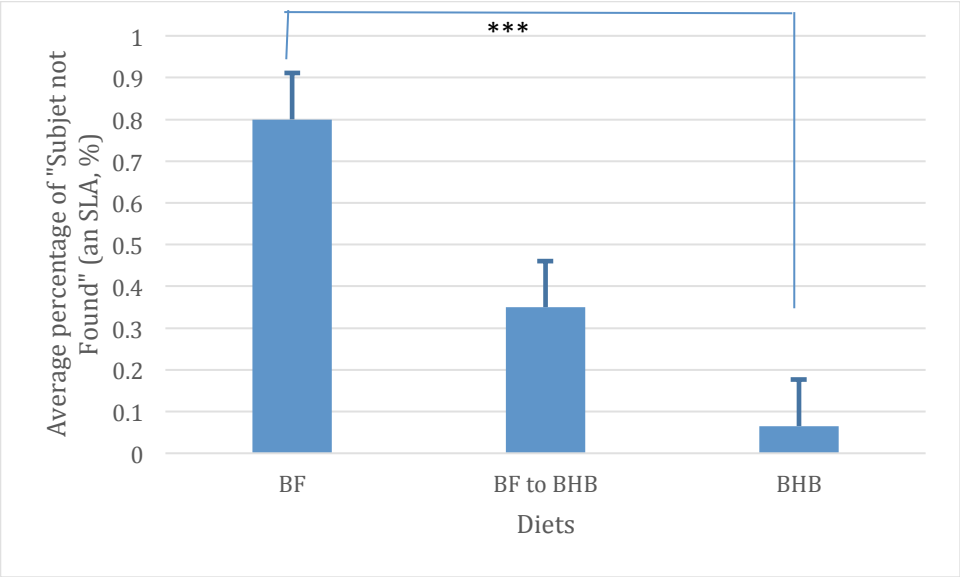


Figure 9. *eas* BS flies reduced average percentage of "Subject not Found," an SLA, with the application of the ketone body (βHB) supplemented diet in third-round experiments. Mean percentage of SLA on βHB supplemented diet (n = 20, mean ± SEM = 0.800 ± 0.111 %) was significant reduced compared to the control diet (n = 20, mean ± SEM = 0.065 ± 0.111 %) using Mann-Whitney U test ($p <$ 0.001). There was no significant difference between the dietary switch diet (BF to βHB) and the control diet, while there was a reduction in mean percentage of SLA on the dietary switch. *** $p< 0.001$.

In addition to the results from Ethovision, I compared *eas*BS mutant flies' average frequency of all observed SLAs between the control diet and the ketone body βHB supplemented diet through performing manual scoring in third-round experiments (Table 11). Because the mean frequency of SLAs were not normally distributed and there was one categorical independent variable – diets containing three groups -- a Kruskal-Wallis test was conducted (n = 60). Differences in mean percentage of SLA were significantly different within *eas*BS mutant flies across three different diets ($p < 0.001$). Pairwise comparisons between each diet in *eas*BS mutant flies using multiple Mann-Whitney U-tests (Table 11) revealed that there were significant differences in SLA between specifically selected diets: BF and βHB ($p < 0.001$), βHB and dietary switch ($p < 0.05$). Mean frequency of SLAs was significantly reduced on the βHB supplemental diet (n = 20, mean = 1.750, SEM = 0.792) compared to either the control diet (n = 20, mean = 6.600, SEM = 0.792) or the dietary switch (n = 20, mean = 4.600, SEM = 0.792) (Figure 10).

To study whether there is a differences in mean duration of each SLA types between the control diet and the βHB supplemented diet in *eas*BS mutant flies, I categorized and calculated mean duration of each SLA in *eas*BS mutant flies between those diets in third-round experiments (Table 12). There were four types of SLAs being evaluated, including "jump," "flip," "disappearances," and "a mix of SLAs (mix)." Because the mean duration of each SLA was not normally distributed and there was one categorical independent variable, diet with

two groups, a two sample Mann-Whitney U test was performed (Table 13). There were significant differences in mean duration of "mix" (n = 40, $p < 0.01$), flip (n = 40, $p < 0.01$), and "jump" (n = 40, $p < 0.05$) behaviors between the control diet and the BHB supplemented diet. More specifically, the mean duration of "mix" behaviors was significantly reduced on βHB supplemented diet (n = 20, mean ± SD = 4.8 ± 5.550 seconds) compared to the control diet (n = 20, mean ± SD = 1.05 ± 1.432 seconds); the mean duration of "jump" behaviors was significantly reduced on the βHB supplemental diet (n = 20, mean ± SD = 1.9 ± 2.269 seconds) compared to the control diet (n = 20, mean ± SD = 0.45 ± 0.686 seconds); and the mean duration of "flip" behaviors was significantly reduced on the βHB supplemental diet (n = 20, mean ± SD = 2.7 ± 3.686 seconds) compared to the control diet (n = 20, mean ± SD = 0.65 ± 0.745 seconds) (Figure 11). The mean duration of "disappearance" behaviors was reduced compared to the control diet, however differences were not significant different (Figure 11).

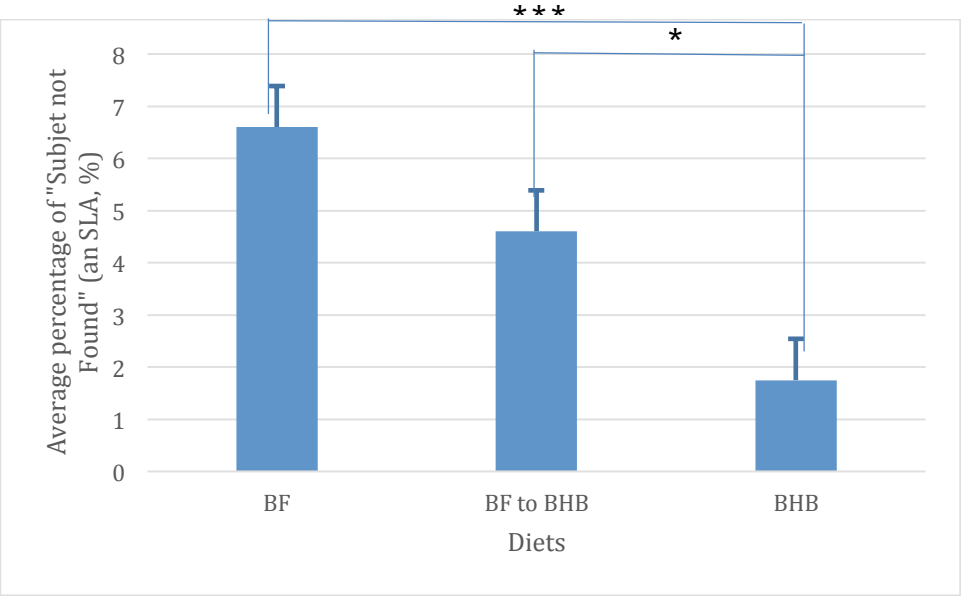


Figure 10.*eas* BS flies reduced average frequency of SLAs with the application of the ketone body (βHB) diet using manual scoring in third-round experiments. There was a significant reduction in mean frequency of SLAs on the βHB supplemented diet (n = 20, mean ± SD = 1.75 ± 0.795) compared to either the control diet (n = 20, mean ± SEM = 6.600 ± 0.792, p< 0.001) or the dietary switch (n = 20, mean ± SEM = 4.600 ± 0.792, $p < 0.05$) using a Kruskal-Wallis test with post hoc Multiple Mann-Whitney U's tests. * $p<0.05$, *** $p< 0.001$.

Table 11. Summary of mean frequency of SLAs in *eas*BS mutant flies fed three different diets (BF, βHB, dietary switch) in the third-round experiments. There was a significant reduction in mean frequency of SLAs on the βHB supplemented diet compared to the control diet in *eas*BS mutant flies using post hoc multiple Mann-Whitney U test (p <0.001). There was no significant reduction in mean frequency of SLAs between the dietary switch and the control diet, where there was a reduction on dietary switch compared to the control diet.

Genotype	Diet	Sample Size	Mean frequency of SLA ± Standard error of the mean	*p*-value	
eas	BF	20	6.600 ± 0.792	-	-
	βHB	20	1.750 ± 0.792	BF vs βHB	0.000
	BF to βHB	20	4.600 ± 0.792	βHB vs BF to βHB	0.016

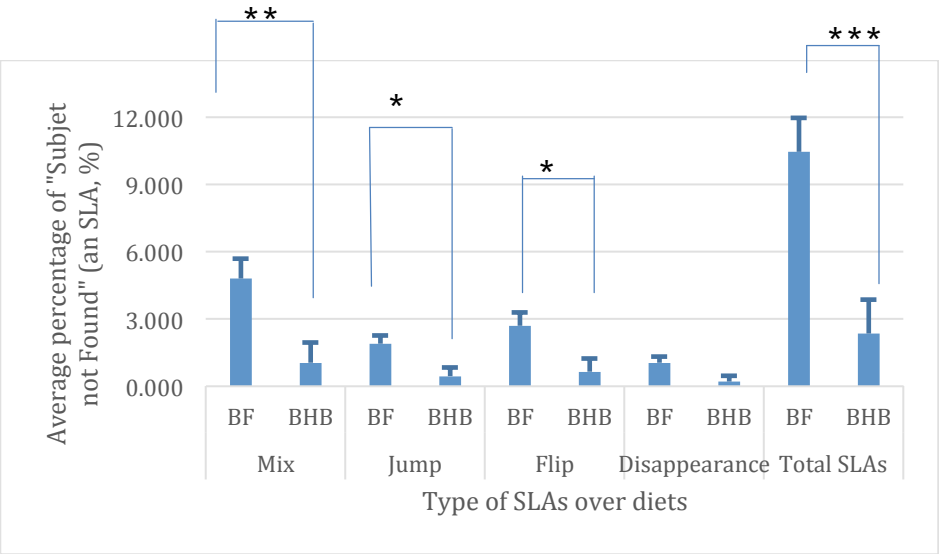


Figure 11. *eas* BS mutant flies reduced average SLA duration in each of the SLA types with the presence of the ketone body (βHB) supplemented diet using manual scoring in third-round experiments. A two sample Mann-Whitney U test shows that there were significant difference in mean duration of "mix" (n = 40, $p< 0.01$), and "flip" (n = 40, $p< 0.01$), and "jump" (n = 40, $p< 0.05$) between the control diet and the βHB supplemented diet in *eas*BS mutant flies. Mean duration of "mix" behaviors was 4.8 seconds (n = 20, SEM = 0.906 seconds) on the control diet and 1.05 seconds (n = 20, SEM = 0.906 second) on the βHB supplemented diet. Mean duration of "jump" behaviors was 1.9 seconds (n = 20, SEM = 0.375 seconds) on the control diet and 0.45 seconds (n = 20, SEM = 0.375 seconds) on the βHB supplemented diet. Mean duration of "flip" behaviors was 2.7 seconds (n = 20, SEM = 0.595 seconds) on the control diet and 0.65 seconds (n = 20, SEM = 0.595seconds) on the βHB supplemented diet. Mean duration of total SLAs was 10.450 seconds (n = 20, SEM = 1.520 seconds) on the control diet and 2.350

seconds (n = 20, SEM = 1.520 seconds) on the βHB supplemented diet. * $p < 0.05$, ** $p < 0.001$, *** $p < 0$.

Table 12. Summary of mean duration of each SLA types in *eas*BS mutant flies fed two different diets (BF, βHB) in the third-round experiments. There were four different SLA types involving "jump," "flip," "disappearance," and "mix" behaviors. A Two Sample Mann-Whitney U's test revealed that there was a significant reduction in mean duration of each of three types of SLAs ("jump," "flip," and "mix" behaviors) and the total SLAs on the βHB supplemented diet compared to the control diet. The difference in mean duration of "disappearance" behavior was not significantly different.

Genotype	SLA	Diet	Sample Size	Mean duration of each SLA types ± Standard error of the mean (seconds)	*p*-value
eas	Mix	BF	20	4.800 ± 0.906	0.002
		βHB	20	1.050 ± 0.906	
	Jump	BF	20	1.900 ± 0.375	0.023
		βHB	20	0.450 ± 0.375	
	Flip	BF	20	2.700 ± 0.595	0.017
		βHB	20	0.650 ± 0.595	
	Disappearance	BF	20	1.050 ± 0.267	0.108
		βHB	20	0.200 ± 0.267	
	Total SLAs	BF	20	10.450 ± 1.520	0.000
		βHB	20	2.350 ± 1.520	

Tolbutamide Effects on SLA

Because there is a well-established role, in *vivo* mouse models, for K_{ATP} channels in mediating ketone body effects on neural activity and SLA (Ma et al., 2007, Tanner et al. 2011, Giminez-Cassina et al., 2012), I compared the average percentage of jumps between the control diet and the βHB + Tol (a K_{ATP} channel blocker) diet (Table 3). I wanted to determine what role, if any, K_{ATP} channels might play in ketone bodies' anticonvulsant effects, of reducing SLA in the *eas* BS mutant *Drosophila* strain. Although there appeared to be a reduction of SLA on the βHB + Tol diet (n = 19, mean = 0.337 %, SEM = 0.114 %), comparison of mean SLA between the control diet and the βHB + Tol diet using multiple Mann-Whitney U's tests ($p = 0.639$) revealed that there was no significant difference (Table 3, Figure 12). Additionally, the average percentage of SLA on the βHB + Tol diet was greater compared to the βHB supplemented diet. Therefore, it seems as though blocking K_{ATP} channels may have partially reversed ketone body's anticonvulsant effects on SLA. I noted that the average percentage of SLA on the Tol diet (n = 20, mean = 0.200 %, SEM = 0.111 %) was also greater than the mean percentage of SLA on the βHB supplemented diet and less than the mean percentage of SLA on the BF control diet, but not significantly so ($p = 0.200$).

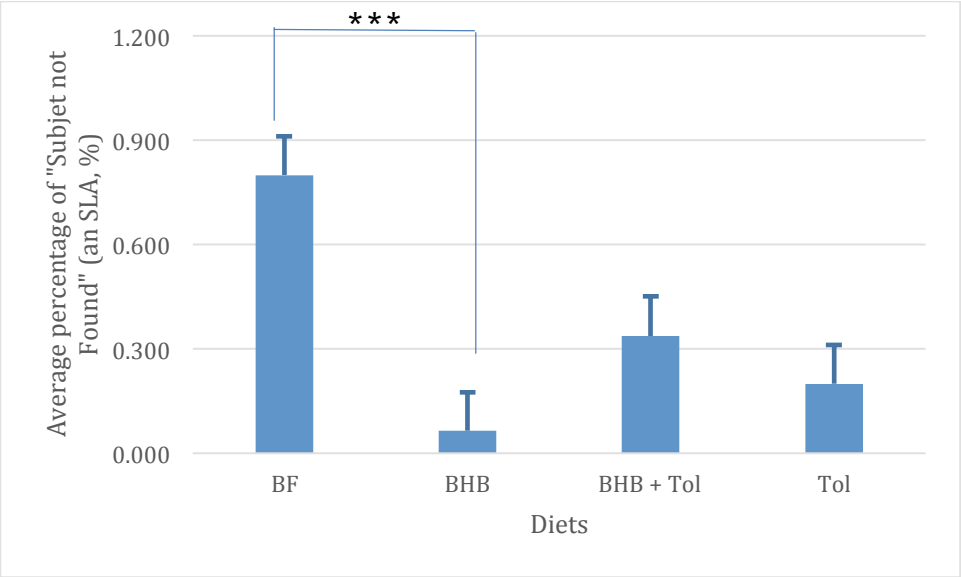


Figure 12. K_{ATP} channel blocker, tolbutamide partially reversed βHB's anticonvulsant effects in third-round experiments. There was no significant difference in SLA incidents (mean ± SEM) between the control diet and the βHB + Tol diet (p = 0.639), and between the control diet and the Tol diet (p = 0.200) using multiple Mann-Whitney U's tests. The mean percentage of SLA on the βHB + Tol diet was 0.337 % (n = 19, SEM = 0.114 %), and the mean percentage of SLA on the Tol diet was 0.200 % (n = 20, SEM = 0.111%). The mean percentage of SLA on the βHB + Tol diet and the Tol diet were less compared to the control diet. *** p< 0.

CGP55485 Effects on SLA

Because there is an established *in vitro* role of $GABA_B$ signaling in ketone bodies' effects on spontaneous neuronal firing (Ma et al., 2007) SLA, I compared the average percentage of SLA between the control diet and the βHB + CGP (a $GABA_B$ receptor blocker) diet (Table 3). Although the comparison of mean SLA between the control diet and the βHB + CGP diet using multiple Mann-Whitney U's tests ($p = 1.000$) shows that there was no significant difference between these two diets (Figure 13), there was a reduction of mean percentage of SLA on the βHB + CGP diet (n = 20, mean = 0.290 %, SEM = 0.111 %) compared to the control diet (Figure 13). Furthermore, the average percentage of SLA on the βHB + CGP diet was greater compared to the βHB supplemented diet (Figure 13). Therefore, blocking the effect of GABA appeared to partially reverse ketone body's anticonvulsant effects, which resulted in a raise SLA in this whole-animal model. Additionally, the average percentage of SLA on the CGP diet (n = 19, mean = 0.137 %, SEM = 0.114) was greater than the mean percentage of SLA on the βHB supplemental diet and was significantly less than the mean of SLA on the BF control diet ($p< 0.05$).

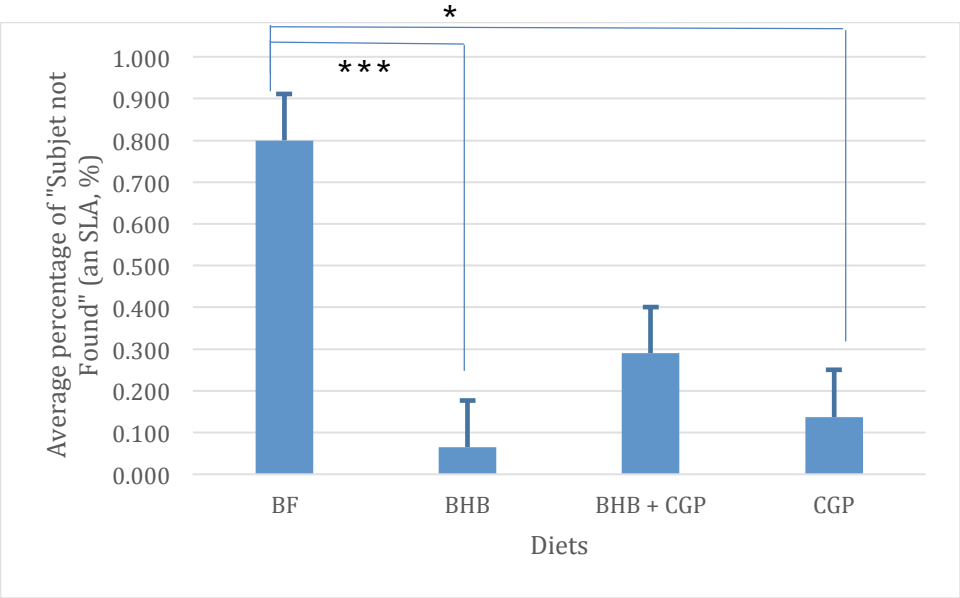


Figure 13. $GABA_B$ blocker, CGP55485 partially reversed βHB's anticonvulsant effect in third-round experiments. There was no significant difference in SLA incidence (mean ± SEM) between the control diet and the βHB + CGP diet (p = 1.000), while there was a significant difference between the control diet and the CGP diet ($p< 0.05$) using multiple Mann-Whitney U's tests. Mean percentage of SLA on the βHB + CGP was 0.290 % (n = 20, SEM = 0.111 %), and mean percentage of SLA on the CGP diet was 0.137 % (n = 19, SEM = 0.114 %). Mean percentages of SLA in both diets were less than the control diet. * $p< 0.05$, *** p < 0.

CHAPTER IV: DISCUSSION

This project was designed to understand the impact of K_{ATP} channels and $GABA_B$ signaling on the ketone body effect in a whole-animal *Drosophila* model. I recoded fly behavior and quantified SLA with Ethovision software using the most reliable reporter for SLA incidences, which was "Subject not Found" (%). This was interpreted as SLA-related flips or jumps. In this project, the *eas* BS mutant strain was chosen as the focal strain to study effects of the K_{ATP} blocker and the $GABA_B$ blocker. My results showed that the ketone body (βHB) supplement reduced SLA, and both the K_{ATP} blocker tolbutamide and the $GABA_B$ blocker CGP55485 partially reversed the anticonvulsant effect of ketone bodies in a whole-animal model.

eas BS mutant was chosen as the focal strain for further experiments

To determine the ideal BS mutant strain for more in-depth experimentation, the mean percentage of "Subject not Found," an SLA, was compared across four genotypes on the control (BF) diet in both first- and second-round experiments (Figure 7 and Figure 8). The first-round experiments (Figure 7) show that there was no significant difference in mean percentage of "Subject not Found" across genotypes on the control diet, while the second-round experiments (Figure 8) display significant differences in mean percentage of "Subject not Found" across four different genotypes on the control diet. The difference between first- and second-round experiments was possibly due to the

length of the videos that were used for the analysis. In the first-round experiments, we recorded videos for three minutes, including 10 seconds for vortexing and 20 seconds for placement of the observation chamber for recording. Therefore, the actual video length used for analysis was two and half minutes in the first-round experiments. In the second-round experiments, I recorded videos for three minutes, including 10 seconds of vortexing time and 5 seconds to place the observation chamber for recording, so the actual video length of behavioral observation in the second-round experiments was two minutes and 45 seconds. Thus the first-round experiments lost the first 20 seconds and the second-round experiments lost only the first 5 seconds of fly behavior. Based on my results, I conclude that the first few seconds of behavior after vortexing were essential because it was the immediate post-stimulation behavior we would like to capture in the experiments.

There was a contradictory result between experiments, in which the mean SLA was reduced in *sesB* BS mutant flies compared to the control flies (Table 5) in the first-round experiment. This was not expected because the *sesB* strain was one of the BS mutant strains, which should exhibit seizure susceptibility in flies (Zhang et al. 1999, Kuebler et al., 2001, Song et al., 2007, Vartiainen et al. 2014). There were two possible reasons for this result. The first reason was that a small sample size of *sesB*BS mutant flies was used due to these mutant flies' low reproduction rate. Previous research found that *sesB* BS mutant flies took a longer time to reach eclosion compared with the other tested genotypes (Vartiainen et al.

2014), which happened in my experiment as well. The second reason was that the first-round experiment lost important immediate post-stimulation behavior. In the second-round experiments (Table 6), the mean percentage of SLA in *tko* BS mutant flies was close to the mean percentage of SLA in *ea*s BS mutant flies. Thus, I could have chosen either *tko* or *eas* BS mutant flies for further experiments. However, compared to the other strains, the *eas*BS mutant strain had the greatest reproductive rate and the greatest mean percentage of SLA in both experiments. Thus, the *eas* BS mutant strain was the best strain for future experiments.

To further study whether the *eas* BS mutant strain would yield interpretable results in the second-round experiments, differences in mean percentage of "Subject not Found" between control flies and *eas* BS mutant flies across four diets (BF, βHB, βHB + Tol and Tol) were compared (Table 8). There was a significant reduction in percentage of "Subject not Found" on the BF diet compared to the BHB supplemented diet in *eas*BS mutant flies ($p < 0.01$, Table 9), which we did not expect because previous research in mouse brain slices found that the firing rate of SNr neurons was reduced in the presence of ketone bodies (e.g., βHB) (Ma, et al. 2007). This slow firing rate in mice experiments suggested possible anticonvulsant effects in the presence of ketone bodies so that I expected to see a reduction in convulsions in these flies on the BHB supplemented diet. But surprisingly, I did not see it in the second-round experiment. In addition, clinical research had shown that the ketogenic diet was

an effective treatment for epileptic patients, especially in children (Neal et al. 2008). I believe that this discrepancy in expected and actual results was due to the way in which I collected the flies. In both first- and second-round experiments, I recorded flies immediately after eclosion in order to obtain juvenile flies. Flies ate the diet during their larval stage for about a week, and then they stopped eating the diet that were provided during their pupa stage for a couple of days before eclosion. Therefore, it is possible that the effect of the diet diminishes or even disappears before eclosion. In conclusion, the *eas* BS mutant strain was the ideal strain to choose as the focal strain for further experiments, but I still needed to optimize the experimental conditions by recording young adult flies three days after eclosion instead of recording immediately after eclosion. This is what was performed for the third-round experiments.

Ketone body *(βHB) supplement* reduced SLA in *eas* BS mutant flies

To determine whether the ketone body (βHB) supplemented diet reduces SLA, I compared seizure behaviors between the control diet and the βHB supplemented diet in *eas* BS mutant flies using Ethovision and manual scoring in the third-round experiment. The mean percentage of "Subject not Found" from Ethovision, mean frequency of SLA and mean duration of each type of SLAs from manual scoring were obtained. The mean percentage of SLA from Ethovision ($p < 0.001$, Figure 9) and the mean frequency of SLA from manual scorings ($p < 0.001$, Figure 10) were significantly reduced for the βHB

supplemented diet compared to the control diet, where the flies were raised on the diet for their entire life spans and tested three days post eclosion. In addition, mean duration of each type of SLA - including "mix," ($p < 0.01$) or "jump," ($p < 0.05$) or "flip" ($p < 0.05$) - and mean duration of total SLA ($p < 0.001$) were significantly reduced in the βHB supplemented diet compared with the control diet (Figure 11). Furthermore, there was a reduction in SLA in flies fed the dietary shift (BF to βHB diet) compared to the control diet in the data collected from both Ethovision and manual scorings. Although the result was not significant for BF → βHB switch, it did follow a trend. Therefore, even if BS mutant flies were not fed the ketone body supplemented diet throughout their entire lives, their SLA could still be reduced after exposure to ketone bodies. In conclusion, BS mutant flies fed with ketone body supplemented diet exhibited the greatest reduction in SLA incidents. Although the way we applied the ketogenic diet was atypical, these results were expected because previous research showed that the application of ketone body supplements on top of a glucose diet reduced the firing rate of neurons in the rodent SNr (Ma et al., 2007). Patients on the ketogenic diet produce increased levels of ketone bodies; therefore, on the ketogenic diet, the main fuel for brain metabolism shifts from glucose to ketone bodies (KBs), resulting in a reduction in seizure behaviors (McNally & Hartman, 2012, Lutas&Yellen, 2012).

Tolbutamideeffected on SLA in *eas* BS mutant flies

Previous researchers have demonstrated a link between ketone bodies and K_{ATP} activity in several mammalian brain regions, and suggest that ketone bodies increase the likelihood of K_{ATP} channel opening, resulting in a slower firing rate of neurons, which may serve to temper seizures (Ma et al., 2007, Tanner et al. 2011). However, the mechanism to explain this phenomenon is unclear. It is possible that ketone bodies either directly open K_{ATP} channels (Lutas and Yellen, 2013), or they operate through less-direct metabolic effects.

Since the ketone body supplemented diet reduced SLA in *eas*BS mutant flies, I investigated whether ketone bodies' anticonvulsant effects were mediated by K_{ATP} channels using the K_{ATP} blocker tolbutamide (Tol). Although there was some reduction in SLA in flies fed with tolbutamide on top of βHB compared with flies fed BF (0.32 % versus 0.8 % subject not found), this difference was not significant ($p = 0.639$), suggesting that tolbutamide partially blocked the effects of the KD metabolite on SLA (Figure 12). Because there was no significant difference between BF and βHB + Tol, these results suggest that K_{ATP}blockade partially reversed the KB effects on SLA. This indicates that ketone bodies reduced SLA by mediating K_{ATP} channels, which was supported by previous research in which the application of tolbutamide to SNrGABAergic neurons incubated in βHB in mouse brain slices prevented the KB-induced slowing of neuronal firing rate (Deransart et al., 2003, Ma et al., 2007).

However, I did not expect to see a reduction in mean percentage of SLA on the Tol diet compared to the control diet (Figure 12). This difference in mean SLA, however, was not significant. Because a previous lab concluded that K_{ATP} channels might be completely closed after the application of tolbutamide to SNrGABAergic neurons in mouse brain slices (Ma et al., 2007), I would expect to see very little or no reduction in SLA, and in fact, I might even have expected an increase. Although this result was unanticipated, there are some reasons to explain it. One explanation is that the research by Ma et al. was conducted using brain slices in mice (2007), and we used fruit flies as a whole-animal model. Therefore, K_{ATP} channels in other parts of body might be blocked by tolbutamide. Another study investigating the correlation between metabolism and seizure susceptibility after feeding BS mutant flies tolbutamide found that *eas* mutants showed a reduction in SLA by upregulating their metabolism, leading to a metabolic shift from carbohydrates to lipids/ketones (Stone et al., 2013). Tolbutamide is a K_{ATP} blocker that binds to the sulfonylurea receptors (SUR) subunits of K_{ATP} channels, which are expressed on corpora cardiac cells which regulate metabolism by releasing adipokinetic hormone (Ashfield et al., 1999, Stone et al., 2013). Due to the likely increased secretion of adipokinetic hormone in flies fed tolbutamide, I would expect a potential increase in lipid metabolism, which might provide neurons with additional metabolic stability that could actually serve to protect against seizures. This altered whole-body metabolism could help explain the reduced SLA in flies fed tolbutmide.

In addition, to study the effect of tolbutamide on flies' seizure susceptibility, I dissolved tolbutamide in ethanol to incorporate Tol into the BF food. Therefore, an EtOH diet was used as a control to study the effect of ethanol on SLA, irrespective of tolbutamide. My data revealed that there was a significant reduction in mean percentage of "Subject not Found" for the EtOH diet compared to the control diet ($p < 0.01$). This result was unexpected, but is likely due to cell death induced by ethanol. Because ethanol can disturb the fluidity of cell membranes, which is necessary for cells to function normally, rats fed ethanol were shown to have stiff membranes by incorporating more cholesterol into their membranes (Goldstein, 1986). Therefore, neurons - including hyperpolarized neurons in BS mutant flies fed the EtOH diet - might not be able to function normally, so less SLA, a maker of neuronal hyperactivity, would be detected, as shown in my results.

CGP55485 effects on SLA in *eas* BS mutant flies

I also investigated whether $GABA_B$ signaling is important for the ketone body's anticonvulsant effect. Ma et al. found that the application of the ketone body reduced the firing rate in vitro, but this effect could be reversed by the blockade of $GABA_B$ receptors with CGP55485 (2007). It had been hypothesized that a ketone body metabolism could lead to increased production of GABA, which would hyperpolarize neurons, resulting in a slower firing rate of neurons (Yudkoff et al. 2007, Melø et al., 2006, Ma et al., 2007). To test whether

$GABA_B$signaling mediates the effect of ketone bodies in a whole-animal model in BS mutant flies, I compared the mean percentage of "Subject not Found" on BF, βHB, βHB + CGP and CGP diets (Figure 13). Although there was some reduction in SLA in flies fed with CGP on top of BHB compared with flies fed BF (0.29 % versus 0.8 % subject not found), this difference was not significant ($p = 1.000$), suggesting that CGP also might partially blocked the effects of the KD metabolite on SLA (Figure 13). In conclusion, GABA signaling is required for the full KB effect, but the exact mechanism is unknown.

However, I did not expect to see a significant reduction in mean percentage of SLA on the CGP diet compared to the control diet ($p < 0.05$, Figure 13). A previous lab demonstrated that after the application of CGP55485 to SNrGABAergic neurons in mouse brain slices, the firing rate was not changed (Ma et al., 2007). Therefore, I would not expect a reduction in SLA on the CGP diet inmy experiment. After a comparison between my experiment and previous experiment done by Ma et al. (2007), there were four main differences. The first of which was that I used a whole-animal model instead of brain slices. The second difference was that I used a non-mammalian model. The third was that I looked at SLA in flies, while they observed spontaneous firing rate of neurons. The last difference was that the BS mutant flies were fed CGP diet for their entire lives, instead of applying the drug during the experiment. This would lead to a biochemical shift to favor increased GABA signaling to make up the loss of

$GABA_B$ receptor function during their development. As a result, BS mutant flies fed on the CGP diet appeared to have less SLA

In addition, I dissolved CGP55485 in dimethyl sulfoxide (DMSO) to incorporate CGP into the BF food. Therefore, the DMSO diet and βHB + DMSO diet were used as controls to study the effect of DMSO on SLA, irrespective of CGP and βHB + CGP respectively. My data revealed that there was a reduction in mean percentage of "Subject not Found" for the DMSO diet compared to the control diet and a reduction in the mean SLA for the βHB diet compared to the βHB + DMSO diet, but not significantly so (Table 4). These unanticipated results were also likely due to the membrane effect resulting in cell death, which was similar to that on the EtOH diet (Notman et al., 2006). Therefore, cells might be unable to function normally and would cause less SLA.

Future Directions

There are factors of the experimental design that need to be modified in future experiments. Based on the results in the first-round and second-round experiments, I noticed that it was necessary to include the immediate post-stimulation behavior after vortexing. Future studies should also be redesigned to allow for faster fly transfer, perhaps within a second.

In my project, the strain of control (Canton-S) flies had been contaminated with unknown transgenes when we obtained them from Bloomington fly center, which should be prevented in the future by using a clean wild-type strain. In third-

round experiments, Ionly collected data for *eas*BS mutant flies fed ten different diets for three days after eclosion. In the future, researchers should test Canton-S (wild-type) flies and other mutant strains as well to see whether results are consistent across mutant strains. In addition, researchers could try to collect videos two days after eclosion, or five days after eclosionto see whether there was a difference in SLA. Mean frequency of SLA and duration of each SLA using manual scoring should also be done in third-round experiments in order to check whether there is consistency between automated (Ethovision) and manual scoring.

In addition, although the Ethovision software was helpful by providing an objective evaluation of seizure behaviors in flies, it was not as specific and accurate as what we observed using manual scoring. Even though we chose "Subject not Found" as the most reliable reporter for SLA incidences, this parameter still did not account for all of the jumps or flips. It would be necessary to find some other parameters in Ethovision, or other software, that could identify flies' seizure behaviors better. Furthermore, it would be also helpful to conduct blind manual scoring to compare SLA phenotypes across genotypes and dietary conditions.

Furthermore, in third-round experiments, ethanol, CGP55485 and DMSO were shown to decrease the mean percentage of "Subject not Found" in *eas* BS mutant flies. Because ethanol and DMSO might cause cell death, researchers could try to put tolbutamide or CGP55485 directly into the fly food. In addition, they could transfer both control flies and BS mutant flies immediately after

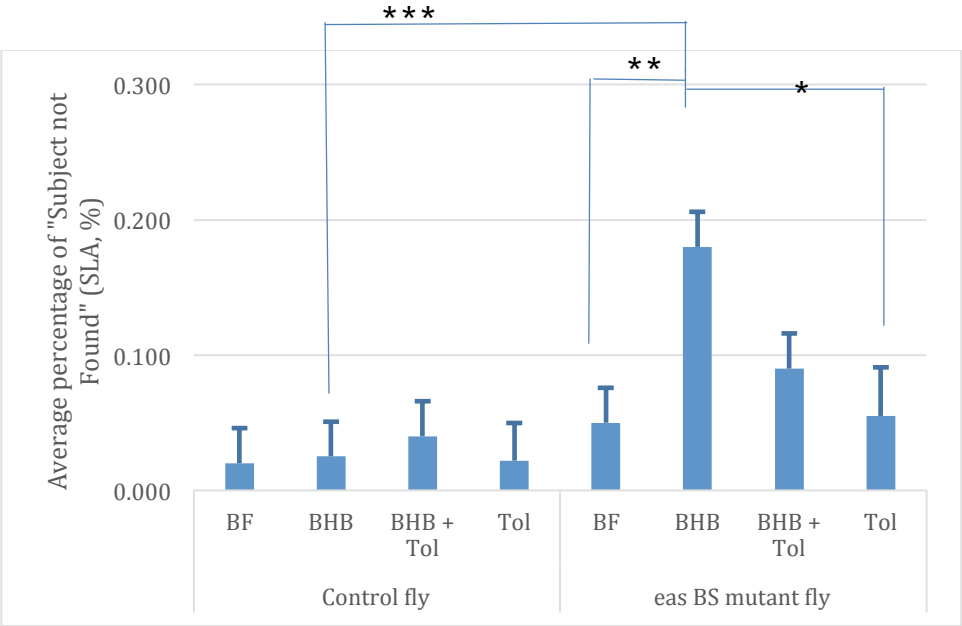
eclosion from the BF diet to the Tol or CGP diet for three days, in order to see whether there is a difference in SLA across diets.

Overall, I chose the eas BS mutant as the ideal strain for the third-round experiments based on its high reproduction rate and elevated incidence of SLA. I found that both the K_{ATP} blocker tolbutamide and the $GABA_B$ blocker CGP55485 could partially reverse the observed anticonvulsant effect of ketone bodies in a whole-animal model. In addition, my project provided a simple way to observe seizure behavior in flies before and after metabolic and pharmacological manipulations with a ketogenic diet.

APPENDIX

The effects of the ketone body supplemented diet and the K_{ATP} blocker diet between Canton-S flies and easBS mutant flies in second-round experiments.

Average percentage of "Subject not Found" (mean ± SEM) were significantly reduced in βHB supplemental diet between Canton-S flies (n = 20) and *eas*BS mutant flies (n= 20) using a factorial ANOVA test with a Bonferroni correction ($p< 0.001$). *eas*BS mutant flies' average percentage of SLA were significantly reduced on both the control diet ($p< 0.01$) and the Tol diet (n = 11, $p< 0.05$) compared to the βHB supplemented diet. * $p<0.05$, ** $p< 0.001$, *** $p< 0$.



All pairwise comparison between all ten diets in eas BS mutant flies in the third-round experiment. There were significant differences in mean percentage of SLA between specifically selected diets: BF and βHB, BF and EtOH, and BF and CGP.

Diet	Diet	*p*-value
βHB	BF	0.000
	BF to βHB	1.000
	βHB + Tol	1.000
	Tol	1.000
	EtOH	1.000
	βHB + CGP	0.124
	CGP	1.000
	DMSO	1.000
	βHB + DMSO	1.000
BF to βHB	BF	0.492
	βHB + Tol	1.000
	βHB + CGP	1.000
βHB + Tol	BF	0.639
	βHB + CGP	1.000
Tol	BF	0.200
	BF to βHB	1.000
	βHB + Tol	1.000
	βHB + CGP	1.000
EtOH	BF	0.005
	BF to βHB	1.000
	βHB + Tol	1.000
	Tol	1.000
	βHB + CGP	1.000
	CGP	1.000
	DMSO	1.000
	βHB + DMSO	1.000
βHB + CGP	BF	1.000
CGP	BF	0.032
	BF to βHB	1.000
	βHB + Tol	1.000
	Tol	1.000
	βHB + CGP	1.000
	DMSO	1.000
	βHB + DMSO	1.000

DMSO	BF	0.131
	BF to βHB	1.000
	βHB + Tol	1.000
	Tol	1.000
	βHB + CGP	1.000
	βHB + DMSO	1.000
βHB + DMSO	BF	0.193
	BF to βHB	1.000
	βHB + Tol	1.000
	Tol	1.000
	βHB + CGP	1.000

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