Epilepsy Research 155 (2019) 106153

Contents lists available at ScienceDirect

Epilepsy Research

journal homepage: www.elsevier.com/locate/epilepsyres

The addition of a lipid-rich dietary supplement eliminates seizure-like activity and paralysis in the drosophila bang sensitive mutants

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ARTICLE INFO

Keywords:
Seizures
Epilepsy
Metabolism
Drosophila

ABSTRACT

Objective: To investigate the effect that a diet supplemented with KetoCal 4:1, a commercially available dietary formula consisting of a 4:1 ratio of fats to carbohydrates plus proteins, had on the seizure-like activity (SLA) and paralysis normally exhibited by the *Drosophila* Bang-sensitive (BS) paralytic mutants following mechanical shock.

Methods: Given that dietary changes are known to reduce seizures in humans and animal models, three BS mutants, *easily-shocked (eas), bang-senseless (para^{bss}),* and *technical knockout (tko)*, were fed a standard cornmeal/yeast/sugar diet supplemented with 10% KetoCal 4:1 (KetoCal-sup diet). Newly eclosed BS flies were fed this diet for 3–7 days and the effect this had on SLA, paralysis, locomotor activity, triglyceride levels, and glucose levels was examined.

Results: All three genotypes displayed significant reductions in SLA and BS sensitivity following mechanical shock. After only 3 days on the diet, 95% of *tko* flies no longer exhibited SLA or paralysis, and near complete suppression of the BS phenotype was seen by day 7. In the case of *eas*, there was a 78% reduction of SLA after 3 days on the diet and SLA was completely suppressed by day 7. The *para^{bss}* flies showed a similar but less robust reduction of SLA on the diet as there was only a 68% reduction of SLA and paralysis following 7 days on the diet. The diet did not suppress activity globally as *tko* flies had increased basal locomotor activity on the diet while the *para^{bss}* and *eas* flies showed no significant change in basal activity. The KetoCal-sup diet did not significantly alter the triglyceride levels or the total glucose levels in the BS mutants. In addition, the SLA and BS suppression was maintained even when the BS mutants were transitioned back to a standard fly diet.

Conclusions: The SLA and paralysis associated with the *Drosophila* BS phenotype can be effectively suppressed by transient exposure to a KetoCal-sup diet. This suppression was not dependent upon long term maintenance of the diet and it was not associated with alterations in total glucose or triglyceride levels in these flies.

1. Introduction

There is a large body of evidence demonstrating that dietary and metabolic factors play a critical role in seizure susceptibility (Martin et al., 2016). For example, multiple studies have shown that a ketogenic diet can reduce seizures significantly, particularly in children that fail to respond to current medications (Clanton et al., 2017; Martin et al., 2016; Theile, 2003; Vining et al., 1998). While the seizure inhibiting effects of the ketogenic diet have been known for nearly a century (Wilder, 1921), the exact mechanism by which this diet suppresses seizures and the role ketone body production has on seizure reduction is still subject to much debate (Simeone et al., 2018). Evidence suggests that the diet may work via multiple mechanisms including but not limited to the alteration of neurotransmitter systems, the enhancement of cellular metabolism, and the reduction of oxidative damage (Clanton et al., 2017; Rho, 2017). Other studies have demonstrated that non-ketogenic dietary changes, such as the addition of omega-3 fatty acids or the modification of the proportion of branched-chained vs. aromatic fatty acids can affect seizure susceptibility (Dallerac et al., 2017). In addition, many studies have found that pharmacological and genetic alterations of metabolic genes can alter seizure susceptibility in animal models (Li et al., 2017; Stone et al., 2013; Voskuyl et al., 1998). One study in mice demonstrated that genetic alterations that reduced glucose metabolism were associated with 1) an increased activity level of K_{ATP} channels in neurons and 2) a reduction in seizure activity (Gimenez-Cassina et al., 2012).

Abbreviations: SLA, ceizure-like activity; BS, bang-sensitive; CS, canton-S; eas, easily-shocked; para^{bss}, paralytic$^{Bang-senseless}$; tko, technical-knockout

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https://doi.org/10.1016/j.eplepsyres.2019.106153
Received 9 January 2019; Received in revised form 19 May 2019; Accepted 11 June 2019
Available online 11 June 2019
0920-1211/

Given that nearly 30% of patients fail to respond adequately to current antiepileptic treatments (Hauser, 2018), a better understanding of how dietary and related metabolic changes affect seizure susceptibility may open new avenues for seizure treatment and anti-epileptic drug development. Toward this end, we examined how dietary changes could affect seizure susceptibility in a group of Drosophila mutants known as the BS mutants. These mutants are susceptible to seizure-like activity (SLA) following a variety of insults (Ganetzky and Wu, 1982; Pavlidis et al., 1994; Pavlidis and Tanouye, 1995; Whelan et al., 2010). The SLA activity is characterized by spastic spinning of the flies coupled with rapid uncoordinated movement of the legs, wings and abdomen. The initial SLA is followed by a variable period of paralysis interspersed with subsequent bouts of SLA, a phenotype that mimics generalized tonic-clonic seizures in humans. Previous work has demonstrated that 1) the SLA exhibited by the BS mutants shares significant similarities with human seizure disorders (Kuebler and Tanouye, 2000; Kuebler et al., 2001; Lee and Wu, 2002; Song and Tanouye, 2008) and 2) metabolic alterations can reduce SLA susceptibility in these mutants (Kemppainen et al., 2016; Li et al., 2017; Stone et al., 2013). These previous studies indicate that the BS mutants represent a valuable model for investigating the relationship between diet, metabolism, and seizure disorders in general.

In this study, we specifically tested the ability of KetoCal 4:1, a human ketogenic dietary supplement, to alter the seizure-susceptible phenotype in the BS mutants following mechanical stimulation. We found that only a few days of feeding flies a diet supplemented with 10% KetoCal 4:1 had a remarkable effect in suppressing the BS phenotype, particularly the SLA associated with the BS behavior.

2. Experimental procedure

2.1. Drosophila stocks

Drosophila melanogaster stocks were maintained on standard cornmeal-molasses-yeast medium (https://bdsc.indiana.edu/information/recipes/molassesfood.html) on a 12 h/12 h light-dark cycle at 25 °C. The BS mutants used in this study were *easily shocked*^{1} (*eas*^{1}), *paralytic*$^{bang\ senseless1}$ (*para*^{bss1}), and *technical knockout*^{25t} (*tko*^{25t}). The *eas* locus encodes an ethanolamine kinase involved in phosphatidyl ethanolamine synthesis (Pavlidis et al., 1994) and the *tko* locus encodes a mitochondrial riboprotein (Royden et al., 1987). The *para*^{bss} mutant has been identified as a gain of function of the *paralytic* (*para*) voltage-gated sodium channel gene (Parker et al., 2011). While other BS strains exhibit some aspects of the BS behavior, only these strains were tested here because they exhibit the most robust example of the stereotypical BS seizure-paralysis-seizure phenotype and the paralysis phenotype is fully penetrant in these strains. The wild-type flies used as controls were Canton-S (CS).

2.2. Feeding and bang-sensitivity testing

To examine the behavioral effects of feeding KetoCal 4:1 to the BS mutants, 10 newly-eclosed male flies were reared on agar only media with 10% w/w KetoCal 4:1 (KetoCal-only). 4.5 g of agar only media was liquified by heating briefly in a microwave and 500 mg of KetoCal 4:1 and 1 ml of dH_2O was added. The solution was mixed and allowed to cool/solidify before using. Because flies could get stuck in the oily layer that formed near the top of the agar if left for a prolonged time in the vial, flies were transferred to fresh vials every 3–4 days. Using an identical method as described above, newly-eclosed flies were also reared on standard media (cornmeal/sucrose/yeast media with propionic acid) supplemented with 10% KetoCal 4:1 (KetoCal-sup).

Flies raised in standard media, KetoCal-only media, or KetoCal-sup media were tested for the BS behavior after 3 or 7 days on the diet. Measurements of BS behavior were performed by gently transferring individual flies into empty 70 ml fly vials without anesthesia. The flies were allowed to acclimate for ~15 min. Individual vials were then vortexed for 10 s on the highest setting (VWR Vortex Genie) and the behavior was observed. The stereotypical BS behavior following mechanical stress is as follows: 1) Immediately after the vortexing, the flies lie motionless at the bottom of the vial for a period of 20–40 secs depending upon the genotype (paralysis). 2) The flies then begin to exhibit bouts of seizure-like activity (SLA), which consists of uncontrolled rapid wing and abdominal motions that cause them to move in sporadic circular patterns at the bottom of the vial. The SLA bouts normally last from 2 to 10 seconds. 3) The flies exhibit a second bout of paralysis, which can be interrupted by additional bouts of SLA. 4) The flies eventually right themselves and resume normal movements (recovery). This recovery occurs between 45 ss and 5 min after the initial mechanical shock and varies depending upon the genotype. The wildtype CS strain did not exhibit any BS behavior following mechanical shock regardless of diet.

Three data points were gathered for each fly examined: presence/absence of paralysis, presence/absence of SLA, and time to recovery. Flies were considered to have undergone SLA if they had an uncontrolled rapid wing and abdominal motions that caused them to move in sporadic circular patterns at the bottom of the vial. This behavior is quite distinct from normal movement patterns. Flies were considered to display BS behavior if they exhibited either 1) paralysis-only or 2) paralysis followed by subsequent SLA. Only flies in the second category were counted as exhibiting SLA. Recovery time was gauged by the amount of time it took flies to return to a standing position. The investigators were not blinded during the assessment of BS sensitivity or SLA but 1) the robustness of the behavior (20 s of paralysis and 2–10 seconds of distinct SLA activity) and the magnitude of the suppression (100% in some cases) minimizes the possibility of investigator bias.

2.3. Locomotor testing

To examine how the KetoCal-sup diet affected behavior, locomotor activity was measured using the DAM2 Drosophila Activity Monitor (TriKinetics). Individual flies were immobilized on ice and then placed into individual 5 mm DAM2 tubes. Each tube contained either a small plug of normal media or media supplemented with 10% KetoCal 4:1 on one end. The flies were given 1 h to acclimate to the new surroundings and then activity was monitored for a 24 -h period. The number of beam crossings was recorded for each individual fly.

2.4. Glucose measurements

The total glucose levels in the flies were also measured (glucose plus enzymatically digested glycogen/trehalose stores) using a protocol described previously (Al-Anzi et al., 2009). Briefly, flies were fed for five days and then frozen in groups of three in a -80° freezer before being homogenized in ice-cold PBS. The homogenate was centrifuged to remove particulates and the supernatant/extract was analyzed. 15uls of the extract was incubated for 1 h at 37^{O}C with 15uls of 8 mg/ml *Rhizopus* amylglycosidase (Megazyme), which catalyzes the conversion of trehalose and glycogen to glucose. The total glucose levels were then quantified using a glucose (HK) assay kit (Sigma) following the manufacturers recommendations. A portion of the extract was assayed for protein levels as described below. Each sample was assayed in duplicate and the data presented as the ratio of glucose to protein content in the flies.

2.5. Triglyceride measurements

Triglyceride measurements were performed on five day old male flies using a method similar to that described previously (Skorupa et al., 2008). After starving the flies for 2 h, flies were frozen in groups of three in a -80° freezer and then ground in a 1.5 ml eppendorf tube with

100uls of cold PBS + 0.05% Tween using a micro-pestle. The homogenate was centrifuged and the supernatant/extract was removed and processed using an EnzyChrom Triglyceride Assay Kit (BioAssay) following the manufacturers recommendations. A separate aliquot of the extract was assayed for protein levels using the BCA protein assay reagent (Pearce). Each sample was assayed in duplicate and data presented as the ratio of triglyceride to protein content in the flies.

2.6. Statistical analysis

In comparing the percentage of flies exhibiting SLA and BS susceptibility between treatments, the Fisher's exact test was used to determine if percentages were significantly different. For comparisons of locomotor activity between groups and treatments, a Kruskal-Wallis test was used as the data was not normally distributed. This test was followed by a Dunn's post-hoc test. For comparisons of triglyceride and glucose levels between treatments and genotypes, a 1-way ANOVA followed by a Tukey's post-hoc test was used to determine if there were significant differences between diets. Prism software was used to perform all statistical calculations.

Power analysis was performed using G*Power version 3.1. For the Fishers Exact Test, a sample size of 60 gave a power of 0.8 to detect a 12 percent reduction in SLA and bang sensitivity in treated groups as compared to controls (assumes 100% SLA and bang sensitivity in controls). In the case of triglyceride comparisons, sample sizes of 7 per group gave a power of 0.8 to detect an effect size of 0.43. For glucose comparisons, sample sizes of 10 per group gave a power of 0.8 to detect an effect size of 0.33. For movement comparisons, sample sizes of 50 per group gave a power of 0.85 to detect an effect size of 0.15.

3. Results

3.1. KetoCal-only diet partially rescues the BS phenotype

Given that high-fat and low-carb diets have the ability to reduce seizure susceptibility in mammals, we examined the effect feeding BS flies KetoCal, a nutrition supplement that has a 4:1 ratio of lipids to carbohydrates plus proteins, had on the BS phenotype. When *eas*, *tko* and *para*^{bss} flies were fed a normal diet for three days, > 98% of them displayed some aspect of the BS phenotype, SLA and/or paralysis, following mechanical shock. When the flies were fed a diet consisting solely of KetoCal, there was a significant suppression of the BS phenotype in *eas* (20.0% suppression) and *tko* (76.7% suppression) flies (Fig. 1a). In contrast, no suppression was seen in the *para*^{bss} flies. The KetoCal-only diet had a more pronounced effect on the reduction of SLA. In the case of *eas*, there was a 56.7% suppression of SLA, while in the *tko* flies there was an 86.7% suppression (Fig. 1b). However, the Keto-Cal only diet did not significantly reduce the level of SLA in the *para*^{bss} flies.

3.2. KetoCal-sup diet shows greater efficacy as compared to KetoCal-Only diet

Given that the *tko* and *eas* flies reared on a KetoCal-only diet displayed a reduction in SLA and BS susceptibility, we examined whether supplementing the standard cornmeal/sugar/yeast diet with 10% KetoCal (w/v) would have any effect on these behaviors. While the KetoCal-only diet had a 4:1 ratio of lipids to carbs/proteins, the normal diet supplemented with 10% KetoCal (KetoCal-sup diet) had roughly a 1:5 ratio of lipids to carbs/proteins. Interestingly, the flies fed the KetoCal-sup diet for 3 days displayed similar or greater reductions in BS susceptibility and SLA as compared to the KetoCal-only diet (Fig. 1). Only 8.9% of the *tko* flies exhibited BS behavior and only 3.3% exhibited SLA on the KetoCal-sup diet as opposed to 23.3% and 13.3% for the KetoCal-only diet. The *eas* flies displayed the largest reduction as only 54.4% exhibited BS susceptibility and 26.7% exhibited SLA on the KetoCal-sup diet as opposed to 80.0% and 43.3% for the KetoCal-only diet. In addition, while the KetoCal-only diet had virtually no effect on reducing BS susceptibility and SLA in *para*^{bss} flies, when fed the Keto-sup diet only 85.5% of flies exhibited BS susceptibility and only 80.0% displayed SLA following mechanical shock.

For flies that did display the temporary paralysis normally associated with the BS behavior, those fed the KetoCal-sup diet tended to recover quicker than those fed the KetoCal-only diet. The recovery times were 204 ± 72.4 s vs 227 ± 106 s for *para*^{bss}, 46.7 ± 8.5 s vs 55.7 ± 16.6 s for *eas*, and 30.5 ± 16.1 s vs 34.0 ± 10.6 s for *tko*. While these differences were consistent across all three genotypes, in no cases were they statistically significant ($p > 0.01$). Given that the KetoCal-sup diet was either as efficacious or more efficacious in reducing SLA and BS susceptibility in these genotypes, the remaining experiments focused on this diet.

3.3. 7-Day KetoCal-Sup diet shows greater efficacy than a 3-Day diet

Given that three days of exposure to the KetoCal-sup diet partially rescued the BS phenotype, we examined if the effect would be augmented by feeding the flies the diet for a prolonged period (seven days). For all three genotypes, flies fed for seven days exhibited lower levels of both BS susceptibility and SLA, although in the case of *tko* this reduction was not statistically significant. In the case of the *tko* flies, after seven days none of the flies exhibited SLA and only one displayed BS susceptibility indicating an almost complete rescue of the BS phenotype. Likewise, after seven days on the diet, the *eas* and *para*^{bss} flies had a significant reduction in BS susceptibility and SLA as compared to three days. For the *eas* flies, the percentage of flies exhibiting BS susceptibility dropped from 54.4% to 16.7%, while for *para*^{bss} BS susceptibility dropped from 85.5% to 33.3% (Fig. 2a). A similar pattern was seen with the reduction in SLA following prolonged exposure to the diet (Fig. 2b).

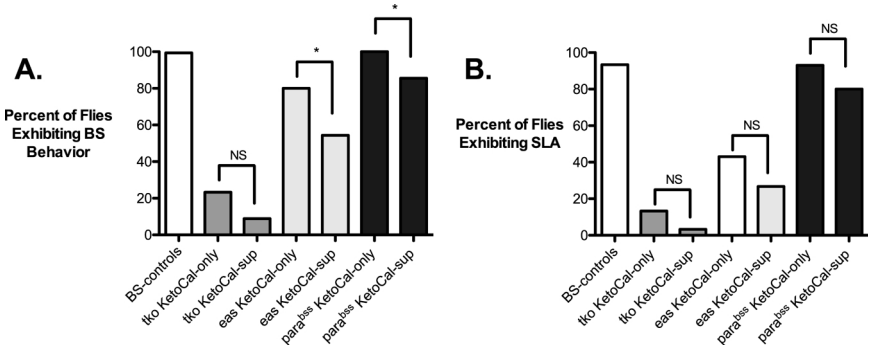

Fig. 1. KetoCal-Only vs KetoCal-sup diets. Individual 3-day old flies were mechanically shocked to induce the BS behavior. A) The percentage of flies that exhibited BS behavior is indicated for each genotype and diet. All treatments showed a significant reduction compared to the flies fed a control diet except for the *para*^{bss} flies. (n = 30 for each genotype on the KetoCal-only diet; n = 90 for each genotype on the KetoCal-sup diet). B) The percentage of flies that exhibited SLA is indicated for each genotype and diet. All treatments showed a significant reduction compared to the control diet except for the *para*^{bss} flies fed the KetoCal-only diet. (n = 30 for each genotype on the KetoCal-only diet; n = 60 for each genotype on the KetoCal-sup diet). (* $p < 0.01$).

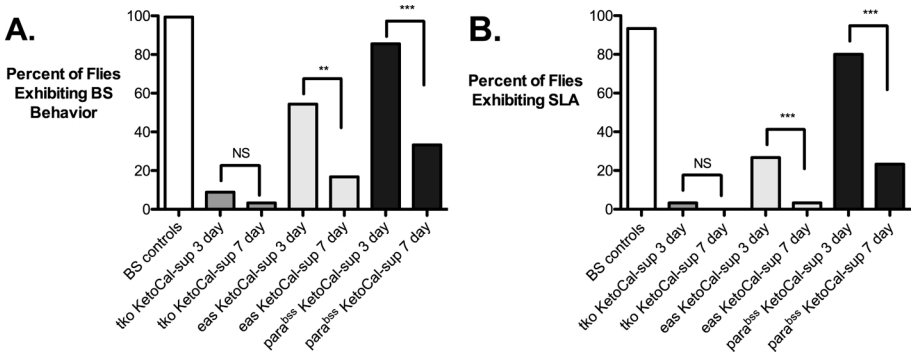

Fig. 2. Three-day vs seven-day KetoCal-sup diets. Individual 3-day or 7-day old flies were mechanically shocked to induce the BS behavior to determine the effect the length of feeding had on BS behavior and SLA. All treatments showed a significant reduction in both BS susceptibility and SLA as compared to the control diet. A) The percentage of flies that exhibited BS behavior is indicated for each genotype and diet. (n = 90 for each genotype on the 3-day KetoCal-sup diet; n = 30 for each genotype on the 7-day KetoCal-sup diet). B) The percentage of flies that exhibited SLA is indicated for each genotype and diet. (n = 60 for each genotype on the 3-day KetoCal-sup diet; n = 30 for each genotype on the 7-day KetoCal-sup diet) (**p < 0.001, *** p < 0.0001).

3.4. Locomotor changes following the KetoCal-Sup diet

The effect the KetoCal-sup diet had on locomotor behavior was examined to determine if the diet's ability to rescue the BS phenotype was caused in part by a general reduction in motor/neural activity. After three days on the KetoCal-sup diet, changes in locomotor behavior were monitored using a beam crossing assay. The amount of beam crosses per 24 h increased in *tko* flies from 661 ± 357 to 872 ± 450 following three days on the KetoCal-sup diet but the increase was not statistically significant (Fig. 3). In the other two BS strains, the number of beam crosses decreased following the KetoCal-sup diet, *eas* decreased from 602 ± 451 to 568 ± 391 and *bss* decreased from 1032 ± 395 to 876 ± 552, but in neither case were these changes statistically significant. Likewise, the wildtype CS flies also displayed a reduction in beam crossings when fed the KetoCal-sup diet, 1484 ± 585 to 1217 ± 497 but again the change was not statistically significant. In general, there was no consistent change in locomotion across genotypes following exposure to the KetoCal-sup diet. However, all three BS strains displayed significantly less locomotor activity in this assay as compared to the CS wildtype controls ($p < 0.05$). This reduction in locomotor activity in the BS mutants as compared to wildtype flies was consistent across both diets. In addition, the *eas* and *tko* flies exhibited lower levels of movement on the control diet than *para*^{bss} flies. When switched to the KetoCal-sup diet, only the *eas* flies exhibited significantly lower levels of movement as compared to the *para*^{bss} flies.

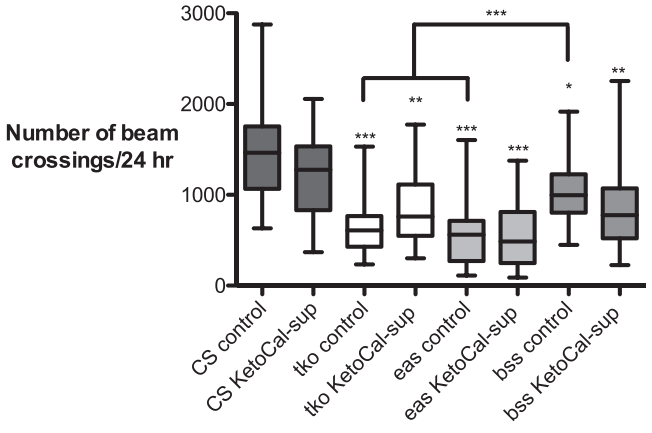

Fig. 3. Locomotor activity levels in flies fed KetoCal-sup diet. As compared to the control diet, none of the genotypes exhibited a significant change in locomotor activity while on the KetoCal-Sup diet ($p > 0.01$). All of the BS mutants fed control or KetoCal-sup diets (grey bars) exhibited reduced locomotor activity as compared to CS flies fed the corresponding diet (*$p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$). In addition, the *tko* and *eas* flies displayed significant lower levels of locomotor activity on the control diet as compared to the *para*^{bss} flies on the control diet (*** $p < 0.0001$). (n = 50 for each genotype/diet combination).

3.5. Changes in total glucose levels following the KetoCal-Sup diet

Given the increase in lipids and corresponding decrease in sugars in the KetoCal-sup diet, we examined whether flies fed the KetoCal-sup diet displayed any significant changes in total glucose levels (free glucose plus stored trehalose and glycogen). After five days on the diet, a time period sufficient to cause significant levels of SLA suppression, the flies displayed no significant change in total glucose levels. While all three strains had slightly lower levels of total glucose/mg protein when fed the KetoCal-sup diet, the reduction was not significant for any of the BS genotypes. The largest difference was seen in the *tko* flies in which the levels were reduced from 0.514 ± 0.353 mg/mg protein (control) to 0.384 ± 0.130 mg/mg protein (KetoCal-sup) (Fig. 4). However, the *para*^{bss} flies had nearly identical total glucose levels on the two diets; 0.482 ± 0.259 mg/mg protein (control) vs. 0.478 ± 0.200 mg/mg protein (KetoCal-sup). In addition, there was no significant difference in total glucose levels when comparing the BS strains to wildtype CS flies.

3.6. Changes in lipid levels following the KetoCal-Sup diet

In addition to examining the glucose levels in the flies following exposure to the KetoCal-sup diet, the triglyceride levels were examined as well. As with glucose levels, there was no significant change in the triglyceride levels following five-day exposure to the KetoCal-sup diet (Fig. 5). When comparing between genotypes, the *para*^{bss} flies had significantly higher triglyceride levels on the control diet, 0.376 ± 0.149 mg/mg protein, than both *tko*, 0.176 ± 0.094 mg/mg protein, and wildtype CS flies, 0.121 ± 0.089 mg/mg protein. This

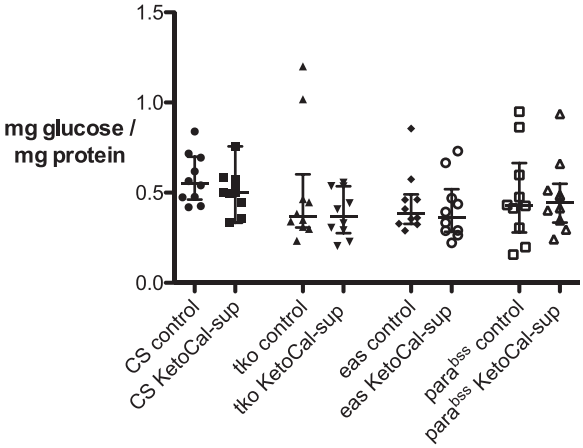

Fig. 4. Glucose levels in KetoCal-sup fed flies. Flies fed the KetoCal-sup diet were assayed in groups of three for total glucose levels (free glucose plus trehalose and glycogen). In none of the genotypes did the KetoCal-sup diet significantly alter the glucose levels. ($p > 0.01$). (n = 10 for each genotype/diet combination).

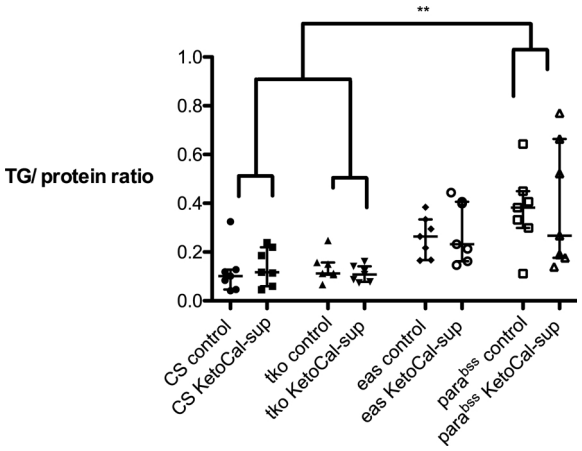

Fig. 5. Triglyceride levels in KetoCal-sup fed flies. Flies fed the KetoCal-sup diet were assayed in groups of three for whole body triglyceride levels. In none of the genotypes did the KetoCal-sup diet significantly alter the triglyceride levels. However, the *para*^{bss} flies had significantly higher triglyceride levels than the CS and *tko* flies on both diets (** $p < 0.001$). (n = 7 for each genoitype/diet combination).

difference in triglyceride levels was consistent across both diets. The *eas* flies displayed intermediate levels of triglycerides that were not significantly different from any of the other three genotypes tested.

3.7. Long-term BS suppression following a short-term KetoCal-Sup diet

After 3–7 days on the KetoCal-sup diet, all three BS strains showed significant levels SLA and BS behavior suppression. Interestingly, this suppression was maintained even after these flies were switched back to a normal diet. Following three days on the KetoCal-sup diet, *tko* flies (n = 26) were switched back to a normal diet for ten days. At the end of this time period, only two of the flies, 7.7%, exhibited BS susceptibility while 100% of control *tko* flies at this age, 13 days post-eclosion, exhibited BS susceptibility (Table 1). Similarly, *para*^{bss} (n = 29) and *eas* (n = 13) flies were fed the KetoCal-sup diet for seven days and then switched back to the control diet for ten days. At the end of this time period, none of the *eas* flies and only eight of the *para*^{bss} flies, 27.6%, exhibited BS susceptibility while control *eas* and *para*^{bss} flies at this age, 17 days post-eclosion, displayed 100% BS susceptibility. The percentage of *eas*, *tko* and *para*^{bss} flies that displayed BS susceptibility at the end of this treatment was similar to the levels seen immediately after three or seven-day exposure to the KetoCal-sup diet indicating that continuing the diet is not essential for maintaining the BS and SLA suppressive effects of the diet (Table 1).

4. Discussion

While often used as a treatment of last resort, the ketogenic diet has shown efficacy in reducing seizure activity in children whose seizures are refractory to current medications (Clanton et al., 2017; Martin et al., 2016). In addition, recent work has begun to explore the ability of the ketogenic diet to reduce seizures in adults (Mosek et al., 2009). The induction of ketosis is thought to be key in generating the seizure suppressive effects of this diet although the mechanism(s) by which the ketogenic diet suppresses seizures remains open to debate (Clanton et al., 2017; Youngson et al., 2017). It is known that ketone bodies can affect diverse cellular functions ranging from metabolism to cell signaling pathways to histone modifications, all of which could impact neuronal excitability (Newman and Verdin, 2014; Shimazu et al., 2013; Taggart et al., 2005).

In this study, we found that BS flies reared on a KetoCal-only diet, a diet that contained > 75% of its caloric content in lipids, displayed a significant reduction in SLA and bang sensitivity. However, when the flies were fed the KetoCal-sup diet, a diet that contained less than 10% of its caloric content from lipids, the flies had even more pronounced SLA and BS suppression. Clearly, a higher lipid content was not associated with improved seizure control. In addition, the KetoCal-sup diet did not correlate with any significant shift in lipid or glucose levels in the flies as compared to a standard control diet. Previous work has found that *eas* flies directly fed ketone bodies displayed a marked reduction in the SLA duration (over 5-fold), although the reduction was less than the complete elimination seen here (Li et al., 2017).

Taken together, this data suggests that ketosis and the generation of ketone bodies may not fully account for the dramatic SLA suppression seen here in the BS flies on the KetoCal-sup diet. However, this interpretation is limited by the fact that 1) ketone levels in the flies were not assessed experimentally and 2) the exact caloric intake of individual flies was not measured. In addition, previous work in Drosophila has found that ATP6^{1} mutants, which exhibit spontaneous SLA with age (Celotto et al., 2011), can be successfully treated by dietary supplementation with ketone bodies even in the presence of normal dietary carbohydrate levels (Fogle et al., 2019). This is similar to the effect of the KetoCal-sup diet seen here in which the KetoCal supplement had a profound effect even in the presence of normal dietary carbohydrate levels.

Interestingly, a number of studies investigating the ketogenic diet have failed to find a correlation between ketone levels and seizure control in both animal models and humans (Augustin et al., 2018; Bough et al., 2000; McNally and Hartman, 2012; Musa-Veloso et al., 2006). This suggests that other factors associated with the ketogenic diet may have the ability to suppress seizures independent of the elevation of ketones in the blood (Augustin et al., 2018). For example, the reduction in the activity of the glycolysis enzyme lactate dehydrogenase (LDH), which is associated with a low carbohydrate ketogenic diet, may contribute to a reduction in seizure occurrence (Dallerac et al., 2017; Sada et al., 2015; Youngson et al., 2017). In fact, low-glycemic-index diets have shown efficacy in reducing seizures in the clinic (Pfeifer and Thiele, 2005). Interestingly, the *tko* flies have well-documented metabolic defects including an upregulation of LDH (Fernandez-Ayala et al., 2010) and a shift toward glycolysis (Kemppainen et al., 2016). It is possible that the lipids supplied by the KetoCal-sup diet may trigger a reduction in LDH expression, which could reduce seizures. However, this seems unlikely given 1) the high level of carbohydrates that remain present in the KetoCal-sup diet, 2) the absence of alterations in carbohydrate and lipid levels in the flies following exposure to the KetoCal-

Table 1
BS flies that were initially sensitive to SLA and paralysis were fed the KetoCal-sup diet for 3 days (*tko*) or 7 days (*eas* and *para*^{bss}), which led to a decrease in both bang-sensitivity (BS) and SLA after mechanical shock. These flies were then switched to normal fly media for ten days and the suppression of BS and SLA was maintained even in the absence of the KetoCal-sup diet. No significant difference in BS sensitivity or SLA was seen for any genotype between testing immediately after the KetoCal-sup diet and testing following the 10-day switch to normal media.

Genotype	Initial Sensitivity (%BS/%SLA)	Sensitivity After 3-7 Days on KetoCal-sup Diet (%BS/%SLA)	Sensitivity After 10 Day Switch to Normal Media (%BS/%SLA)
tko	100%/100%	13.6%/0%	7.7%/0%
eas	100%/100%	0%/0%	0%/0%
para^{bss}	100%/100%	34.2%/31.6%	27.6%/20.6%

sup diet, and 3) the fact that previous work with ATP6[1] flies has shown that reduction of glycolysis was not associated with SLA reduction (Fogle et al., 2019).

A more likely possibility is that other nutritional components of the KetoCal 4:1 product have anti-convulsant effects. A number of studies in both humans and animal models have shown that polyunsaturated fatty acids (PUFAs), which compose roughly 20% of the caloric content of the KetoCal product, may have the ability to suppress seizures. It is known that PUFAs have a neuroprotective effect in *Drosophila* (Ziegler et al., 2015). In addition, recent work has found that a diet containing medium-chain fatty acids, polyunsaturated fatty acids, low glycemic index carbohydrates, and a high branched-chained/aromatic amino acid ratio had the ability to suppress seizures activity in a chronic kainate (KA) mouse model of epilepsy (Dallerac et al., 2017). Other studies have found that PUFAs alone could inhibit seizures to some extent in mouse and rat models (Porta et al., 2009; Voskuyl et al., 1998). In addition, PUFA supplementation was shown to be effective in reducing seizures in humans with drug resistant epilepsy (DeGiorgio et al., 2015) and other CNS diseases (Schlanger et al. 2002). In a systematic review, however, no strong association was found between omega-3 PUFA supplementation and seizure reduction in humans (Pourmasoumi et al., 2018).

While there is conflicting evidence regarding the efficacy of PUFAs to suppress seizures in humans, little is known regarding the possible mechanism(s) by which PUFAs could reduce seizures. I*n vitro* studies have shown that the external application of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to mouse hippocampal slices have the ability to raise stimulatory thresholds of CA1 neurons (Xiao and Li, 1999) and that EPA application can reduced Na^+ currents in patch clamp experiments (Xiao et al., 1995). In whole animal studies in rats, linolenic acid (LA) dietary supplementation reduced seizures but it had no effect on body fat composition or membrane phospholipid composition in the brain. However, it did increase the level of DHA and LA in the plasma (Porta et al., 2009).

If the PUFAs have an ability to suppress SLA in these flies, it is interesting that even though similar amounts of PUFAs were in both diets, the KetoCal-sup diet had a greater efficacy. This could be due to a variety of factors. First, the high amount of carbohydrates in the KetoCal-sup diet could spare the metabolic breakdown of PUFAs, thereby effectively elevating the PUFA levels in these flies as opposed to those fed the KetoCal-only diet. Secondly, the KetoCal-sup diet may have an increased palatability due to the presence of carbohydrates, thereby elevating the dietary intake of PUFAs. Finally, the effect of PUFAs (as well as ketone bodies) may be augmented by other metabolites found or produced by flies fed a normal diet supplemented with KetoCal. Future studies that investigate the metabolome of flies fed these two diets are necessary to address these possibilities.

While the mechanism by which the KetoCal-sup diet suppresses the SLA and BS behavior remains unclear, it did display a broad efficacy across the different BS genotypes, although the efficacy varied by genotype. The diet was close to 100% efficacious in the *tko* and *eas* flies in eliminating SLA after 7 days on the diet, while the efficacy dropped to roughly 75% in the $para^{bss}$ mutants. Previous work has found that the BS phenotype in the *tko* flies is exacerbated by a high sugar diet (Kemppainen et al., 2016), a result that is consistent with the reduction in SLA and BS behavior on the slightly reduced sugar diet seen here. In addition, the reduced effect in the $para^{bss}$ mutant correlates with the fact that this mutant has the most drastic BS phenotype, taking 2–3 times as long to recover from mechanical and electrical shock as compared to the other mutants tested here (Kuebler and Tanouye, 2000).

Interestingly, the seizure suppressive effects of the KetoCal-sup diet were maintained even after removing the flies from the KetoCal-sup diet. Even after returning to a normal diet for ten days, the KetoCal-sup fed flies still remained resistant to SLA. This result indicates that short-term exposure to the diet can trigger lasting changes to the nervous system of the BS flies, changes that are maintained in the absence of the dietary supplement. A similar phenomenon is seen in humans who have become seizure-free on the ketogenic diet. Previous research has shown that eighty percent of children who became seizure-free on the ketogenic diet remained seizure-free following discontinuation of the diet (Martinez et al., 2007).

Given the response of the Drosophila BS mutants to the KetoCal-sup diet and the similarity in the results to mammalian and human studies, the BS mutants represent an excellent model system to examine further the ability of dietary modifications to reduce neuronal excitability defects. The ability to manipulate the diet easily in these flies and the variety of seizure and excitability mutants available can help facilitate future studies aimed at identifying 1) optimal dietary strategies to reduce seizure susceptibility and 2) the physiological mechanisms behind those strategies.

Declarations of interest

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

The authors would like to thank Denise Lombard for help with maintaining fly stocks and Kris Burner for preliminary work on this project.

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