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Ketogenic and anaplerotic dietary modifications ameliorate seizure activity in *Drosophila* models of mitochondrial encephalomyopathy and glycolytic enzymopathy

Keri J. Fogle^{*,a,b}, Amber R. Smith^b, Sidney L. Satterfield^{a,b}, Alejandra C. Gutierrez^{a,b}, J. Ian Hertzler^{a,b}, Caleb S. McCardell^{a,b}, Joy H. Shon^b, Zackery J. Barile^{a,b}, Molly O. Novak^{a,b}, and Michael J. Palladino^{a,b}

^aDepartment of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

^bPittsburgh Institute for Neurodegenerative Diseases (PIND), University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

Abstract

Seizures are a feature not only of the many forms of epilepsy, but also of global metabolic diseases such as mitochondrial encephalomyopathy (ME) and glycolytic enzymopathy (GE). Modern anti-epileptic drugs (AEDs) are successful in many cases, but some patients are refractory to existing AEDs, which has led to a surge in interest in clinically managed dietary therapy such as the ketogenic diet (KD). This high-fat, low-carbohydrate diet causes a cellular switch from glycolysis to fatty acid oxidation and ketone body generation, with a wide array of downstream effects at the genetic, protein, and metabolite level that may mediate seizure protection. We have recently shown that a *Drosophila* model of human ME (*ATP6^L*) responds robustly to the KD; here, we have investigated the mechanistic importance of the major metabolic consequences of the KD in the context of this bioenergetics disease: ketogenesis, reduction of glycolysis, and anaplerosis. We have found that reduction of glycolysis does not confer seizure protection, but that dietary supplementation with ketone bodies or the anaplerotic lipid triheptanoin, which directly replenishes the citric acid cycle, can mimic the success of the ketogenic diet even in the presence of standard carbohydrate levels. We have also shown that the proper functioning of the citric acid cycle is crucial to the success of the KD in the context of ME. Furthermore, our data reveal that multiple seizure models, in addition to *ATP6^L*, are treatable with the ketogenic diet. Importantly, one of these mutants is *TPP^{sugarkill}*, which models human glycolytic enzymopathy, an incurable metabolic disorder with severe neurological consequences. Overall, these studies reveal widespread success of the KD in *Drosophila*, further cementing its status as an excellent model for studies of KD treatment and mechanism, and reveal key insights into the therapeutic potential of dietary therapy against neuronal hyperexcitability in epilepsy and metabolic disease.

*Corresponding author. kjf38@pitt.edu (K.J. Fogle).

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Keywords

Drosophila; Mitochondrial encephalomyopathy (ME); Triose phosphate isomerase (TPI) deficiency; Ketogenesis; Anaplerosis; Seizures

1. Introduction

Epilepsy, a disease characterized by recurring seizures resulting from abnormal, highly synchronous activity in the brain, is one of the most common neurological disorders, affecting approximately 1% of people worldwide. In addition, many disorders that are not specifically classified as epilepsies can exhibit seizures as part of their symptomatic profile [1–3]. There are dozens of modern anti-epileptic drugs (AEDs) available to seizure patients, and many people experience reduction or cessation of seizures with single drug or drug combination therapy. However, a significant number of patients experience refractory or breakthrough seizures, achieving little to no benefit with standard pharmacological protocols. Additionally, AEDs may cause undesirable side effects, and many are teratogens, posing long-term risk for the children of patients. For these reasons, clinical interest in the ketogenic diet (KD), which has long been known to effectively reduce seizures in refractory patients, has risen dramatically in recent years [4–7]. This high-fat, low-carbohydrate diet drastically reduces glycolysis in favor of maximizing fatty acid oxidation as the primary source of cellular fuel for the citric acid cycle, and increases synthesis of ketone bodies, which can be used as an alternate source of crucial intermediates for mitochondria.

We recently showed that the ketogenic diet is highly successful in treating the seizures of *Drosophila ATP6^l* [8], a model of human mitochondrial encephalomyopathies (ME) including Maternally Inherited Leigh Syndrome (MILS) and Familial Bilateral Striatal Necrosis (FBSN). MEs are characterized by defects in mitochondrially-encoded genes or nuclear encoded genes of the electron transport chain, and result in a deficiency of ATP produced via oxidative phosphorylation (OXPHOS). This deficit in energy supply manifests in severe dysfunction in energy demanding neuromuscular tissues. In humans, symptoms of these progressive diseases include cognitive and sensory decline, neuro- and muscular degeneration, cardiomyopathies, multi-organ system failure, and a high percentage of refractory seizures [1,2,9–11]. In flies, the *ATP6^l* mutation affects the proton passing subunit of Complex V of the electron transport chain (ATP synthase) resulting in a shortened lifespan, progressive locomotor impairment, stress induced seizures, and increased glycolysis and ketogenesis [12,13] [14]. We found that the KD ameliorated *ATP6^l* seizures up to 90% even in late stage disease and, as in other animal seizure models, this success was contingent upon the presence of the ATP-sensitive potassium channel (K_{ATP}) [8]. However, the contributions of other molecular targets and cellular processes to the effectiveness of the KD in the context of an already perturbed metabolic landscape remain unknown.

We show here that AEDs are largely ineffective in treating the seizures of *ATP6^l*, which is also a common difficulty in human ME patients. This highlights the importance of dietary modification as a therapy for ME-associated seizures. We show that blocking glycolysis pharmacologically does not mimic the KD or prevent its success, despite reduced glycolysis

being a major consequence of the diet. In contrast, we find that interference with the function of the citric acid cycle is highly disruptive to the effectiveness of the KD, and that dietary supplementation with an anaplerotic (citric acid cycle replenishing) lipid triheptanoin is seizure suppressive in multiple metabolic disease states and can “rescue” the effects of introducing a citric acid cycle defect into *ATP6^l* flies. We also show that the ketogenic diet is effective in a second metabolically compromised mutant, the glycolytic enzymopathy model *TPF^{sugarkill}* (*TPF^{sgk}*), and thus may represent a novel treatment for patients of TPI deficiency. Overall, our results highlight the importance of the citric acid cycle in mediating the effects of the ketogenic diet in metabolic disease states and demonstrate that ketogenic and anaplerotic supplementation is a promising new avenue for dietary therapy for metabolic disease associated seizures.

2. Methods

2.1. Flies and genetics

Strains, matings, and experimental flies were maintained on standard media comprised of agar, corn meal, dextrose, sucrose, dry yeast, and molasses, with propionic acid, phosphoric acid, and Tegosept added as fungicides. Flies are maintained in a light and temperature controlled room under a 12:12LD schedule. In all reported genotypes, “*ATP6^l*” or “*ATP6* [1]” refers to the mitochondrially encoded gene mutation in *ATP6* and is denoted first, separated from the nuclear chromosomes by standard semi-colon notation. The *ATP6^l* strain used in all experiments was a mitochondrial recombinant generated by the O’Farrell lab and described previously [15,16].

The classical bang sensitive alleles (*eas*, *cpo*, *kcc*, *kdn*), neuronal driver (*elav-GAL4*), isocitrate dehydrogenase gene disruption line, and RNAi lines (citrate synthase, isocitrate dehydrogenase) were obtained from the Bloomington Stock Center (Bloomington, IN). *TPF^{sgk}* results from a missense mutation at codon 80 leading to a Met to Thr substitution referred to as M80T herein. The construct used here was generated by a genetic engineering (GE) procedure described previously [17,18]. Multi-gene experimental lines were generated using standard mating schemes. Complete genotypes are provided in the appropriate figure legends.

2.2. Behavioral testing

Stress sensitivity or bang sensitivity (BS) experiments to assay stress-induced seizures were conducted as originally described by Ganetzky and Wu [19] with some modifications that have been described in detail previously [8]. Briefly, flies are placed in empty plastic, cotton-plugged vials in groups of 3–5, then vortexed at maximum speed for 20s. Bang sensitive flies exhibit paralysis, convulsions, or a combination of the two, while wild type flies recover immediately following the stimulus. *ATP6^l* and *TPF^{sgk}* flies exhibit progressively longer periods of paralysis and convulsions; due to this phenotype, their seizure severity is measured by the parameter time to recovery (TTR), which assays how long it takes each fly to resume purposeful movements after paralysis and convulsion. The classical bang sensitive alleles exhibited shorter but repeated seizures, and therefore the parameter “seizures per fly” was used to quantitatively assess their seizure severity. Each

seizure observed within a defined two-minute post-vor-texting period was noted, and then divided by the total number of flies in the vial.

2.3. Pharmacological tests

All anti-epileptic drugs, glycolysis blocking agents, and ketone body supplements tested in these experiments were obtained from Sigma Aldrich (St. Louis, MO). Compounds were administered by mixing a stock solution into heat-softened standard media to the final concentration indicated. The initial concentration tested was generally the highest possible that could be administered, based on the upper limit of either drug solubility or vehicle tolerance, if the vehicle was not water. The control flies were fed the equivalent amount of vehicle mixed into standard media and tested in parallel. Vehicle for valproate, gabapentin, vigabatrin, and phenytoin was water; for ethosuximide and carbamazepine was ethanol, and for topiramate and lamotrigine was DMSO. Flies were placed on drug media on day 10 post-eclosion then evaluated at day 18 for any change in their seizure phenotype (BS).

2.4. Dietary treatment

Administration of the ketogenic diet was performed as described previously [8]. Briefly, a standard vial was filled with an agar plug for hydration while the ketogenic media paste was provided in a 1 cm plastic cup embedded in the agar plug for nutrition. The ketogenic paste was comprised of a 3:1 ratio by mass of coconut oil:carbohydrates (sucrose, dextrose, 20% of the yeast, and 80% of the corn meal). Triheptanoin (BOC Sciences, Shirley, NY) and soybean oil were also administered in an agar-plugged vial and 1-cm cup. These oils were mixed with standard media at 35% by mass, which is a saturating concentration that creates an extremely viscous oil-based homogenous mixture for the flies to feed on. Large amounts of each specialty media were made simultaneously to reduce potential variability within each batch and across experiments. For *ATP6^l* and *TPP^{gk}* genotypes, the flies were placed on these diets at day 7 post eclosion and behavioral testing began at day 12. For classical alleles, flies were placed on the diet at day 3 and tested at days 7, 12, and 18.

2.5. Analysis/statistics

Data are presented as mean $\pm$ standard error (Fig. 1) or as box and whisker plots with a bold red mean line and each data point included (Figs. 2–5). Two-way comparisons were performed by *t*-test with a two-tailed *p*-value, and group comparisons were done using one-way ANOVA followed by the Tukey post-hoc test. All statistical tests were performed in SigmaPlot13.

3. Results

To determine whether anti-epileptic drugs can reduce the seizure phenotype of *ATP6^l* flies, we tested eight drugs of this class, chosen for their clinical relevance and mechanism of action, at multiple concentrations. Six of these eight drugs (carbamazepine, gabapentin, phenytoin, lamotrigine, topiramate, ethosuximide) had no significant effect on seizure phenotype at any tested concentration (Fig. 1B–G). Vigabatrin made seizure recovery significantly worse, even at a low concentration (1H), which may indicate that altering

GABA metabolism has a deleterious effect in ME. In contrast, valproic acid (VPA) confers modest benefit at a high concentration (Fig. 1A).

In contrast to the low efficacy of AEDs, the ketogenic diet is highly effective at reducing seizures in the *ATP6^l* model of ME, improving time to recovery by up to 90% even in late-stage disease [8]. One of the most fundamental mechanistic questions surrounding the seizure-suppressive effects of this dietary treatment is whether they arise from a suppression of glycolysis or the increased availability of fatty acids and ketone bodies; many mechanisms have been implicated downstream of both phenomena at the gene and/or protein level. To begin to address this question in the case of ME, we performed ketone body supplementation, adding beta-hydroxybutyrate and acetoacetate directly to standard sucrose and dextrose-containing fly media. We found that even in the presence of a normal sugar supply, directly providing ketone bodies confers significant benefit to *ATP6^l* (Fig. 2). In contrast, we supplemented standard media with 2-deoxyglucose and iodoacetate, two pharmacological inhibitors of glycolysis, and found that neither treatment changes seizure behavior (Fig. 2).

These results indicate that ketone bodies are conferring anti-seizure properties and that their production may underlie some of the benefit of the ketogenic diet. Suppressive mechanisms involving the direct modulatory action of ketone bodies have been proposed, such as modulation of the ATP-sensitive potassium channel [20], regulation of the mitochondrial permeability transition [21], and influence on the synthesis of GABA [22–24]. These are all intriguing and non-mutually exclusive mechanistic possibilities; however, an additional metabolic role for ketone bodies is their potential for re-conversion to acetyl-coA, a metabolite that enters the citric acid cycle. This replenishment of the citric acid cycle (anaplerosis) is also accomplished as fatty acids are oxidized to acetyl-coA. This led us to ask whether KD support of the citric acid cycle is a significant contributor to the neurological benefits in *ATP6^l*. We reasoned that this was a viable hypothesis especially in an energetically compromised disease state, given that citric acid cycle can produce GTP with each turn that can be converted into ATP by Nucleoside-diphosphate kinases (NDKs). In an animal that derives no energy from OXPHOS, this typically less significant ATP source may have an outsized role.

To test the role of the citric acid cycle in the KD in *ATP6^l*, we expressed RNAi constructs against *citrate synthase* (*csyn*) and *isocitrate dehydrogenase* (*idh*; the rate-limiting enzyme of the citric acid cycle) in the brain of *ATP6^l* flies to determine the effect on seizures and the success of the KD. Expression of *idh* RNAi worsened seizures; expression of both of these citric acid cycle RNAi's prevented the ketogenic diet from working (Fig. 3A). Given these findings, we wanted to test the interaction between *ATP6^l* and *idh* more thoroughly, so we introduced a global genetic disruption construct into the *ATP6^l* genetic background (*ATP6^l-idh-DR*). At every time point tested, the global knockout of *idh* worsened *ATP6^l* seizures and prevented or reduced the benefit of the ketogenic diet (Fig. 3B).

The *Drosophila melanogaster* genetic toolkit contains several models of epilepsy, together known as the “classical bang sensitive mutants.” The etiologies of these models are distinct and result from mutations affecting highly disparate protein functions including

ethanolamine kinase (*easily shocked, eas*), a potassium chloride co-transporter (*ka-zachoc, kcc*), an RNA binding protein (*couch potato, cpo*), and citrate synthase (*knockdown, kdn*). One of these, *eas*, has previously been shown to respond to ketone body supplementation [25]. We treated these four bang sensitive mutants with the ketogenic diet in order to discern whether a seizure phenotype that arises specifically from a citrate synthase mutation is responsive to the KD. We used *eas* as a positive control and the other two mutants (*kcc* and *cpo*) to help us establish the range of possible benefit and give us a sense of the overall applicability of the KD in treating *Drosophila* seizures. As shown in Fig. 3F, *knockdown* was the only classical bang sensitive mutant tested that did not benefit from the KD. Intriguingly, it was also the only bang sensitive mutant that did not survive the duration of the experiment when fed the KD – no flies survived to the day 18 time point, perhaps indicating that the perturbed citric acid cycle in this mutant prevented utilization of the diet as a viable nutrition source. In contrast, survival of the *kdn* flies on regular media was 78%, and survival of the other BS mutants on the KD was > 60% in each case (data not shown).

These results demonstrate that the KD is beneficial to a wide range of *Drosophila* seizure models including those caused by global energetic deficit due to mitochondrial dysfunction, but that perturbing the citric acid cycle renders it ineffective. Having now tested the effect of the KD in the face of mitochondrial and citric acid cycle dysfunction, we next wanted to test the efficacy of the KD on a bang sensitive mutant in which glycolysis is compromised. Triose phosphate isomerase (TPI) is a crucial enzyme of the glycolysis pathway that converts dihydroxyacetone (DHAP) to glyceraldehyde-3-phosphate (G3P). It is thus responsible for the production of glycolytic ATP; without it, DHAP cannot be metabolized and there is no net ATP gained from glycolysis. In humans, mutations in the *TPI* gene cause a glycolytic enzymopathy (GE) called TPI deficiency (Df), which is characterized by a shortened lifespan, hemolytic anemia, and neurological dysfunction. *TPF^{sgk}* is a *Drosophila* model of TPI deficiency caused by a point mutation resulting in a methionine to threonine substitution at position 80 (M80 T). Like *ATP6^l*, *TPF^{sgk}* has a progressive stress-sensitive seizure phenotype, although it is generally less severe than that of *ATP6^l*. We reasoned that while one major result of the KD – reduced glycolysis – is already present in *TPF^{sgk}*, the increased presence of ketone bodies, fatty acids, and citric acid cycle intermediates due to the KD could be hypothesized to provide seizure protection. When we tested the ability of the ketogenic diet to ameliorate these seizures, we found that it is successful throughout the TPI Df disease progression (Fig. 4).

The finding that the KD improves mitochondrially- and glycolytically-derived neurological hyperexcitability, but citric acid cycle dysfunction abolishes these effects, suggests that anaplerosis may be a critically important component underlying KD benefit, and that supporting anaplerosis directly may be a fruitful therapeutic strategy. The lipid triheptanoin, which is comprised of three 7-carbon fatty acids, is highly anaplerotic due to its ease of catabolism to acetyl-CoA and propionyl-coA, which is then metabolized to succinyl-coA. It has been shown to provide anti-seizure benefits to induced seizure models in mice [26]. We tested its ability to reduce seizures in our two models of human metabolic disease, *ATP6^l* and *TPF^{sgk}*. Saturating triheptanoin (35%) mixed into standard fly media improved seizures at every tested time point for *ATP6^l* (Fig. 5A). This benefit was not mimicked by treatment with 35% soybean oil (Fig. 5E). *TPF^{sgk}* (Fig. 5F) also responded robustly to

triheptanoin feeding. Together, these findings have promising implications for its use as a therapy for the neurological consequences of metabolic disease. We next tested triheptanoin in *ATP6^I-Idh*-DR flies, which did not respond to the KD (Fig. 3B), reasoning that since this lipid replenishes the citric acid cycle downstream of *idh*, it might yield success where the KD did not. Intriguingly, this OXPHOS-TCA doubly compromised mutant experienced significant benefit from saturating triheptanoin at every stage except end-stage disease (Fig. 5B). This was a much greater benefit than these mutants received from the KD. In contrast, *ATP6^I* with an intact citric acid cycle and *TPF^{gk}* derive greater benefit from the KD, while triheptanoin treatment is more modestly successful (Fig. 5C, D).

4. Discussion

Metabolic disorders including mitochondrial encephalomyopathies and glycolytic enzymopathies are complex diseases that are incredibly difficult to treat with currently available strategies. Even drug treatments that are typically successful in other disease profiles often fail in the face of a profound bioenergetics defect. Anti-epileptic drugs, for example, are clinically helpful to a wide range of epilepsy patients, but they have a lower success rate among patients of mitochondrial disease, especially as the disease progresses. This lack of sustained success may reflect the atypical seizure etiology due to metabolic perturbation, demonstrating the need for alternate drug targets and therapies. Here we have shown that, like in human ME patients, *ATP6^I* flies do not respond robustly to common AEDs no matter their mechanism of action. One exception is valproate, which is able to confer mild benefit at high dosage. This may be due to its multi-faceted mechanisms of action, which are proposed to include enhancement of GABA transmission and blockade of excitatory ion channels. Additionally, evidence has emerged that this drug provides anti-epileptic benefits at the epigenetic level, including modifying histone deacetylases (HDAC) [27,28]. This suggests epigenetic modifications may be a promising new area of study for refractory seizures such as those of metabolic disease. It is also possible that drug combination therapy would increase the success of the AED profile shown here; however, some studies have reported that use of AEDs including valproate may be contraindicated in mitochondrial disease [29]. Thus, this therapeutic avenue is complex and must be approached with caution.

In contrast, dietary therapy with the KD offers robust protection from seizures in ME model flies [8]. Identifying the mechanism(s) by which the diet is quelling seizures in our model of ME is therefore of high priority. The mechanism by which the ketogenic diet suppresses seizure activity is under intense scrutiny; many key insights have been revealed in the past decade [5,6,30–34]. Physiological changes that mediate its success may stem from either reduced glycolysis or the increased availability of ketones and lipids, and may include ion channel or receptor modulation by ketone bodies, free fatty acids, or redox changes [20,25,35–39]; altered gene expression in oxidative stress pathways and the inflammasome [40–43]; mitochondrial biogenesis [44]; changes in neurotransmitter availability [45]; and even dietary influence on gut microbiota [46]. It is thus increasingly likely that no single mechanism can be pinpointed to explain the totality of seizure suppression by the KD, but rather that it results from the multifaceted outcomes of this profound cellular metabolic change. Nonetheless, elucidating the crucial molecular targets and key cellular effects is

highly desirable, as compliance with the diet is challenging and its long-term effects are unknown, especially in the context of metabolic dysfunction. An intriguing but potentially confounding possibility is that the mediators of KD success in a particular case may be linked to the etiology of the disease – for example, that different effectors downstream of the metabolic switch may be important in the relief of seizures resulting from a channelopathy versus those characterizing a multifaceted metabolic disease.

As described above, the KD induces major changes in cellular energy sources, and one of the most fundamental mechanistic questions is whether the relevant changes in seizure behavior are downstream of the reduction in glucose/glycolysis/glycolytic ATP or the increased production of fatty acids, acetyl CoA, and ketone bodies. We have shown here that supplementing the flies' normal diet with ketone bodies offered substantial seizure protection, especially with beta-hydroxybutyrate, which was more effective than acetoacetate in advanced disease. This discrepancy may be due to the use of the lithium salt of acetoacetate in our experiments, which could have a neuronal depolarizing effect offsetting the calming effects of the ketone body itself. Future studies utilizing alternate forms of acetoacetate could be designed to test this hypothesis. Nonetheless, the effectiveness of supplementing media with these KBs suggests that ketone bodies themselves are necessary and sufficient for at least some of the benefit derived from the KD, even in the presence of normal levels of dietary sugars. This finding has exciting implications for patients of metabolic disease given promising recent results of ketone ester administration in animal and clinical studies. Oral administration of these compounds, including 1,3-butanediol acetoacetate diester and glyceryl-tris-3-hydroxybutyrate, have been found to increase human plasma ketone body levels to those comparable to a stringent ketogenic diet [47]. In a mouse model of Angelman syndrome, treatment with ketone esters improved motor function and memory, and reduced convulsions [48]. Other studies suggest ketone esters could be effective in treating neurodegenerative disease and obesity [47,49]. In mechanistic terms, our previous report that K_{ATP} channels are crucial to KD success presents the intriguing possibility that ketone bodies could be conferring their benefit via this channel, a finding that agrees with previously published results in mouse slice physiology [36]. Future experiments will be designed to test the mechanistic possibilities of KB modulation in *ATP6^d*.

Blocking glycolysis pharmacologically has been shown previously to induce ketogenesis and slow epilepsy progression [50,51]. However, treating *ATP6^d* flies with glycolytic inhibitors 2-DG or IAA in our studies did not mimic the seizure protection of the KD. This is somewhat surprising given that we have previously demonstrated that reduction of sugar in the flies' media confers seizure amelioration [8]. However, there are several complicating factors with these experiments that should be noted. The first is the possible multiplicity of the effects of iodoacetic acid, which is often used as a GAPDH inhibitor but can also alter glutathione levels when present at millimolar concentrations [52]. Since it is difficult to know with certainty the final concentration of IAA in the fly after ingestion, it cannot be ruled out that redox perturbations confound any positive effects due to reduced glycolysis. It is also true that *ATP6^d* upregulates glycolysis as a compensatory mechanism [14], and it is reasonable to conclude that eliminating it entirely without an alternate source of energy outweighs any benefits that may be derived. Furthermore, blocking the progression of

glycolysis has dramatic implications for pathways downstream, including the citric acid cycle, which would suffer a lack of pyruvate and reducing equivalents when glycolysis is pharmacologically compromised. Thus, we cannot rule out the possibility that a reduction in glycolysis or glycolytically-derived ATP is contributing some seizure protective benefit, but only when paired with high lipid availability. One mechanistic aspect worth considering is that local pools of glycolytic ATP are hypothesized to be critical for certain physiological functions, including axonal transport [53], synaptic function [54–56], and channels and transporters in the plasma membrane that complex with glycolytic enzymes, including the K_{ATP} channel [57,58]. The pathophysiological contribution of glycolytically derived ATP in *ATP6^l* warrants further study.

Anaplerosis has been suggested as a contributing mechanism of the ketogenic diet [34]. While the citric acid cycle is typically and correctly thought of as a source of key metabolites for downstream pathways and reducing equivalents for the electron transport chain, it is often overlooked as a direct source of energy production: the synthesis of GTP by succinyl-coA synthetase, which can then be converted to ATP by nucleoside diphosphate kinase. Furthermore, as seizures are highly metabolically demanding themselves, TCA intermediates and the metabolites derived from them (including neurotransmitters) can be depleted, causing further physiological stress and imbalance [59]. The ketogenic diet supplies acetyl-coA via fatty acid oxidation; *ATP6^l*'s ability to benefit from this diet and from ketone bodies that can be processed to produce acetyl-coA suggests its anaplerotic properties may be therapeutically relevant in this model. Indeed, we show that interfering with citrate synthase or isocitrate dehydrogenase precludes KD benefit in the *ATP6^l* background. The failure of the classical bang sensitive mutant *kdn*, a citrate synthase mutant, to respond to or indeed survive on the KD suggests this dependence upon the citric acid cycle is not unique to *ATP6^l*. Metabolomics studies analyzing the intermediates of the citric acid cycle throughout ME progression can shed light on whether perturbation of this cycle contributes to pathophysiology.

In light of these findings, we supplemented the flies' diet with triheptanoin, a highly anaplerotic lipid that has been shown to suppress seizures in mouse induced-seizure models [26,60] and in human patients with glucose transporter type I deficiency [61]. For *ATP6^l*, saturating triheptanoin offered modest to moderate benefit at multiple time points in disease progression. At each time point, saturating triheptanoin was not as effective as the ketogenic diet in reducing seizure severity. This indicates that while anaplerosis might be a relevant component of the ketogenic diet, it is unlikely to serve as a replacement for the KD. Future metabolomics studies can be used to determine which citric acid cycle intermediates, if any, are bolstered by triheptanoin supplementation in *ATP6^l*. Intriguingly, although *ATP6^l* with genetically disrupted isocitrate dehydrogenase (*ATP6^l-Idh-DR*) did not respond robustly to the KD, it derived significant benefit from triheptanoin supplementation. This could be due to triheptanoin's direct replenishment of succinyl-CoA, which is an intermediate downstream of idh.

Finally, our results have highlighted the potential of dietary therapy as a treatment for the neurological aspects of a second devastating, incurable human metabolic disease. TPI deficiency is characterized by the inability to convert DHAP to G3P, cutting production of

pyruvate and NADH in half while eliminating the net gain of ATP from glycolysis. By providing *TPF^{sgk}* flies with anaplerotic supplements such as the KD or triheptanoin, we may be effectively circumventing the defective glycolysis pathway and directly providing intermediates to the citric acid cycle, increasing its ability to produce energy, metabolites, and reducing equivalents. While KD or triheptanoin treatment were efficacious in the *Drosophila* TPI Df model improving neurologic function, such treatments would be predicted to increase the anemia and could lead to hemolytic crisis in humans with TPI Df, and thus must be considered with caution.

5. Conclusions

We have shown that the success of the KD in a model of mitochondrial encephalomyopathy is dependent upon the presence of ketone bodies and the correct functioning of the citric acid cycle, and that supporting it via anaplerotic supplementation is a promising therapeutic avenue. We also show that the ketogenic diet is successful for multiple *Drosophila* epilepsy and metabolic disease models, including the human glycolytic enzymopathy model *TPF^{sgk}*. Overall, these findings highlight several promising therapeutic avenues for the neurological consequences of metabolic diseases and further establish *Drosophila* as an excellent model for the study of dietary therapy against seizure pathophysiology.

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Fig. 1.

Anti-epileptic drugs have limited benefit against the seizure of the *ATP6^l* model of mitochondrial encephalomyopathy. A) Valproate treatment provides modest benefit as measured by time to recovery at high concentration (1 mM) at day 15 ($p = .003$) and at day 18 ($p = .0002$) vs. vehicle, but not at a lower concentration (0.2 mM). B-G) Phenytoin (PHNT), carbamazepine (CMZ), topiramate (TPR), lamotrigine (LMT), gabapentin (GBP), and ethosuximide (ETH) have no effect on seizure recovery at any tested concentration. H) Treatment with vigabatrin exacerbates seizure phenotype at 0.25 mM ($p = .049$) and 1.0 mM ($p = .031$). All flies tested were *ATP6^l*. All bars represent at least $n = 20$ flies.

Fig. 2.

Supplementation of ketone bodies in standard media provides seizure protection to *ATP6^l*. Flies whose diets were supplemented with 5 mM of either beta hydroxybutyrate (BHB) or lithium acetoacetate (AcAc) showed significant benefit in the time to recovery parameter: $p < .0001$ vs. standard media control (35.3 ± 2.8 , $n = 178$ for day 15 and 82.9 ± 2.5 , $n = 177$ for day 18); mean for BHB at day 15 (3.5 ± 0.7 , $n = 61$) and day 18 (24.0 ± 3.6 , $n = 60$) and for AcAc at day 15 (8.04 ± 1.7 , $n = 58$) and day 18 (53.9 ± 5.1 , $n = 57$). In contrast, treatment with glycolysis blocker 2-deoxyglucose (2DG, 2.0 mM; 27.4 ± 4 , $n = 71$ at day 15 and 87.2 ± 2.9 , $n = 35$ at day 18) or iodoacetate (IAA, 1.0 mM; 41.5 ± 5.3 , $n = 44$ at day 15 and 64.6 ± 6.3 , $n = 44$ at day 18) for the same length of time did not provide seizure relief ($p = .32, 0.46, 0.39, 0.25$). Flies were *ATP6^l*[1]. Bold red line indicates mean. All data points included in the mean are depicted.

Fig. 3.

Perturbation of the citric acid cycle renders the ketogenic diet inefficient in *ATP6^l* and the classical bang sensitive mutant *knockdown*. A) *ATP6^l* with RNAi knockdown of citrate synthase and isocitrate dehydrogenase on standard media vs. 3:1 ketogenic media. The ketogenic diet reduces TTR significantly for *ATP6^l* flies (26.9 ± 0.5 standard vs. 2.63 ± 0.5 KD, $p < .0001$), but fails to do so for *ATP6^l; w; elav-GAL4/+; UAS-RNAi/+* (27.01 ± 4.2 vs. 25.0 ± 6.2 $p = .79$ for citrate synthase and 54.1 ± 6 vs. 51.4 ± 7.3, $p = .78$ for isocitrate dehydrogenase RNAi, respectively). *Idh* RNAi knockdown worsens the phenotype significantly ($p < .0001$) vs. standard media control. B) Introducing a global genetic disruption (*ATP6^l; Idh-DR/Idh-DR*) of the isocitrate dehydrogenase gene leads to worsened seizure phenotype relative to control at day 12 (20.7 ± 4 $p < .0001$), day 15 (30.6 ± 4.2, $p = .0002$), and day 18 (76.0 ± 8.1, $p < .0001$). Treatment of the ketogenic diet does not provide benefit relative to the regular media *idh* phenotype at day 12 (22.5 ± 6.7, $p = .$

86), day 15 (23.2 ± 9.6 , $p = .28$) day 18 (62.1 ± 12.7 , $p = .44$), or day 21 (107.4 ± 30.7 , $p = .71$). CC–F) Classical bang sensitive fly mutants were provided with the KD and tested for seizure phenotype as quantified by seizures per fly at days 7, 12, and 18. At day 7, *eas* (1.07 ± 0.08 regular vs. 0.4 ± 0.001 KD, $p < .0001$), *cpo* (2.06 ± 0.12 regular vs. 1.3 ± 0.13 KD, $p = .0002$), and *kcc* (1.26 ± 0.1 regular vs. 0.9 ± 0.3 KD, $p = .046$) benefitted from the KD, while *kdn* (2.81 ± 0.2 regular vs. 2.6 ± 0.16 KD $p = .6$) did not. At day 12, *eas* (1.58 ± 0.14 regular vs. 0.58 ± 0.08 KD, $p < .0001$), *cpo* (2.01 ± 0.19 regular vs. 1.45 ± 0.04 KD, $p = .0002$), and *kcc* (1.35 ± 0.04 regular vs. 0.83 ± 0.02 KD, $p = .046$) benefitted from the KD, while *kdn* (2.72 ± 0.09 regular vs. 3.02 ± 0.34 KD $p = .6$) did not. At day 18, *eas* (1.61 ± 0.2 regular vs. 0.82 ± 0.04 KD, $p < .0001$), and *kcc* (1.66 ± 0.06 regular vs. 1.27 ± 0.03 KD, $p = .046$) benefitted from the KD, while *cpo* no longer responded (1.63 ± 0.1 regular vs. 1.36 ± 0.1 KD, $p = .0002$), and *kdn* flies on KD did not survive. All plots represent at least 15 flies. In C-F, each point represents a vial of 3–8 flies.

Fig. 4.

The ketogenic diet improves seizure phenotype of *TPF^{sugarkill}*, a model of human glycolytic enzymopathy Triose Phosphate Isomerase (TPI) Deficiency. *TPF^{sugarkill}* exhibits progressively worsening seizures, the severity of which are quantified by time to recovery (TTR) after mechanical overstimulation. At each time point tested, chronic treatment with a 3:1 ketogenic diet kept time to recovery at disease onset levels, providing significant benefit at day 15 (15.5 ± 1.9 regular vs. 8.65 ± 1.5 KD, $p = .01$), day 18 (19.9 ± 3.9 regular vs. 9.5 ± 1.8 KD, $p = .009$), and day 21 (29.9 ± 6.3 regular vs. 11.5 ± 2.7 KD, $p = .003$). Flies were w¹¹¹⁸; GE-M80 T. $n > 40$ for each condition presented.

Fig. 5.

Dietary supplementation with triheptanoin improves seizures in mitochondrial, citric acid cycle, and glycolytic mutants. A) The progressive seizures exhibited by *ATP6^l* are improved by treatment with saturating (35% by mass) triheptanoin (6.5 ± 0.6 regular vs. 5.6 ± 1.2 THP, $p = .23$ at day 12; 16.0 ± 1.7 regular vs. 5.6 ± 1.0 THP, $p < .0001$ at day 15; 42.9 ± 3.7 regular vs. 26.7 ± 4.3 THP, $p = .01$ at day 18; and 85.7 ± 4 regular vs. 67.5 ± 6.8 THP, $p < .0001$ at day 21). B) When global genetic disruption of isocitrate dehydrogenase is introduced into the background of *ATP6^l* (*ATP6^l; w⁻; Idh-DR/Idh-DR*), triheptanoin is still seizure-reducing (20.72 ± 4.0 regular vs. 9.1 ± 2 THP, $p = .0001$ at day 12, 30.6 ± 4.2 regular vs. 15.1 ± 2.7 THP, $p < 0.001$ at day 15, 76.0 ± 8.1 regular vs. 37.6 ± 2.7 THP, $p = .05$ at day 18, and 114.3 ± 14 regular vs. 85.8 ± 28 THP, $p = .018$ at day 21). C,D) Comparison of the effects of the ketogenic diet vs. triheptanoin supplementation for *ATP6^l* and *ATP6^l-Idh-DR*. *ATP6^l* alone sustains dramatic benefit from the KD, which is more

modestly mimicked by THP. In contrast, *ATP6^l-Idh-DR* is helped only modestly by the KD while sustaining 50–100% more benefit from THP. E) Treatment with soybean oil (SBO) does not rescue seizures of *ATP6^l*. 46.1 ± 4.0 regular vs. 56.9 ± 21 SBO, $p = .0001$ at day 12, 58.3 ± 6.5 regular vs. 49.5 ± 13.5 SBO, $p = . < 0.001$ at day 15, 87.5 ± 8.3 regular vs. 88.1 ± 19 SBO, $p = .0.05$ at day 18, and 106.5 ± 9 regular vs. 100.3 ± 51 SBO, $p = .0.18$ at day 21). F) *TPF^{sgk}* (M80 T) benefits from treatment with saturating THP (11.6 ± 1.6 regular vs. 6.8 ± 1.1 THP, $p = .041$ at day 12, 15.5 ± 1.9 regular vs. 7.2 ± 2.1 THP, $p = .001$ at day 15, 19.9 ± 3.9 regular vs. 12.7 ± 6.6 THP, $p = .032$ at day 18, and 29.9 ± 6.3 regular vs. 19.9 ± 8.5 THP, $p = .25$ at day 21). N values are > 20 for A-D and F and > 10 for E.