

RESEARCH ARTICLE

Ketogenic diet reduces early mortality following traumatic brain injury in *Drosophila* via the PPAR γ ortholog Eip75B

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Abstract

Traumatic brain injury (TBI) is a common neurological disorder whose outcomes vary widely depending on a variety of environmental factors, including diet. Using a *Drosophila melanogaster* TBI model that reproduces key aspects of TBI in humans, we previously found that the diet consumed immediately following a primary brain injury has a substantial effect on the incidence of mortality within 24 h (early mortality). Flies that receive equivalent primary injuries have a higher incidence of early mortality when fed high-carbohydrate diets versus water. Here, we report that flies fed high-fat ketogenic diet (KD) following TBI exhibited early mortality that was equivalent to that of flies fed water and that flies protected from early mortality by KD continued to show survival benefits weeks later. KD also has beneficial effects in mammalian TBI models, indicating that the mechanism of action of KD is evolutionarily conserved. To probe the mechanism, we examined the effect of KD in flies mutant for Eip75B, an ortholog of the transcription factor PPAR γ (peroxisome proliferator-activated receptor gamma) that contributes to the mechanism of action of KD and has neuroprotective effects in mammalian TBI models. We found that the incidence of early mortality of Eip75B mutant flies was higher when they were fed KD than when they were fed water following TBI. These data indicate that Eip75B/PPAR γ is necessary for the beneficial effects of KD following TBI. In summary, this work provides the first evidence that KD activates PPAR γ to reduce deleterious outcomes of TBI and it demonstrates the utility of the fly TBI model for dissecting molecular pathways that contribute to heterogeneity in TBI outcomes.

Introduction

Traumatic brain injury (TBI) is a major health issue worldwide [1]. It is a leading cause of disability and death, and its clinical management is challenging because the physical, behavioral, cognitive, and emotional sequelae are highly variable. Variation in sequelae among TBI patients stems from heterogeneity of primary injuries to the brain as well as heterogeneity of

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genetic and environmental factors such as physical activity, sleep, and diet that promote tissue repair or exacerbate tissue damage through secondary injury mechanisms [2, 3]. Cellular and molecular mechanisms associated with secondary injuries include ionic imbalance, excitotoxicity, oxidative stress, inflammation, and mitochondrial dysfunction that disrupt cellular metabolism leading to neuronal dysfunction and cell death [4].

Glucose is the main energy source for the brain, but following TBI, glucose uptake and use by the brain is progressively reduced [5, 6]. Under these circumstances, ketone bodies, derived from fatty acid oxidation in the liver, become the main energy source for the brain [7, 8]. Ketone bodies such as β -hydroxybutyrate, acetone, and acetoacetate improve mitochondrial metabolism, reduce production of reactive oxygen species and proinflammatory proteins, and have broad neuroprotective effects [8, 9]. Elevated levels of ketone bodies in the blood, a state known as ketosis, can be induced by fasting and by high-fat, low-carbohydrate, low-protein ketogenic diet (KD). KD reduces seizures in refractory childhood epilepsy and ameliorates detrimental outcomes in mammalian models of neurological disorders, including TBI [10, 11]. In rat TBI models, KD reduces apoptosis, contusion volumes, and anxiety- and depressive-like behaviors and improves motor and cognitive performance [12–18]. However, much remains to be learned about the influence of KD in TBI, including the extent to which genetic background modulates its beneficial effects.

The ligand-dependent transcription factor PPAR γ (peroxisome proliferator-activated receptor gamma) contributes to the mechanism of action of KD and has anti-inflammatory and neuroprotective effects in mammalian models of neurological disorders, including TBI [19, 20]. Activation of PPAR γ by fatty acids inhibits inflammation by a variety of mechanisms, including by reducing the activity of nuclear factor-kappa B (NF- κ B) transcription factors that promote expression of inflammatory genes encoding cytokines, chemokines, and adhesion molecules [21]. In rodent TBI models, the PPAR γ agonist pioglitazone is protective against mitochondrial dysfunction, cognitive impairment, cortical tissue loss, inflammation, dendritic morphological changes, and long-term memory loss [22–25]. However, it is not yet known if PPAR γ mediates the beneficial effects of KD in TBI.

To investigate the role of genetic and environmental factors in TBI outcomes, we developed a *Drosophila melanogaster* model of TBI [26, 27]. The fly TBI model uses a High-Impact Trauma (HIT) device to deliver blunt force injuries to the head and body of unanesthetized flies. Behavioral outcomes of TBI shared between flies and humans include temporary incapacitation, ataxia, abnormal sleep, early mortality, and reduced lifespan [26–31]. Cellular and molecular outcomes are also shared, including progressive neurodegeneration, disruption of the blood-brain barrier and the intestinal barrier, transient hyperglycemia, and prolonged activation of innate immune response pathways [26, 28, 29, 32, 33]. Using the Mortality Index at 24 hours (MI₂₄)—the normalized percent of flies that die within 24 h after strikes from the HIT device—as a readout, we previously found that genetic background plays a substantial role in determining TBI outcomes. For example, the MI₂₄ of flies injured at 0–7 days old varies from 7 to 58 among 179 inbred lines in the *Drosophila* Genetic Reference Panel (DGRP) [28, 34]. Additionally, the MI₂₄ is reduced by heterozygosity for a mutation of the NF- κ B innate immune response transcription factor Relish [33]. Age and diet also play substantial roles in determining outcomes of TBI in flies. The MI₂₄ of flies injured at a younger age is lower than at an older age, and the MI₂₄ is lower for flies fed water versus high-carbohydrate diets during the 24 h following primary injuries [28, 29]. Furthermore, using the HIT device, Lee et al. (2019) demonstrated that β -hydroxybutyrate, a metabolite of KD, reduces TBI-induced aggression in flies [35]. Thus, to further explore the utility of the fly TBI model, we examined the effect of KD on the MI₂₄ and lifespan following TBI. We found that, relative to high-carbohydrate diets, high-fat KD reduced the MI₂₄ and increased lifespan following TBI and that

Eip75B, an ortholog of PPAR γ , was necessary to mediate the beneficial effect of KD on the MI₂₄.

Materials and methods

Fly lines and culturing

Flies were maintained in humidified incubators at 25°C on solid CMYD. DGRP lines and *Eip75B* mutant fly lines were obtained from the Bloomington Stock Center (Indiana University).

Diets

Solid CMYD contains 30 g Difco granulated agar (Becton-Dickinson, Sparks, MD), 44 g YSC-1 yeast (Sigma, St. Louis, MO), 328 g cornmeal (Lab Scientific, Highlands, NJ), 400 ml unsulfured Grandma's molasses (Lab Scientific), 3.6 L water, 40 ml propionic acid (Sigma), and tegosept (8 g Methyl 4-hydroxybenzoate in 75 ml of 95% ethanol) (Sigma). YD contains YSC-1 yeast (Sigma) in water. KD is commercial mouse Teklad ketogenic diet (TD.96355) (Envigo) that contains 173.3 g/Kg casein, 2.6 g/Kg DL-methionine, 586.4 g/Kg vegetable shortening (Crisco), 86.2 g/Kg corn oil, 88.0 g/Kg cellulose, 13.0 g/Kg vitamin mix (Teklad 40060), 2.5 g/Kg choline bitartrate, 0.1 g/Kg tertiary butylhydroquinone (TBHQ), 20.0 g/Kg mineral mix (calcium phosphate deficient), 19.3 g/Kg dibasic calcium phosphate, 8.2 g/Kg calcium carbonate, and 0.4 g/Kg magnesium oxide. KD at 0.3 cal/200 μ l was prepared by adding 1.1 g of Teklad ketogenic diet to 5 ml of water and stirring the solution for 1 min at about 95°C. [Table 1](#) provides the caloric contribution of carbohydrate, protein, and fat for each diet as well as the amount of each diet used to make 0.3 cal/200 μ l solutions. Flies were fed water and diluted diets by placing 200 μ l on a filter paper disc at the bottom of a vial.

MI₂₄ and lifespan assays

Flies were injured using a HIT device as described by Katzenberger et al. [26, 27]. Vials containing 60 flies at 0–7 days old were injured by 4 strikes at 5 min intervals with the spring deflected to 90°. Vials with mixed sex flies had approximately 30 males and 30 females. The Mortality Index at 24 h (MI₂₄) was calculated by subtracting the percent of uninjured flies that died from the percent of injured flies that died during the 24 h following TBI. The lifespan of adult flies that survived 24 h following TBI flies was determined using vials with 20 flies each. The number of surviving flies was counted daily until all flies had died. Flies were transferred to new vials approximately every 3 days. Flies were considered dead if they did not show obvious locomotor activity. Statistical analysis of survival by the Kaplan-Meier Fisher's Exact Test was performed using OASIS 2 (Online Application for Survival Analysis 2) [36].

Table 1. Caloric content of diets used in Figs 1–4.

Diet	Percent calories from:			cal/g	0.3 cal/200 μ l g diet/ml water
	Protein	Carbohydrate	Fat		
KD	9.2	0.3	90.5	6.70	0.22
CMYD	4.9	92.7	2.4	3.31	0.45
YD	41.0	42.0	17.0	3.25	0.46
Glucose	0	100.0	0	3.74	0.40
Sucrose	0	100.0	0	3.94	0.38

KD, ketogenic diet; CMYD, cornmeal-molasses-yeast diet; YD, yeast diet.

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Results

Ketogenic diet following TBI reduces the incidence of early mortality

We previously found that the diet consumed directly after TBI in *Drosophila* substantially affects the incidence of early mortality [28, 29]. Flies fed cornmeal-molasses-yeast diet (a standard laboratory fly diet) or simple carbohydrates (i.e., sucrose, glucose, and fructose) during the 24 h following TBI have a significantly higher MI₂₄ than flies fed water. To further explore the effect of diet on the MI₂₄, we examined different concentrations of cornmeal-molasses-yeast diet (CMYD), yeast diet (YD, *S. cerevisiae*), and ketogenic diet (KD, a commercial mouse ketogenic diet). Based on caloric content, CMYD is high in carbohydrate and low in protein and fat, YD is high in carbohydrate and protein and low in fat, and KD is high in fat and low in carbohydrate and protein (Table 1). Diets were dissolved in water at 0.5 g/ml, serially diluted by 2-fold increments in water down to 0.0625 g/ml, and 200 μ l was absorbed onto a filter paper disc that was placed at the bottom of a vial. 0–7 day old, mixed sex *w*¹¹¹⁸ flies cultured on solid CMYD (i.e., undiluted CMYD), were subjected to four strikes from the HIT device with 5 min between strikes and transferred to vials with diets at different concentrations.

We found that the MI₂₄ increased to a similar extent with increasing concentrations of CMYD and YD (Fig 1A). In contrast, the MI₂₄ was not affected by increasing concentrations of KD. We also examined the effect of CMYD, YD, and KD as well as glucose and sucrose at approximately the same caloric content (0.3 cal/200 μ l) (Table 1). MI₂₄ values were similar for flies fed CMYD, YD, glucose, or sucrose and they were significantly higher than the MI₂₄ of flies fed water (Fig 1B). In contrast, the MI₂₄ of flies fed KD was the same as that of flies fed water. These data support our prior finding that ingestion of carbohydrate after TBI increases the MI₂₄ and demonstrate that ingestion of fat after TBI does not increase the MI₂₄ compared with water.

An alternative interpretation of the data in Fig 1A and 1B is that flies did not consume KD, suggesting that starvation and water have equivalent effects on the MI₂₄. To test this possibility, we determined the MI₂₄ of 0–7 day old, mixed sex *w*¹¹¹⁸ flies that were starved by placing them in vials with a dry filter paper disc following TBI. In contrast with flies fed KD, the MI₂₄ of starved flies was substantially higher than that of flies fed water (Fig 1C), demonstrating that consuming KD rather than starvation was beneficial. As an additional approach to test if flies consumed KD, we examined the lifespan of uninjured, mixed sex *w*¹¹¹⁸ flies cultured on solid CMYD to 0–7 days old and thereafter on water or 0.3 cal/200 μ l CMYD or KD. The median and maximum lifespans of flies cultured on KD (14.7 \pm 1.1 days and 37 days, respectively) were longer than those of flies cultured on water (10.6 \pm 0.1 days and 13 days, respectively) and shorter than those of flies cultured on CMYD (23.4 \pm 0.6 days and 74 days, respectively), indicating that flies examined in Fig 1A and 1B consumed KD (Fig 1D). Further support for this conclusion is provided in Fig 4B.

Ketogenic diet is similarly beneficial following TBI in females versus males and in different genetic backgrounds

To investigate whether KD has sex-specific effects on TBI outcomes, we compared effects of KD, water, and solid CMYD on the MI₂₄ of 0–7 day old female, male, and mixed sex *w*¹¹¹⁸ flies. In each case, solid CMYD resulted in a significantly higher MI₂₄ than both water and KD, and water and KD had equivalent MI₂₄ values (Fig 2A). Moreover, comparisons of male, female, and mixed sex flies, revealed that KD as well as solid CMYD and water had similar effects on the MI₂₄. Therefore, sex does not alter the effects of KD, water, and solid CMYD on secondary injury mechanisms that cause early mortality following TBI.

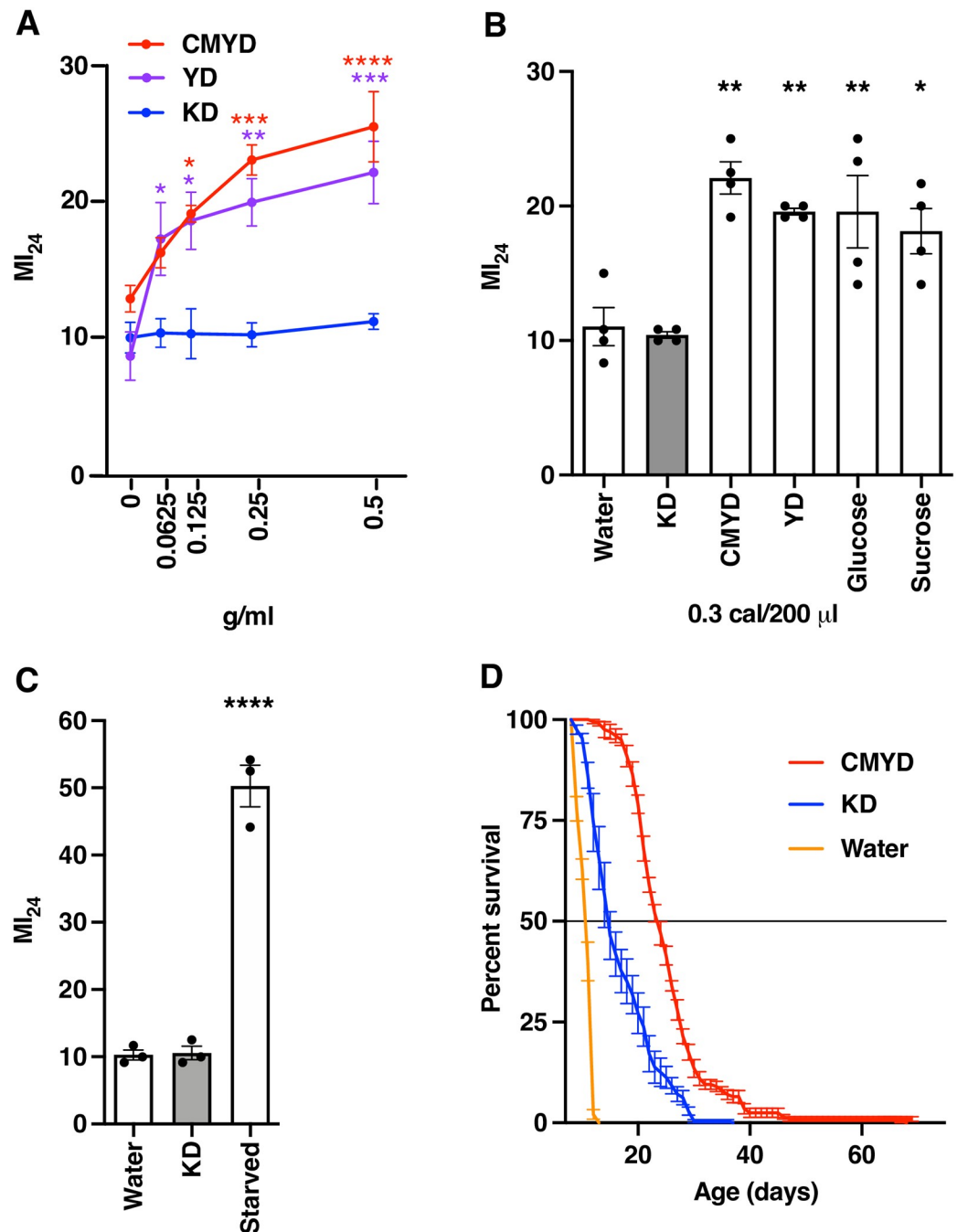


Fig 1. Analysis of the effect of CMYD, YD, and KD on the MI₂₄. (A) Dose-response analysis of the effect of CMYD, YD, and KD on the MI₂₄. The MI₂₄ represents the percent mortality of injured flies minus the percent mortality of uninjured flies 24 h following TBI. MI₂₄ values were determined for 0–7 day old, mixed sex *w¹¹¹⁸* flies fed CMYD, YD, or KD at the indicated concentrations following TBI. At least three biological replicates of 60 flies were tested for each condition. Dots indicate the average MI₂₄, and error bars indicate the standard error of the mean (SEM). (B) MI₂₄ values were determined for 0–7 day old, mixed sex *w¹¹¹⁸* flies fed water or CMYD, YD, KD, glucose, or sucrose at 0.3 cal/200 µl following TBI. Dots indicate biological replicates of 60 flies, bars indicate averages, and error bars indicate the SEM. (C) MI₂₄ values were determined for 0–7 day old, mixed sex *w¹¹¹⁸* flies fed water, 0.3 cal/200 µl KD, or no food or water (starved) following TBI. Dots indicate biological replicates of 60 flies, bars indicate averages, and error bars indicate the SEM. (D) Percent survival was determined for uninjured 0–7 day old, mixed sex *w¹¹¹⁸* flies fed water (*n* = 240) or 0.3 cal/200 µl CMYD (*n* = 200) or KD (*n* = 239) over the course of the experiment. Error bars indicate the SEM, and the horizontal line at 50% indicates the median lifespan. Significance for panels A, B, and C was determined using ordinary one-way ANOVA with Dunnett's Multiple Comparison test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

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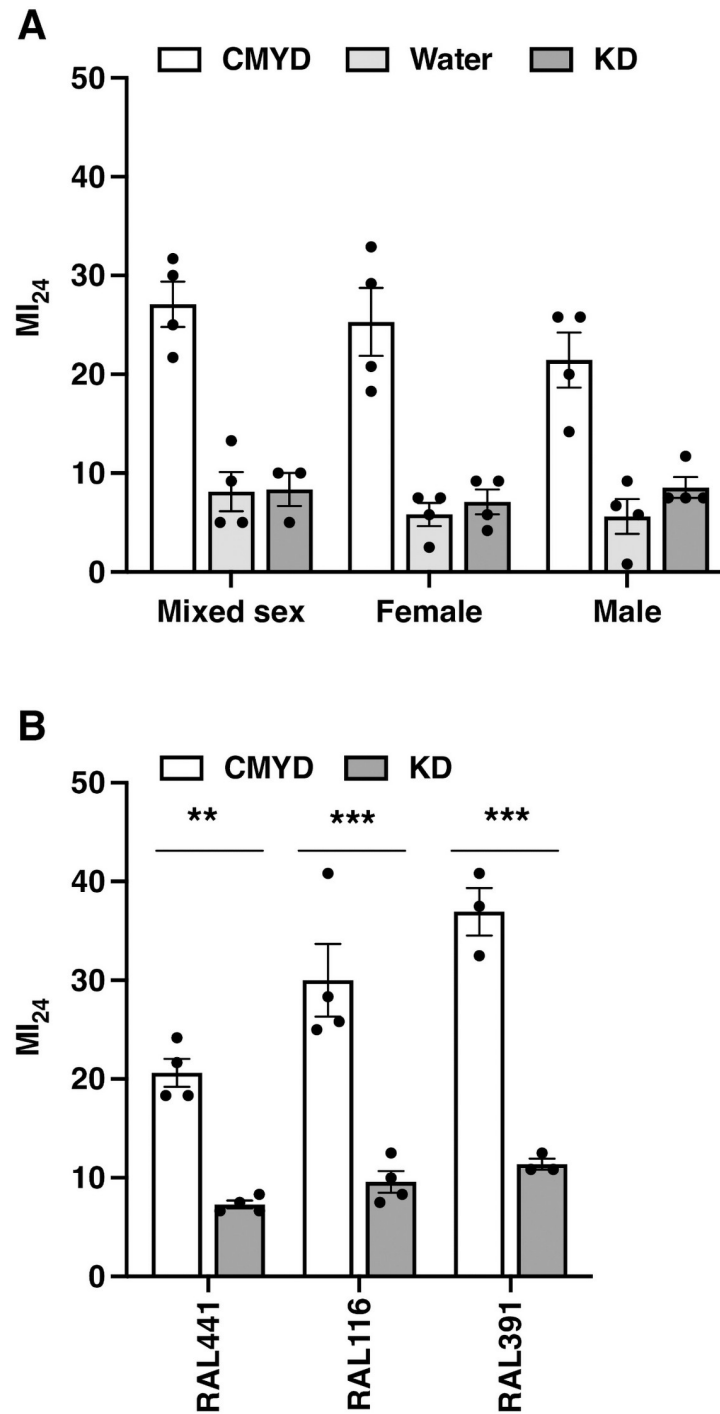


Fig 2. The beneficial effect of KD on early mortality after TBI is equivalent in females and males and is conserved in different genetic backgrounds. (A) MI₂₄ values were determined for 0–7 day old female, male, and mixed sex *w¹¹¹⁸* flies fed solid CMYD, water, or 0.3 cal/200 μ l KD. The MI₂₄ represents the percent mortality of injured flies minus the percent mortality of uninjured flies 24 h following TBI. (B) MI₂₄ values were determined for 0–7 day old, mixed sex fly lines (RAL441, RAL116, and RAL391) from the DGRP fed 0.3 cal/200 μ l CMYD or KD following TBI [34]. Dots indicate biological replicates of 60 flies, bars indicate averages, and error bars indicate the SEM. Significance was determined using ordinary one-way ANOVA with Dunnett's Multiple Comparison test. ** $p < 0.01$ and *** $p < 0.001$.

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We previously found that the MI₂₄ of flies fed solid CMYD varied significantly among fly lines with different genetic backgrounds, including inbred fly lines from the *Drosophila* Genetic Reference Panel (DGRP) [26, 28, 29, 34]. To determine the extent to which genetic background affects the MI₂₄ of flies fed KD following TBI, we examined three lines from the DGRP that have different MI₂₄ values when fed solid CMYD following TBI [28, 29]. We fed 0–7 day old, mixed sex flies 0.3 cal/200 μ l KD or CMYD for 24 h following TBI and determined the MI₂₄ of each line. For all three DGRP lines, the MI₂₄ of flies fed KD was lower than the MI₂₄ of flies fed CMYD (Fig 2B). Moreover, the *w¹¹¹⁸* line and the DGRP lines fed KD had comparable MI₂₄ values, whereas these values varied among the same fly lines when fed CMYD (Figs 1B and 2B). These results indicate that the beneficial effect of KD on the MI₂₄ does not depend on the starting value of the MI₂₄ (on CMYD) in different fly lines, leading to uniformly low MI₂₄ values for flies fed KD. However, it does appear that the beneficial effect of KD has a limiting threshold beyond which it cannot act further, resulting in a proportionally greater rescuing effect for lines with higher MI₂₄ values on CMYD.

Ketogenic diet following TBI has beneficial long-term effects on lifespan

For both humans and flies, individuals that survive TBI manifest a variety of long-term consequences, including reduced lifespan, as a result of secondary injuries triggered by primary injuries to the brain. The exact connection between primary injuries and secondary injuries is complex, and the details are still poorly understood. The fact that the MI₂₄ is reduced in flies fed KD immediately after TBI, raises the question of whether the beneficial effects of KD extend to longer-term pathological consequences of TBI. We examined this possibility using lifespan as a readout. Lifespan was determined for 0–7 day old, mixed sex *w¹¹¹⁸* flies fed 0.3 cal/200 μ l KD or CMYD for 24 h following TBI with surviving flies subsequently cultured on solid CMYD. As we reported previously, flies fed CMYD for 24 h after injury had a reduced lifespan relative to uninjured controls (Kaplan-Meier Fisher's Exact Test, $p = 4.1 \times 10^{-9}$ at 50%) (Fig 3) [26, 29]. The same was true for flies fed KD (Kaplan-Meier Fisher's Exact Test, $p = 1.7 \times 10^{-7}$ at 50%). However, notably, injured flies fed KD rather than CMYD for 24 h after injury had a significantly longer median lifespan (40.3 ± 0.2 days vs. 37.8 ± 0.28 days, Kaplan-Meier Fisher's Exact Test, $p = 1.0 \times 10^{-4}$ at 50%). Moreover, the difference in median lifespan between injured flies and uninjured controls was much narrower for KD-fed than for CMYD-fed flies (40.3 ± 0.2 days vs. 44.2 ± 0.8 days for KD; 37.8 ± 0.28 days vs. 48.2 ± 1.0 days for CMYD). Thus, flies that avoid early mortality following TBI because of the beneficial effects of KD during a 24 h window after primary injuries continue to manifest long-term benefits of this diet weeks later.

Beneficial effects of KD on early mortality are mediated by the PPAR γ ortholog Eip75B

In mammals, the mechanism of action of KD is mediated by the transcription factor PPAR γ , which has neuroprotective effects in a number of progressive neurological disorders, including TBI [19, 20]. Because KD exerts a protective effect following TBI in flies as well as mammals, we hypothesized that the underlying mechanism is conserved as well. If so, the protective effect in flies should depend on the transcription factor Eip75B (ecdysone-induced protein 75B), the *Drosophila* ortholog of PPAR γ . The orthologous relationship is inferred both from amino acid sequence identity (i.e., Eip75B is the most significant match to human PPAR γ in a BLAST search of the *Drosophila* proteome) and from common activation by the PPAR γ agonist pioglitazone [37–39]. Under this hypothesis, mutational loss of *Eip75B* should result in loss of the beneficial effect of KD. Thus, we examined the effect of water and 0.3 cal/200 μ l KD on the

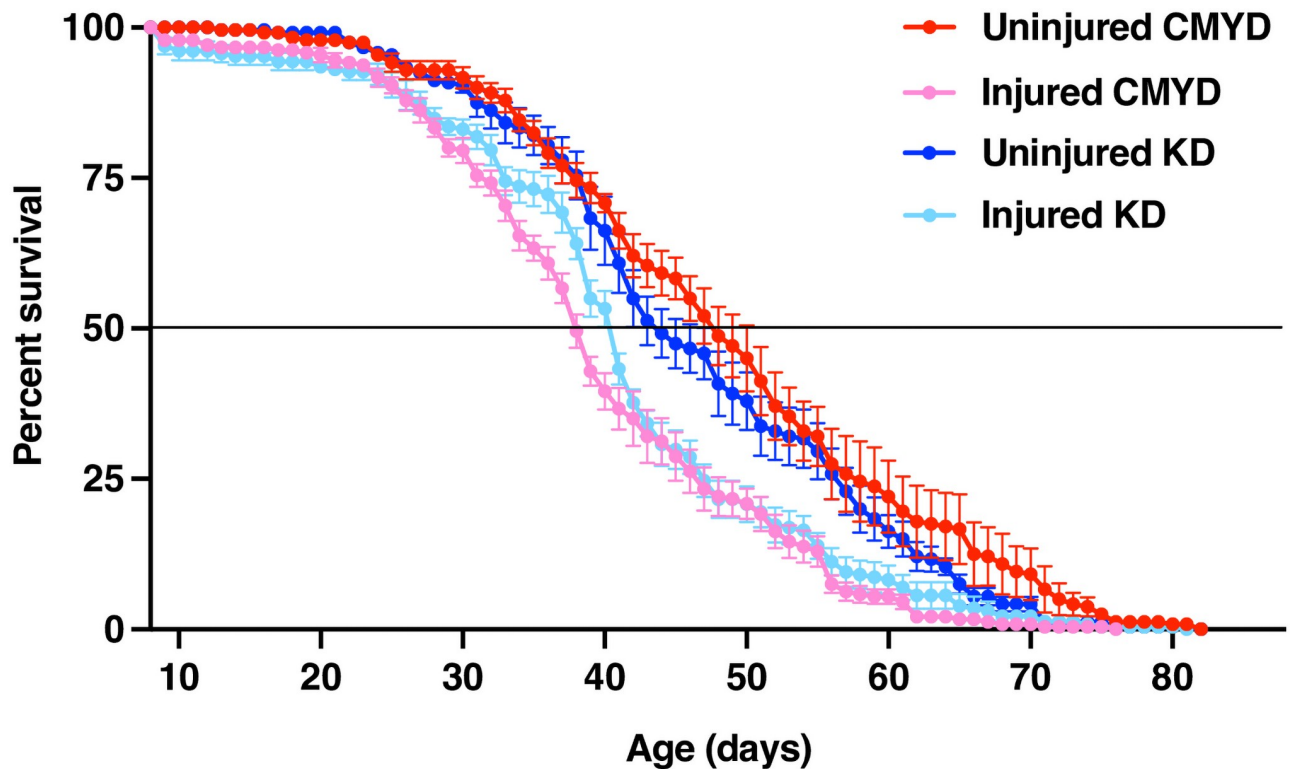


Fig 3. KD has a long-term beneficial effect on lifespan following TBI. Percent survival was determined for uninjured and injured 0–7 day old, mixed sex w^{1118} flies fed 0.3 cal/200 μ l CMYD or KD for 24 h following TBI and solid CMYD thereafter, that is, flies in the experiment that survived 24 h feeding on 0.3 cal/200 μ l CMYD or KD were fed solid CMYD throughout the rest of their lifespan. At least 230 flies were examined for each condition. Error bars indicate the SEM, and the horizontal line at 50% indicates the median lifespan.

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MI₂₄ of 0–7 day old, mixed sex *Eip75B* mutant flies. Three hypomorphic alleles of *Eip75B* (*Eip75B*^{MI04895}, *Eip75B*^{KG04491}, and *Eip75B*^{BG02576}) containing transposon insertions within the transcribed region were examined (Fig 4A). As in Fig 1, the MI₂₄ was comparably low in control w^{1118} flies fed either water or KD (ordinary one-way ANOVA with Dunnett's Multiple Comparison test, $p = 0.835$) (Fig 4B). In contrast, although water-fed *Eip75B*^{KG04491}, *Eip75B*^{BG02576}, and *Eip75B*^{MI04895}/*Eip75B*^{BG02576} flies still had low MI₂₄ values comparable to that of water-fed w^{1118} controls, MI₂₄ values were higher in KD-fed *Eip75B* mutants (ordinary one-way ANOVA with Dunnett's Multiple Comparison test, $p = 0.078$, $p = 0.033$, and $p = 0.006$, respectively), indicating that the beneficial effect of KD was impaired in these mutants. Furthermore, higher MI₂₄ values in KD-fed versus water-fed mutants provides further evidence that flies consumed KD. The beneficial effect of KD was, however, retained in *Eip75B*^{MI04895} homozygotes (ordinary one-way ANOVA with Dunnett's Multiple Comparison test, $p = 0.999$), which we attribute to a presumptive weaker loss of function of *Eip75B* caused by this mutation. *Eip75B*^{MI04895} only disrupts three of the seven *Eip75B* pre-mRNA isoforms, whereas *Eip75B*^{KG04491} and *Eip75B*^{BG02576} disrupt four and five isoforms, respectively (Fig 4A). Thus, while it remains possible that differences in genetic background underlie differences in MI₂₄ values for *Eip75B* mutant flies fed water versus KD, the data support the conclusion that activation of Eip75B/PPAR γ by KD triggers mechanisms that reduce early mortality following TBI.

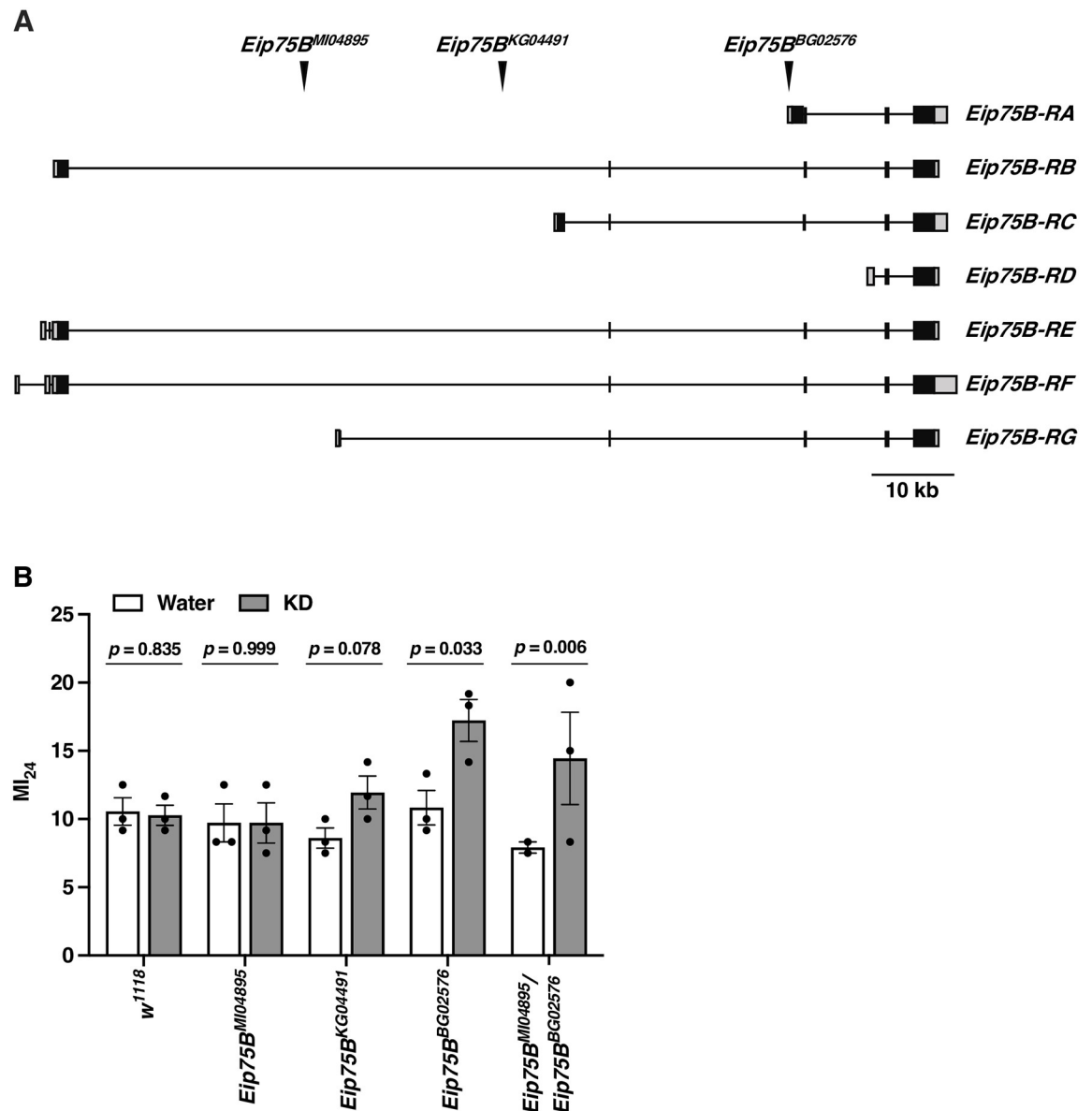


Fig 4. The beneficial effect of KD on early mortality following TBI requires *Eip75B*. (A) Transposon insertion locations (arrowheads) relative to the seven *Eip75B* transcripts drawn 5' to 3' (<http://flybase.org>). Gray boxes, black boxes, and lines indicate 5' and 3' untranslated regions, exons, and introns, respectively. (B) MI₂₄ values were determined for 0–7 day old, mixed sex *w*¹¹¹⁸ and *Eip75B* mutant flies fed water or 0.3 cal/200 μ l KD following TBI. The MI₂₄ represents the percent mortality of injured flies minus the percent mortality of uninjured flies 24 h following TBI. Dots indicate biological replicates of 60 flies, bars indicate averages, and error bars indicate the SEM. Significance was determined using ordinary one-way ANOVA with Dunnett's Multiple Comparison test.

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Discussion

TBI patients face a spectrum of neurobehavioral sequelae initiated by primary injuries to the brain and mediated by the interplay of genetic and environmental factors that control pathophysiological cascades. Here, we discovered that an interaction between the genetic factor *Eip75B*/PPAR γ and the environmental factor KD affects early mortality in a fly TBI model. In particular, whereas flies fed high-carbohydrate CMYD or YD exhibited a dose-dependent

increase in early mortality compared with flies fed water, flies fed high-fat KD showed no increase in early mortality (Fig 1A and 1B). The beneficial effect of KD on early mortality was equivalent in males and females, conserved in different genetic backgrounds, and had long-term beneficial effects on lifespan as well (Figs 2 and 3). However, the beneficial effect of KD on early mortality was diminished in flies mutant for Eip75B, a transcription factor orthologous to mammalian PPAR γ , suggesting that KD exerts its effect through Eip75B (Fig 4B). These data provide a mechanistic link between KD and PPAR γ in modifying TBI outcomes and demonstrate the utility of the fly TBI model for dissecting interactions between genetic and environmental factors that affect TBI outcomes.

The KD-Eip75B/PPAR γ pathway may reduce early mortality following TBI by inhibiting Relish/NF- κ B

Our data suggest that KD reduces early mortality following TBI by activating Eip75B/PPAR γ . However, it remains to be determined what occurs downstream of Eip75B/PPAR γ to exert this effect. One possibility is that Eip75B/PPAR γ controls expression of genes involved in inflammation. In mammals, activation of PPAR γ by dietary fatty acids mitigates neuroinflammation by inhibiting NF- κ B, a transcriptional activator of cytokine, chemokine, and adhesion genes downstream of Toll-like receptor (TLR)/Interleukin-1 receptor (IL-1R) and Tumor necrosis factor- α receptor (TNFR) innate immune response signaling pathways [19]. PPAR γ inhibits NF- κ B by a variety of mechanisms, including ubiquitination and degradation, export from the nucleus, competition for cofactors, and steric inhibition of DNA binding [40]. In mammalian TBI models, reduced NF- κ B activity resulting from treatment with the PPAR γ agonist pioglitazone or other pharmacological agents improves outcomes [22–25, 41–46]. Reducing NF- κ B activity also improves TBI outcomes in flies [33]. Heterozygosity for a null mutation of *Relish*, one of three NF- κ B genes in *Drosophila*, reduces early mortality and increases lifespan following TBI. Relish functions in the Immune-deficiency (Imd) pathway that is homologous to the TNFR pathway in mammals and controls transcription of numerous antimicrobial peptide genes (AMPs) that produce resistance to infection [47, 48]. A confirmed transcriptional target of Relish in TBI is the AMP gene *Metchnikowin* (*Mtk*), which when mutated reduces early mortality and increases lifespan following TBI [32]. Thus, KD-mediated activation of Eip75B/PPAR γ may reduce early mortality following TBI by inhibiting Relish/NF- κ B. This could be tested in the fly TBI model by examining effects of KD and pioglitazone on the MI₂₄, lifespan, and expression of AMP genes in wild type as well as *Relish* and *Mtk* mutant flies.

KD and water appear to reduce early mortality following TBI by different mechanisms

Genetically diverse fly lines fed KD or water following TBI consistently had a lower incidence of early mortality relative to flies fed high-carbohydrate diets (Figs 1A and 1B and 2) [29]. These data suggest that KD and water might activate the same protective pathways. Water is a fasting condition where the amount of available carbohydrate is decreased, forcing a switch to the use of fatty acids as a nutrient supply through beta-oxidation and ketogenesis [49]. Ketogenesis converts acetyl-CoA to ketone bodies (e.g., β -hydroxybutyrate, acetone, and acetoacetate) that are used by the brain and other tissues to produce energy. Flies with impaired mitochondrial ATP synthase activity produce elevated amounts of β -hydroxybutyrate, indicating that ketogenesis operates in flies [50]. Additionally, aggressive behaviors and early mortality induced by TBI in flies are reduced when flies are raised on high-carbohydrate diet supplemented with β -hydroxybutyrate relative to high-carbohydrate diet alone, indicating that β -hydroxybutyrate operates in the fly TBI model [35]. Nonetheless, several lines of evidence

argue that KD and water act by distinct mechanisms to reduce early mortality following TBI. First, the MI_{24} of *Eip75B* mutants differed depending on whether they were fed KD or water, indicating that water acts independently of Eip75B/PPAR γ (Fig 4B). Second, we previously found that flies fed water versus CMYD exhibited increased expression of AMP genes following TBI, suggesting that the beneficial effects of water are not mediated by inhibition of Relish, which activates the transcription of AMP genes [29]. Third, while heterozygosity for a mutation of *Relish* reduced the incidence of early mortality for flies fed CMYD following TBI, it did not affect the incidence of early mortality for flies fed water following TBI, suggesting that water functions either downstream or independently of Relish [33].

In conclusion, our observations indicate that KD signals through PPAR γ to improve TBI outcomes in flies. Thus, the fly TBI model offers considerable potential for understanding the cellular and molecular mechanisms that underlie the beneficial effects of KD and may ultimately facilitate development of therapeutic intervention for TBI in humans.

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References

1. Johnson WD, Griswold DP. Traumatic brain injury: a global challenge. *Lancet Neurol.* 2017; 16: 949–950. [https://doi.org/10.1016/S1474-4422\(17\)30362-9](https://doi.org/10.1016/S1474-4422(17)30362-9) PMID: 29122521
2. Wagner AK. A rehabilitomics framework for personalized and translational rehabilitation research and care for individuals with disabilities: perspectives and considerations for spinal cord injury. *J Spinal Cord Med.* 2014; 37: 493–502. <https://doi.org/10.1179/2045772314Y.0000000248> PMID: 25029659

3. Cortes D, Pera MF. The genetic basis of inter-individual variation in recovery from traumatic brain injury. *NPJ Regen Med.* 2021; 6: 5. <https://doi.org/10.1038/s41536-020-00114-y> PMID: 33479258
4. Thapa K, Khan H, Singh TG, Kaur AJ. Traumatic brain injury: mechanistic insight on pathophysiology and potential therapeutic targets. *J Mol Neurosci.* 2021; 71: 1725–1742. <https://doi.org/10.1007/s12031-021-01841-7> PMID: 33956297
5. Salim A, Hadjizacharia P, Dubose J, Brown C, Inaba K, Chan LS, et al. Persistent hyperglycemia in severe traumatic brain injury: an independent predictor of outcome. *Am Surg.* 2009; 75: 25–29. PMID: 19213392
6. McKenna MC, Scafidi S, Robertson CL. Metabolic alterations in developing brain after injury: Knowns and unknowns. *Neurochem Res.* 2015; 40: 2527–2543. <https://doi.org/10.1007/s11064-015-1600-7> PMID: 26148530
7. White H, Venkatesh B. Clinical review: ketones and brain injury. *Crit Care.* 2011; 15: 219. <https://doi.org/10.1186/cc10020> PMID: 21489321
8. Yang H, Shan W, Zhu F, Wu J, Wang Q. Ketone bodies in neurological diseases: Focus on neuroprotection and underlying mechanisms. *Front Neurol.* 2019; 10: 585. <https://doi.org/10.3389/fneur.2019.00585> PMID: 31244753
9. Jensen NJ, Wodschow HZ, Nilsson M, Rungby J. Effects of ketone bodies on brain metabolism and function in neurodegenerative diseases. *Int J Mol Sci.* 2020; 21: 8767. <https://doi.org/10.3390/ijms21228767> PMID: 33233502
10. Greco T, Prins ML. Traumatic brain injury and diet. *J Child Neurol.* 2013; 28: 983–998. <https://doi.org/10.1177/0883073813487594> PMID: 23670252
11. McDougall A, Bayley M, Munce SE. The ketogenic diet as a treatment for traumatic brain injury: a scoping review. *Brain Inj.* 2018; 32: 416–422. <https://doi.org/10.1080/02699052.2018.1429025> PMID: 29359959
12. Prins ML, Fujima LS, Hovda DA. Age-dependent reduction of cortical contusion volume by ketones after traumatic brain injury. *J Neurosci Res.* 2005; 82: 413–420. <https://doi.org/10.1002/jnr.20633> PMID: 16180224
13. Appelberg KS, Hovda DA, Prins ML. The effects of a ketogenic diet on behavioral outcome after controlled cortical impact injury in the juvenile and adult rat. *J Neurotrauma.* 2009; 26: 497–506. <https://doi.org/10.1089/neu.2008.0664> PMID: 19231995
14. Hu ZG, Wang HD, Jin W, Yin HX. Ketogenic diet reduces cytochrome c release and cellular apoptosis following traumatic brain injury in juvenile rats. *Ann Clin Lab Sci.* 2009; 39: 76–83. PMID: 19201746
15. Hu ZG, Wang HD, Qiao L, Yan W, Tan QF, Yin HX. The protective effect of the ketogenic diet on traumatic brain injury-induced cell death in juvenile rats. *Brain Inj.* 2009; 23: 459–465. <https://doi.org/10.1080/02699050902788469> PMID: 19408168
16. Zhang F, Wu H, Jin Y, Zhang X. Proton magnetic resonance spectroscopy (H1-MRS) study of the ketogenic diet on repetitive mild traumatic brain injury in adolescent rats and its effects on neurodegeneration. *World Neurosurg.* 2018; 120: e1193–e1202. <https://doi.org/10.1016/j.wneu.2018.09.037> PMID: 30236814
17. Salberg S, Weerwardhena, Collins R, Reimer RA, Mychasiuk R. The behavioral and pathophysiological effects of the ketogenic diet on mild traumatic brain injury in adolescent rats. *Behav Brain Res.* 2019; 376: 112225. <https://doi.org/10.1016/j.bbr.2019.112225> PMID: 31518660
18. Thau-Zuchman O, Svendsen L, Dyllal SC, Paredes-Esquivel U, Rhodes M, Priestley JV, et al. A new ketogenic formulation improves functional outcomes and reduces tissue loss following traumatic brain injury in adult mice. *Theranostics.* 2021; 11: 346–360. <https://doi.org/10.7150/thno.48995> PMID: 33391479
19. Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim Biophys Acta.* 2011; 1812: 1007–1022. <https://doi.org/10.1016/j.bbadis.2011.02.014> PMID: 21382489
20. Knowles S, Budney S, Doedhar M, Matthews SA, Simeone KA, Simeone TA. Ketogenic diet regulates the antioxidant catalase via the transcription factor PPAR γ 2. *Epilepsy Res.* 2018; 147: 71–74. <https://doi.org/10.1016/j.eplepsyres.2018.09.009> PMID: 30261354
21. Wahli W, Michalik L. PPARs at the crossroads of lipid signaling and inflammation. *Trends Endocrinol Metab.* 2012; 23: 351–363. <https://doi.org/10.1016/j.tem.2012.05.001> PMID: 22704720
22. Sauerbeck A, Gao J, Readnower R, Liu M, Pauly JR, Bing G, et al. Pioglitazone attenuates mitochondrial dysfunction, cognitive impairment, cortical tissue loss, and inflammation following traumatic brain injury. *Exp. Neurol.* 2011; 227: 128–135. <https://doi.org/10.1016/j.expneurol.2010.10.003> PMID: 20965168

23. Deng Y, Jiang X, Deng X, Chen H, Xu J, Zhang Z, et al. Pioglitazone ameliorates neuronal damage after traumatic brain injury via the PPAR γ /NF- κ B/IL-6 signaling pathway. *Genes & Diseases*. 2019; 7: 253–265. <https://doi.org/10.1016/j.gendis.2019.05.002> PMID: 32215295
24. Ratliff WA, Delic V, Pick CG, Citron BA. Dendritic arbor complexity and spine density changes after repetitive mild traumatic brain injury and neuroprotective treatments. *Brain Res*. 2020; 1746: 147019. <https://doi.org/10.1016/j.brainres.2020.147019> PMID: 32681835
25. Ratliff WA, Qubty D, Delic V, Pick CG, Citron BA. Repetitive mild traumatic brain injury and transcription factor modulation. *J Neurotrauma*. 2020; 37: 1910–1917. <https://doi.org/10.1089/neu.2020.7005> PMID: 32292111
26. Katzenberger RJ, Loewen CA, Wassarman DR, Petersen AJ, Ganetzky B, Wassarman DA. A *Drosophila* model of closed head traumatic brain injury. *Proc Natl Acad Sci USA*. 2013; 110: E4152–E4159. <https://doi.org/10.1073/pnas.1316895110> PMID: 24127584
27. Katzenberger RJ, Loewen CA, Bockstruck RT, Woods MA, Ganetzky B, Wassarman DA. A method to inflict closed head traumatic brain injury in *Drosophila*. *J Vis Exp*. 2015; 100: e52905. <https://doi.org/10.3791/52905> PMID: 26168076
28. Katzenberger RJ, Chtarbanova S, Rimkus SA, Fischer JA, Kaur G, Seppala JM, et al. Death following traumatic brain injury in *Drosophila* is associated with intestinal barrier dysfunction. *eLife*. 2017; 4: e04790.
29. Katzenberger RJ, Ganetzky B, Wassarman DA. Age and diet affect genetically separable injuries that cause acute mortality following traumatic brain injury in *Drosophila*. *G3*. 2016; 6: 4151–4166. <https://doi.org/10.1534/g3.116.036194> PMID: 27754853
30. Hill CS, Sreedharan J, Loreto A, Menon DK, Coleman MP. Loss of highwire protects against the deleterious effects of traumatic brain injury in *Drosophila melanogaster*. *Front Neurol*. 2020; 11: 401. <https://doi.org/10.3389/fneur.2020.00401> PMID: 32477254
31. van Alphen B, Semenza ER, Yap M, van Swinderen B, Allada R. A deep sleep stage in *Drosophila* with a functional role in waste clearance. *Sci Adv*. 2021; 7: eabc2999. <https://doi.org/10.1126/sciadv.abc2999> PMID: 33523916
32. Swanson LC, Rimkus SA, Ganetzky B, Wassarman DA. Loss of the antimicrobial peptide Metchnikowin protects against traumatic brain injury outcomes in *Drosophila melanogaster*. *G3*. 2020; 10: 3109–3119. <https://doi.org/10.1534/g3.120.401377> PMID: 32631949
33. Swanson LC, Trujillo EA, Thiede GH, Katzenberger RJ, Shishkova E, Coon JJ, et al. Survival following traumatic brain injury in *Drosophila* is increased by heterozygosity for a mutation of the NF- κ B innate immune response transcription factor Relish. *Genetics*. 2020; 216: 1117–1136. <https://doi.org/10.1534/genetics.120.303776> PMID: 33109529
34. Mackay TF, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, et al. The *Drosophila melanogaster* genetic reference panel. *Nature*. 2012; 482: 173–178. <https://doi.org/10.1038/nature10811> PMID: 22318601
35. Lee DC, Vali K, Balswin SR, Divino JN, Feliciano JL, Fequiere JR, et al. Dietary supplementation with the ketogenic diet metabolite beta-hydroxybutyrate ameliorates post-TBI aggression in young-adult male *Drosophila*. *Front Neurosci*. 2019; 13: 1140. <https://doi.org/10.3389/fnins.2019.01140> PMID: 31736687
36. Han SK, Lee D, Lee H, Kim D, Son HG, Yang JS, et al. OASIS 2: online application for survival analysis 2 with feature for the analysis of maximal lifespan and healthspan in aging research. *Oncotarget*. 2016; 7: 56147–56152. <https://doi.org/10.18632/oncotarget.11269> PMID: 27528229
37. Bridgham JT, Eick GN, Larroux C, Deshpande K, Harms MJ, Gauthier MAE, et al. Protein evolution by molecular tinkering: diversification of the nuclear receptor superfamily from a ligand-dependent ancestor. 2010; *PLoS Biol*. 8: e1000497. <https://doi.org/10.1371/journal.pbio.1000497> PMID: 20957188
38. Joardar A, Menzl J, Podolsky TC, Manzo E, Estes PS, Ashford S, et al. PPAR gamma activation is neuroprotective in a *Drosophila* model of ALS based on TDP-43. *Hum Mol Genet*. 2015; 24: 1741–1754. <https://doi.org/10.1093/hmg/ddu587> PMID: 25432537
39. Zipper L, Jassmann D, Burgmer S, Görlich, Reiff T. Ecdysone steroid hormone remote controls intestinal stem cell fate decisions via the PPAR γ -homolog Eip75B in *Drosophila*. *eLife* 2020; 9: e55795. <https://doi.org/10.7554/eLife.55795> PMID: 32773037
40. Cai W, Yand T, Liu H, Han L, Zhang K, Hu X, et al. Peroxisome proliferator-activated receptor γ (PPAR γ): a master gatekeeper in CNS injury and repair. *Prog Neurobiol*. 2018; 163–164: 27–58. <https://doi.org/10.1016/j.pneurobio.2017.10.002> PMID: 29032144
41. Su X, Wang H, Zhao J, Pan H, Mao L. Beneficial effects of ethyl pyruvate through inhibiting high-mobility group box 1 expression and TLR4/NF- κ B pathway after traumatic brain injury in the rat. *Mediators Inflamm*. 2011; 2011: 807142. <https://doi.org/10.1155/2011/807142> PMID: 21772666

42. Feng Y, Cui Y, Gao JL, Li MH, Jiang XH, Tian YX, et al. Resveratrol attenuates neuronal autophagy and inflammation injury by inhibiting the TLR4/NF-kappaB signaling pathway in experimental traumatic brain injury. *Int J Mol Med*. 2016; 37: 921–930. <https://doi.org/10.3892/ijmm.2016.2495> PMID: 26936125
43. Chen X, Chen C, Fan S, Wu S, Yang F, Fang Z, et al. Omega-3 polyunsaturated fatty acid attenuates the inflammatory response by modulating microglia polarization through SIRT1-mediated deacetylation of the HMGB1/NF-kB pathway following experimental traumatic brain injury. *J Neuroinflammation*. 2018; 15: 116. <https://doi.org/10.1186/s12974-018-1151-3> PMID: 29678169
44. Tao L, Li D, Liu H, Jiang F, Xu Y, Cao Y, et al. Neuroprotective effects of metformin on traumatic brain injury in rats associated with NF-kB and MAPK signaling pathway. *Brain Res Bull*. 2018; 140: 154–161. <https://doi.org/10.1016/j.brainresbull.2018.04.008> PMID: 29698747
45. Caglayan B, Kilic E, Dalay A, Altunay S, Tuzcu M, Erten F, et al. Allyl isothiocyanate attenuates oxidative stress and inflammation by modulating Nrf2/HO-1 and NF-kB pathways in traumatic brain injury in mice. *Mol Biol Rep*. 2019; 46: 241–250. <https://doi.org/10.1007/s11033-018-4465-4> PMID: 30406889
46. Shao X-F, Li B, Shen J, Wang Q-F, Chen S-S, Jiang X-C, et al. Ghrelin alleviates traumatic brain injury-induced acute lung injury through pyroptosis/NF-kB pathway. *Int Immunopharmacol*. 2020; 79: 106175. <https://doi.org/10.1016/j.intimp.2019.106175> PMID: 31918060
47. Lemaitre B, Hoffmann. The host defense of *Drosophila melanogaster*. *Annu Rev Immunol*. 2007; 25: 697–743. <https://doi.org/10.1146/annurev.immunol.25.022106.141615> PMID: 17201680
48. Ganesan S, Aggarwal K, Paquette N, Silverman N. NF-kB/Rel proteins and the humoral immune responses of *Drosophila melanogaster*. *Curr Top Microbiol Immunol*. 2011; 349: 25–60. https://doi.org/10.1007/82_2010_107 PMID: 20852987
49. Morris AAM. Cerebral ketone body metabolism. *J Inherit Metab Dis*. 2005; 28: 109–121. <https://doi.org/10.1007/s10545-005-5518-0> PMID: 15877199
50. Celotto AM, Chiu WK, Van Voorhies W, Palladino MJ. Modes of metabolic compensation during mitochondrial disease using the *Drosophila* model of ATP6 dysfunction. 2011; *PLoS One*. 6: e25823. <https://doi.org/10.1371/journal.pone.0025823> PMID: 21991365