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The ATP-sensitive K channel is seizure protective and required for effective dietary therapy in a model of mitochondrial encephalomyopathy

Keri J. Fogle, J. Ian Hertzler, Joy H. Shon, and Michael J. Palladino

Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA; Pittsburgh Institute for Neurodegenerative Diseases (PIND), University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

Abstract

Effective therapies are lacking for mitochondrial encephalomyopathies (MEs). MEs are devastating diseases that predominantly affect the energy-demanding tissues of the nervous system and muscle, causing symptoms such as seizures, cardiomyopathy, and neuro- and muscular degeneration. Even common anti-epileptic drugs which are frequently successful in ameliorating seizures in other diseases tend to have a lower success rate in ME, highlighting the need for novel drug targets, especially those that may couple metabolic sensitivity to neuronal excitability. Furthermore, alternative epilepsy therapies such as dietary modification are gaining in clinical popularity but haven't been thoroughly studied in ME. Using the *Drosophila ATP6^I* model of ME, we have studied dietary therapy throughout disease progression and found that it is highly effective against the seizures of ME, especially a high fat/ketogenic diet, and that the benefits are dependent upon a functional K_{ATP} channel complex. Further experiments with K_{ATP} show that it is seizure-protective in this model, and that pharmacological promotion of its open state also ameliorates seizures. These studies represent important steps forward in the development of novel therapies for a class of diseases that is notoriously difficult to treat, and lay the foundation for mechanistic studies of currently existing therapies in the context of metabolic disease.

Keywords

ATP6; *Drosophila*; mitochondria; seizure

Introduction

Class I mitochondrial encephalomyopathies (MEs), defined as disorders that arise due to mutation in the mitochondrial DNA or affecting components of the electron transport chain (Leonard & Schapira, 2000), particularly distress the energy-demanding tissues of the nervous and muscular systems. These diseases affect up to 1 in 5000 people worldwide,

Michael J. Palladino, Department of Pharmacology & Chemical Biology and the Pittsburgh Institute for Neurodegenerative Diseases (PIND), University of Pittsburgh School of Medicine, 3501 Fifth Ave., Pittsburgh, PA, USA, Tel: (412) 383-5900; Fax: (412) 648-7029; mjp44@pitt.edu.

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causing symptoms such as reduced lifespan, cardiomyopathy, cognitive and sensory defects, and neuro- and muscular degeneration (Di Donato, 2009; DiMauro & Schon, 2003, 2008; DiMauro et al., 1989; Finsterer, 2004; Schapira & Cock, 1999). With no effective pharmacological treatments currently available to target the mitochondrial defects themselves, clinicians often must rely on trial and error to formulate a so-called “mito cocktail” of vitamins, supplements, and antioxidants for a given patient, which often has limited success even when optimized (DiMauro, Schon, Carelli, & Hirano, 2013; Santorelli, Tanji, Shanske, & DiMauro, 1997). Furthermore, pharmacologic treatments that are efficacious in other disorders, such as anti-epileptic drugs for the treatment of seizures, are often refractory in patients with mitochondrial disease; the unrelenting seizures worsen quality of life and correlate with early mortality (Canafoglia et al., 2001; DiMauro et al., 1989; Jung et al., 2007).

That the common, generally successful anti-epileptic drugs (AEDs), are less effective in mitochondrial disease may suggest that their targets – typically sodium and calcium channels or GABAergic transmission – are not the primary arbiters of the seizure pathophysiology in ME, or that treating them is insufficient unless other defects are also addressed. Given the significant metabolic perturbation that results from dysfunctional oxidative phosphorylation, proteins which couple the ability to regulate membrane excitability with an intrinsic sensitivity to metabolic state represent logical targets for mechanistic investigation and pharmaceutical intervention in ME.

One such candidate is the ATP-sensitive potassium channel (K_{ATP}). The K_{ATP} ion channel complex is an octamer comprised of four inwardly-rectifying potassium channel subunits ($K_{IR}6.1$ or 6.2) each paired with a cytosolic sulfonylurea receptor (SUR1, 2A, or 2B) (Inagaki et al., 1995; Inagaki, Gonoi, & Seino, 1997; Sakura, Ammala, Smith, Gribble, & Ashcroft, 1995). At physiological voltages these channels pass potassium current outward, resulting in an excitability-dampening hyperpolarization (Ashcroft, 2007; Seino, 1999). Their modulation involves interplay between inhibition via ATP binding to the pore-forming K_{IR} subunit (Ashcroft & Kakei, 1989; Tucker, Gribble, Zhao, Trapp, & Ashcroft, 1997) and activation by Mg-ADP binding at the SUR subunit (Dunne & Petersen, 1986; Gribble, Tucker, & Ashcroft, 1997; Nichols et al., 1996), rendering them exquisitely sensitive to metabolic state. They are also regulated by lipids, including phosphatidylinositol-4,5-bisphosphate (PIP2) (Shyng & Nichols, 1998) and long chain acyl-CoAs (Gribble, Proks, Corkey, & Ashcroft, 1998; Larsson, Deeney, Branstrom, Berggren, & Corkey, 1996). K_{ATP} is found in many tissues, including pancreatic β cells where their contribution to insulin release makes them drug targets in diabetes (Ashcroft, 2007; Hibino et al., 2010) and hyperinsulinism (Dunne, Cosgrove, Shepherd, Aynsley-Green, & Lindley, 2004). They are also found in the cardiac sarcolemma where they have a well-known cardioprotective role in ischemic preconditioning (Ashcroft, 2007; Tinker, Aziz, & Thomas, 2014), in vascular smooth muscle, in the mitochondrial membrane, and in the plasma membrane of neurons, where they provide protection during hypoxia (H. S. Sun & Feng, 2013; H. S. Sun, Feng, Miki, Seino, & French, 2006) and physically interact with several glycolytic enzymes (Dhar-Chowdhury et al., 2005; Dhar-Chowdhury, Malester, Rajacic, & Coetzee, 2007; Hong et al., 2011).

The K_{ATP} channel is also a leading molecular candidate to contribute to the neuroprotective properties of dietary restriction therapy or “dietary modification” (DM). Commonly used before the advent of the modern age of AEDs (and perhaps even in antiquity) to treat seizures (Gano, Patel, & Rho, 2014), these modified approaches to nutrition include caloric restriction (fasting), and diets high in fat but low in carbohydrates and protein, such as the ketogenic diet (KD) and the related but less stringent medium chain triglyceride (MGT) and low glycemic index (LGI) diets. These diets emphasize under-utilization of glycolysis, providing most cellular energy through fatty acid oxidation resulting in a dramatic increase in the cellular concentration of ketone bodies acetoacetate, β -hydroxybutyrate, and acetone. These nutritional strategies are increasing in clinical popularity in the modern age, especially for pediatric patients of intractable epilepsy, yielding highly effective seizure control in many cases where AEDs do not (Freeman & Vining, 1998; Neal et al., 2008; Stafstrom & Rho, 2012; Vining et al., 1998). DM, however, has not been thoroughly investigated for effectiveness and safety in ME.

One of the few stable animal models of ME is *Drosophila ATP6^l*, in which the mitochondrially-encoded ATP6 gene possesses a missense mutation resulting in a G to E substitution at position 116. As a result, the crucial hydrogen ion passing subunit of Complex V (ATP synthase) of the electron transport chain is dysfunctional, resulting in the absence of oxidative phosphorylation. This model displays phenotypes that correlate with human disease mutations affecting *ATP6* such as Maternally Inherited Leigh Syndrome, Neuropathy, Ataxia, and Retinitis Pigmentosa, and Familial Bilateral Striatal Necrosis (Celotto et al., 2006; Palladino, 2010). One of these phenotypes is seizure activity in response to sensory hyperstimulation, which is assayed in flies by the well-established “bang sensitivity” (BS) protocol (Benzer, 1971; Ganetzky & Wu, 1982) (and see Methods and Discussion). Metabolically, *ATP6^l* upregulates the use of ketogenesis and glycolysis to compensate for its deficiency in oxidative phosphorylation (Celotto, Chiu, Van Voorhies, & Palladino, 2011).

Here we have used this model to test the effectiveness of the dietary modification strategies of caloric restriction and the ketogenic diet against the seizures of ME. We have also tested whether the K_{ATP} channel has a role in the therapeutic mechanism in these DMs and in seizure protection, and have tested its known drug effector diazoxide at the behavioral and neuronal levels for therapeutic potential. We show that caloric restriction and the ketogenic diet are effective at abrogating seizures in *ATP6^l*. However, these benefits are only realized if K_{ATP} is present, as its removal eliminates the benefit of DM. These findings reveal K_{ATP} 's neuroprotective role in ME such as the *ATP6^l* disease state. Furthermore, we show that the potassium channel opener (KCO) diazoxide significantly reduces seizures and lowers excitability in neurons in *ATP6^l* flies with advanced ME.

Methods

Fly strains and Genetics

Strains, matings, and experimental flies were maintained in standard media comprised of agar, corn meal, dextrose, sucrose, dry yeast, and molasses, with propionic acid and

Tegosept added as fungicides, unless otherwise noted. Flies are maintained in a light and temperature controlled room under a 12:12LD schedule.

In all reported genotypes, “*ATP6^h*”, “*ATP6^{IR}*”, or “*ATP6[1R]*” refers to the mitochondrially encoded gene mutation in *ATP6* and is denoted first, separated from the nuclear chromosomes by standard semi-colon notation. For physiology experiments, female *ATP6^l; sesB^l* flies, where *sesB^l* is a recessive mutation in the adenosine nucleotide transporter (Celotto et al., 2006), were mated with the recombinant strain *CRYGAL4-UAS-NC1*. NC1 is a non-conducting potassium channel GFP fusion for visualization of the large lateral ventral neurons (Fogle, Parson, Dahm, & Holmes, 2011; Sheeba, Gu, Sharma, O'Dowd, & Holmes, 2008). This mating results in the experimental genotype *ATP6^l; sesB^l/w^{*}; CRYGAL4-UAS-NC1/+; +*, wherein, due to its recessive nature, *sesB^l* will have no effect on physiological properties. “Wild type” flies were generated by the reciprocal mating, resulting in the same nuclear genome but a wild type mitochondrial *ATP6⁺*.

The *ATP6^{IR}* strain, used in all behavior (BS) experiments described herein, was generated by the O'Farrell lab (H.Ma & O'Farrell, 2015, 2016) by mitochondrial genome recombination and does not possess the recessive *sesB^l* mutation. Controls were *ATP6⁺; w¹¹¹⁸*.

Bloomington strain 25638 (*w[1118]; MiET1Sur[MB07175]*) was designated *K_{ATPdr-1}*. The *SUR* gene has been disrupted, as part of the Gene Disruption Project, by Minos, a member of the Tc1/mariner family of transposable elements (Bellen et al., 2011; Metaxakis, Oehler, Klinakis, & Savakis, 2005). Bloomington Stock Center line 27966 (*y[1] w[*]; Pw[+mC]=EPSur[G7604]*) was designated *K_{ATPdr-2}*, and here the *SUR* gene is disrupted by a P element. *ATP6^{IR}; w¹¹¹⁸*, *K_{ATPdr-1}/K_{ATPdr-1}* and *ATP6^{IR}; w¹¹¹⁸; K_{ATPdr-2}/K_{ATPdr-2}* strains were generated using standard methods.

Behavior Experiments

Stress sensitivity or bang sensitivity (BS) experiments to assay stress-induced seizures were conducted as originally described by Ganetzky and Wu (Ganetzky & Wu, 1982) with minor modifications (Celotto et al., 2006). Flies were placed in empty plastic, cotton-plugged vials in groups of 3-5, then vortexed at maximum speed for 20s. *ATP6^l* and *ATP6^{IR}* flies, by the age of 15 days, uniformly experience paralysis immediately after this stimulation. During the timed period, some flies also experience intermittent convulsions which cause involuntary wing beating and uncontrolled movement of the fly across the bottom and into the sides of the vial. Wild type flies never paralyze or exhibit convulsive movements. Time to recovery (TTR) is measured as the time from the end of the vortexing to the time the fly rights itself and makes a purposeful movement such as grooming, walking, or climbing. Every fly tested is included in the reported values, even if there is no phenotype (TTR=0) or it takes several minutes to recover, unless there is an obvious confounding factor such as incidental leg or abdomen injury or media components on the body obstructing the animal's movement.

Dietary therapy

Flies undergoing dietary modifications were provided with their respective diets in normal plastic media vials. Flies subjected to 24-hour caloric restriction (CR) were placed in vials containing a water-soaked filter paper to prevent dehydration followed immediately by stress sensitivity assays. The ketogenic diet experiments consisted of a regular vial with a solid but damp pure agar-water plug to maintain hydration which was supplemented with 1cm diameter plastic cup filled with the nutrient-containing ketogenic diet media with either 2:1, 3:1, or 4:1 ratio of fat:carbohydrates by weight. The carbohydrate content of this media was calculated by adding grams of sucrose, grams of dextrose, 20% of the grams of yeast, and 80% of the grams of corn meal in the media. The weight of coconut oil added was calculated by multiplying this value by 2, 3, or 4 as appropriate. Molasses was omitted from this mixture. Large amounts of each mixture were made simultaneously to reduce potential variability within each batch and across experiments. The flies lived on this media every day of their 6-day stress sensitivity time course.

Sugar-restricted media was made by reducing the sucrose, dextrose, and molasses from the normal media mixture by 25%, with all other aspects of production identical to that of the standard media.

Drug treatments

For diazoxide experiments, we used day 17 flies which had a significant seizure phenotype but were not so far into disease progression that poor coordination, muscle degeneration, or other non-specific effects of the disease would likely confound the experiment. Aged flies were separated at random and incubated with either drug or vehicle. Stock diazoxide was made at 36mM in DMSO. A vehicle toxicity test was run prior to the experiments to find the maximum concentration of DMSO flies could tolerate with 100% survival for 72 hours; this was chosen as the maximum drug treatment concentration which was 2.5% DMSO with 900µM diazoxide in normal media. The lower concentrations used were thus 1.875% and 1.25% DMSO containing 675µM and 450µM diazoxide, respectively. Flies were assayed after 24 hours in drug or vehicle, then returned to the same vial and assayed again at 72 hours (age 20 days). This same timing protocol was used for tolbutamide treatments. Tolbutamide was dissolved directly into the media at a concentration of 120mM. Diazoxide and tolbutamide were acquired from Sigma-Aldrich.

Electrophysiology

Whole brains were dissected from ice-immobilized aged adult flies (21-24 days) and cleaned in a solution containing 20 units/mL papain dissolved in standard external solution (see below). Brains were transferred to a RC-22C recording chamber (Warner Instruments) and held in place with a platinum-framed nylon mesh. Standard external recording solution (101mM NaCl, 1mM CaCl₂, 4mM MgCl₂, 3mM KCl, 5mM glucose, 1.25mM NaH₂PO₄, and 20.7mM NaHCO₃, 250 mOsm, pH 7.2, aerated by a gas mixture of 95% O₂-5% CO₂) was gravity perfused over the specimen at a rate of ~1mL/min. Standard-wall borosilicate capillary tubes were pulled into patch pipettes (11-13mΩ) by a Narishige PP-83 electrode puller and filled with standard internal solution (102 K-gluconate, 0.085 CaCl₂, 1.7 MgCl₂, 17 NaCl, 0.94 EGTA, 8.5 HEPES, 235 mOsm, pH 7.2), then guided to GFP+ neurons by a

Sutter MP-225 micromanipulator. Whole-cell current-clamp recordings were performed using an Olympus Fluoview FV1000 upright confocal scanning microscope, CV201A headstage, Axopatch 200A Integrating Patch-Clamp amplifier, DigiData 1322A 16-bit Data Acquisition System and PClamp 10 software (Molecular Devices, Sunnyvale, CA). All recordings were made with no overhead light or microscope light sources due to the light sensitivity of the lateral ventral neurons, and were made in the flies' subjective early day (ZT2-ZT6) to avoid circadian variation in excitability. After whole-cell configuration was achieved and the recording stabilized, data were collected for basal firing frequency, which was calculated by hand counts divided by length (in seconds) of the analyzed region, which always covered at least 120s to account for any variability. For light response measurements, bright white light from the halogen light source was turned on at 80% power for 25-40s then turned off again. The firing frequency is allowed to stabilize again before any further experimental manipulations are made. The light response is calculated as a ratio of the firing frequency when the light is on divided by an average of the firing frequencies in the 60s before and after the light exposure. Similarly, for the depolarization evoked-excitability assay, 8-second pulses of +20 and 25pA current were injected via a protocol written in pClamp10, and the change in firing frequency is presented as a ratio of the FF during the pulse and the FF before and after. For physiology data, n values indicate number of assays (i.e. light cycles or depolarizations), of which there may be multiple acquired for a given cell, in order to account for any physiological or experimental variability. Data in all cases is recorded from at least three different cells, each in a different brain, and no brain is represented by more than 3 cycles in a given condition. Diazoxide was acquired from Sigma, and solution was prepared in ethanol to a stock concentration of 14mM, then diluted in standard external solution to an experimental concentration of 100 μ M. Analysis of electrophysiological data was performed using ClampFit10 (Molecular Devices/Axon Instruments).

Statistics

Data are presented as mean $\pm$ standard error. 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.

Results

For our experiments, we used the endogenous mitochondrial *ATP6^L* mutant strain identified and characterized in our lab (Celotto et al., 2011; Celotto et al., 2006; Palladino, 2010) but whose mitochondrial genome has been recombined to replace a deleted segment in the non-coding region. This recombinant (designated here as *ATP6^{LR}*) leaves the disease-causing missense mutation (G116E codon change) in the *ATP6* gene intact but eliminates any non-specific effects that may arise from non-coding mutations (H. Ma & O'Farrell, 2015, 2016). We characterized the seizure behavior of this mitochondrial recombinant throughout disease progression. As in our previously-published use of the bang sensitivity (BS) assay with *ATP6^L* flies, seizure behavior consisted primarily of paralysis. We have previously shown using strobe-light induced seizures in *ATP6^L* flies that an initial convulsive spasm typically occurs during the hyperstimulation. Approximately half of the flies experience spasms

intermittently or immediately prior to recovering. Seizure activity onset in *ATP6^{IR}* occurs at approximately two weeks of age and progresses rapidly throughout the third week of life (Figure 1A); this is followed by extremely poor coordination and death in the fourth week. Therefore, we have designated days 15, 18, and 21 after adult eclosion as early, intermediate, and advanced disease, and assayed mechanical stress-induced seizures (BS) at each of these time points by measuring the flies' times to recovery from seizure and paralysis (time to recovery, TTR). At all time points measured, nuclear genetic background-matched wild type mitochondrial ("wtmito") flies showed no seizure activity (TTR <1s, $p < .0001$ vs. *ATP6^{IR}* at each age, 1A and Supplemental Figure 1).

We next assayed the ability of caloric restriction to ameliorate the seizures of *ATP6^{IR}*. The simplest method to force fasting in flies is to remove their food source entirely for a block of time (time-restricted feeding). We raised flies on standard media until 14 days of age, then moved them to a culture vial containing only water-soaked filter paper to deprive them of nutrients and prevent dehydration. After 24 hours, the flies were BS assayed and then returned to normal media until 24 hours before the next assay point. Calorically restricted flies had lower average TTR values at early, intermediate, and advanced time points, but these values only reached significance at day 18 (Figure 1B). We hypothesized that while the flies were deriving some benefit from this method of caloric restriction, a full 24-hour starvation block might not be well-tolerated in diseased flies. Therefore, we reduced the starvation time to 12 hours; these flies showed no improvement in their TTR at any time point (data not shown). Next, we altered our method of caloric restriction from forced fasting to *ad libitum* feeding but on nutrient-poor media. When flies were provided media with 25% less sugar content than standard media, TTR values were significantly reduced at all time points (Figure 1B).

To provide a high-fat or ketogenic diet to flies, we supplemented their media with coconut oil, a medium-chain triglyceride which is easily metabolized into ketone bodies, at a ratio of 4:1, 3:1, and 2:1 by weight to carbohydrates. As shown in Figure 1C, all three of the ratios provided were successful in reducing the TTR values at early, intermediate, and advanced disease timepoints. Even at 21 days of age, when seizures are most severe, a 2:1 and 3:1 ratio offered 80-90% protection compared to flies on a normal diet.

In addition to time to recovery from seizures, we analyzed a second quantitative parameter, the bang sensitivity penetrance (BSP), a measurement of how many flies display the seizure phenotype. When we analyzed this parameter for *ATP6^{IR}* we found that the penetrance of the BS mutant on standard media is 80.5% and 88.5% at days 15 and 18, respectively, and reaches 100% by day 21, highlighting the severity of the disease at the advanced time point (Figure 1D). Dietary modification was successful at improving this aspect of the seizure phenotype as well. The two methods of caloric restriction lowered the BSP to less than 50% in early disease, while the ketogenic diet (3:1 and 2:1) reduced seizure penetrance to between 50 and 65% in early and intermediate disease, and even spared up to 9% of flies from seizures at day 21.

While the mechanisms underlying the seizure- and neuroprotective benefits of DM are under intense investigation (see Discussion), at least two prominent hypotheses center around the

K_{ATP} channel complex: that it is positively modulated by ketone bodies, and that it is preferentially closed by glycolytically-derived ATP, promoting depolarization and excitability. Furthermore, as a metabolic state sensor that can influence membrane excitability, this channel complex is potentially critical to study in the context of altered mitochondrial metabolism. Therefore, we tested K_{ATP} for seizure-protective properties and for a role in DM in our model of ME.

We chose two constructs engineered to genetically disrupt the SUR subunit (Bloomington Drosophila Stock Center, see Methods). Since SUR is necessary for trafficking of the channel, failure to generate functional SUR will eliminate the entire complex (Zerangue, Schwappach, Jan, & Jan, 1999). We tested these constructs for basal seizure activity considering the well-known seizure- and neuroprotective qualities of this channel complex. We found that the BSP and the TTR are higher than for *w¹¹¹⁸* flies, possibly indicating a mild neuromuscular dysfunction, but the average TTR is less than 2s at all time points for both constructs (Figure S1). We therefore consider these flies to be seizure-free on the scale that is relevant to *ATP6^{IR}* phenotypes. We next generated *ATP6^{IR}; K_{ATPdr-1}* and *ATP6^{IR}; K_{ATPdr-2}* double mutants for analysis. Figure 2A shows that at our early and intermediate disease time points (day 15 and 18), both *ATP6^{IR}; K_{ATPdr-1}* (yellow bars) and *ATP6^{IR}; K_{ATPdr-2}* (orange bars) show a drastically elevated TTR, and *ATP6^{IR}; K_{ATPdr-1}* is still elevated in advanced disease. We added an earlier assay at day 12 and demonstrated that at this typically pre-pathological age, *ATP6^{IR}; K_{ATPdr-1}* and *ATP6^{IR}; K_{ATPdr-2}* double mutant flies already show significant seizure activity. Furthermore, at all time points including day 12, the BSP of these double mutants is 100% penetrant.

Since K_{ATP} channels have a well-defined pharmacology, we next attempted to mimic the effect of genetically removing K_{ATP} function using the sulfonylurea drug tolbutamide, a K_{ATP} antagonist that binds to the SUR subunit and promotes closing of the channel. Indeed, after 24 and 72 hours of exposure to tolbutamide-containing media, 18-20 day old flies showed more severe seizures than their ethanol vehicle-treated counterparts (Figure 2B). Tolbutamide did not induce seizures in wild type flies. Together, these data suggest that functional K_{ATP} is seizure-protective in ME.

In several non-ME studies on dietary modification, K_{ATP} has proven to be necessary for the organism to achieve the seizure-protective benefit of the diet (Kawamura, Ruskin, Geiger, Boison, & Masino, 2014; Lutas, Birnbaumer, & Yellen, 2014; Yang et al., 2013; Yellen, 2008). Therefore, we tested TTR after 24-hour caloric restriction and a 3:1 fat supplemented diet in *ATP6^{IR}-K_{ATPdr-1}* and *dr-2* double mutant flies. Figures 2C (*dr-1*) and 2D (*dr-2*) reveal that indeed, removal of the functional K_{ATP} complex from our ME flies also removes the benefit of caloric restriction and a high-fat diet. At day 18, in fact, which is the time point at which caloric restriction by fasting was significantly effective in *ATP6^{IR}* (compare to Figure 1B), the flies lacking functional K_{ATP} perform significantly worse, with a higher TTR than their counterparts that were fed normally. Caloric restriction and the high fat diet also significantly worsen the performance of *ATP6^{IR}-K_{ATPdr-2}* in advanced disease (2D). No dietary modification improved the BSP for these double mutants, which remained at 100%.

Diazoxide is a potassium channel opener (KCO) that is best known for its cardioprotective ability, facilitating ischemic preconditioning via opening of K_{ATP} channels in the cardiac sarcolemma and/or the mitochondrial membrane. Diazoxide is also used to treat neonatal hyperinsulinism and, in special circumstances, high blood pressure through K_{ATP} channels in pancreatic β cells and vascular smooth muscle, respectively. Given the seizure-protective properties and the apparent role we discovered in DM of K_{ATP} , we tested the ability of diazoxide to abrogate seizures in $ATP6^lR$. We found that 900 μ M diazoxide was able to significantly reduce seizures compared to vehicle in 18- and 20-day old flies, after 24 and 72 hours of treatment (Figure 3A, center and right panels, square symbols). At 675 μ M, 24 hours of treatment failed to improve TTR but a longer, 72-hour treatment was successful (circles). But at 450 μ M, diazoxide was ineffective at reducing seizures (triangles). The pre-treatment TTR values for the flies that would go on to be tested with DMSO vs. diazoxide did not differ within any group (Figure 3A, left panel). The dosage- and treatment length-dependency of diazoxide effectiveness is summarized in Figure 3B.

Because diazoxide has been reported to have effects on targets in addition to K_{ATP} (Coetzee, 2013) (and see Discussion), we also tested its ability to ameliorate seizures in $ATP6^lR$ - $K_{ATPdr-2}$ double mutant animals. We chose to test the maximum concentration after 24 hours, since this was the most effective protocol for $ATP6^lR$ (Figure 3A, B). Before treatment, the groups that would be incubated with drug vs. vehicle did not differ (4A, orange symbols). After 24 hours of treatment, the groups incubated with drug vs. vehicle still did not differ (4A, grey/green symbols). The effectiveness of diazoxide in the presence vs. absence of K_{ATP} is presented side by side in 4B. These data support the conclusion that diazoxide is acting to abrogate seizures via K_{ATP} channel opening. Both DMSO and diazoxide-treated flies had 100% BSP, possibly owing to toxicity of the vehicle.

We next tested whether the KCO diazoxide could change neuronal properties in adult flies with either WT mitochondria or bearing $ATP6^l$ mutant mitochondria. To do this, we used whole cell patch clamp electrophysiology from the large lateral ventral neurons (l-LNV) of the fly circadian circuit. These neurons serve as excellent tools for investigating adult *Drosophila* neuropathophysiology because they are easily accessible, spontaneously firing, and electrophysiologically well-characterized (Fogle et al., 2011; McCarthy et al., 2011; Sheeba et al., 2008). Representative recording from an $ATP6^l$ fly with advanced disease (age 22 days) demonstrates that the firing rate noticeably decreases when diazoxide is applied (Figure 5A). On average, this effect is small but significant for $ATP6^l$ (Figure 5B, right bars), and while it is also qualitatively present in wild type mitochondrial flies of the same age, the effect does not reach significance (Figure 5B, left bars).

The l-LNV possess an inherent, cell-autonomous responsiveness to light owing to the cytosolic protein cryptochrome, which is relevant to their roles in the circadian circuit and in influencing the animal's night time arousal level. Experimentally, the effect most prominently manifests as an increase in firing frequency when the electrophysiological prep is exposed to bright light (Fogle et al., 2015; Fogle et al., 2011). This acute and easily measurable phenomenon can be used as a measure of evoked excitability as illustrated in Figure 6A. In the top trace, the firing rate of this aged $ATP6^l$ neuron (age 23 days) increases noticeably when the light is switched on (white bar) from total darkness (dark bar). In the

bottom trace, after the light has been turned off and the firing frequency returned to darkness level, the same cell was exposed to 100 μ M diazoxide for approximately ten minutes and then again exposed to light. While firing frequency can vary throughout a recording, the important metric is that acute light exposure increased the firing frequency over baseline to a lesser extent after exposure to the KCO. This effect is quantified and presented as a ratio of firing frequency with the lights on / firing frequency with the lights off (Figure 6B). *ATP6^l* flies before diazoxide perfusion have a robust light response that is significantly reduced afterwards (Figure 6B, right bars). The diazoxide-induced reduction of the light response is not seen in age-matched wild type neurons (Figure 6B, left bars).

We used depolarizing current injections as a second evoked excitability parameter. After acquiring baseline firing frequency data, the cell is injected with +25pA and the evoked firing rate is measured. Figure 7A shows one example *ATP6^l* cell exposed to this protocol without diazoxide and another with diazoxide treatment. Quantification of the increase in firing frequency over baseline is presented in 7B. With diazoxide treatment, both wild type mitochondrial and *ATP6^l* depolarization responses are significantly reduced. Perhaps notably, in both evoked excitability metrics, treatment with diazoxide reduced an elevated *ATP6^l* response approximately to wild type levels. Together, the data in Figure 4 illustrate that the KCO diazoxide reduces both basal and evoked excitability in the adult *Drosophila* brain in ME flies.

Discussion

Mitochondrial encephalomyopathies (MEs) are notoriously difficult to diagnose, treat, and study due to high variability in age of onset, affected tissues, primary symptoms, and overall severity. The high rate of failure of a class of compounds –anti-epileptic drugs or AEDs – that generally have good success with other seizure-associated diseases suggests that the potential of any therapies for ME must be viewed through the lens of a perturbed metabolic landscape. Yet clinical and basic science studies of alternative therapies such as novel drugs or dietary modification like the ketogenic diet in ME are rare. While one small-scale clinical study of pediatric patients of mitochondrial disease showed a promising response to the ketogenic diet (Kang, Lee, Kim, Lee, & Slama, 2007), other trials indicate that for some patients, there may be undesirable side effects (Kossoff et al., 2009). This highlights the potential for complications when making sweeping dietary changes within metabolic disease. Thus it is crucial to study the effectiveness, metabolic consequences, and mechanisms of DM therapies and novel drugs for the treatment of seizures in a model of ME.

Drosophila is well-established as a model organism for the study of seizures. The widely-used bang sensitivity assay, in which several seconds of mechanical stimulation stereotypically induces a pattern of spasm, paralysis, spasm, and recovery (Lee & Wu, 2002; Parker, Howlett, Rusan, & Tanouye, 2011), has been employed for decades to examine seizures at the behavioral and genetic level, helping uncover mechanistic causes of seizures as well as genetic predispositions to and protections from their susceptibility (Benzer, 1971; Ganetzky & Wu, 1982; Howlett, Rusan, Parker, & Tanouye, 2013; Kuebler & Tanouye, 2000; Parker, Padilla, Du, Dong, & Tanouye, 2011; Pavlidis, Ramaswami, & Tanouye, 1994;

Stone, Evans, Coleman, & Kuebler, 2013). Furthermore, these behavioral and genetic studies have often been followed up or complemented with electrophysiological studies on the giant fiber (GF) pathway, enabling the understanding of *Drosophila* seizures at the cellular and circuit level (Elkins & Ganetzky, 1990; Engel & Wu, 1992; Kuebler & Tanouye, 2000; Pavlidis & Tanouye, 1995; Tanouye & Wyman, 1980; Trimarchi & Murphey, 1997). With the burgeoning field of patch clamp electrophysiology in the brain of adult *Drosophila*, seizure pathophysiology in the fly can and has been studied there (Schutte et al., 2014; L. Sun et al., 2012), establishing a powerful combination of seizure genetics, behavior, and neurophysiology in this model organism.

Using these methods and our stable animal model of ME, we have identified two promising avenues of therapy for the seizures of mitochondrial encephalomyopathy. The first is dietary modification that restricts either the amount of food or the type of food the flies may access. A ketogenic or high fat diet with either a 3:1 or 2:1 ratio of fat to sugar and protein was extremely effective, reducing seizures by 80-90% in some cases. More modest improvements were seen with a 4:1 ratio, and caloric restriction by sugar reduction and by 24-hour fasting. It will be important to address the underlying causes of the lower effectiveness of the indicated treatments, because in some cases any benefit of the dietary choices at the cellular level may be erased by the emergence of a secondary problem. For example, are flies with a progressive neuromuscular disease too sick to tolerate a full day of food deprivation? Does a 4:1 ratio of fat to other nutrients force the flies to ingest more fat than they can utilize at the expense of glucose and protein, leading to nutrient deprivation? Are certain metabolic pathways deprived while others are overwhelmed? Further studies are needed to address which of these concerns could affect patients.

Despite these remaining questions, the 2:1 and 3:1 high fat diets are clearly extremely effective even in advanced disease, which implies that there is a benefit to using fat as the primary nutrient source. *ATP6^I* is known to upregulate ketogenesis as a compensatory mechanism in the face of its mitochondrial failure early in the disease (Celotto et al., 2011), so one explanation for the improvement could be that we are providing a much higher amount of a preferred energy source, leading to improved neurological function. However, the mechanism of the neuroprotective benefits of the KD is undergoing intense scrutiny in many healthy and diseased animal models and there are many intriguing hypotheses for the basis of the benefits, including: the antioxidant properties of ketone bodies (Gasior et al., 2007; Jarrett, Milder, Liang, & Patel, 2008), increased mitochondrial biogenesis (Bough et al., 2006), changes in adenosine signaling (Danial, Hartman, Stafstrom, & Thio, 2013; Greene, 2011; Masino, Kawamura, & Ruskin, 2014; Masino et al., 2011), an altered balance between glutamate and GABA synthesis (Gano et al., 2014; Hartman, Gasior, Vining, & Rogawski, 2007), and inhibition of glutamatergic transmission via altering the chloride modulation of vGLUT (Juge et al., 2010).

Two other hypotheses that have gained traction involve K_{ATP} channels, of note in our case because our data suggests K_{ATP} needs to be present for the benefits of DM to *ATP6^I* to emerge. The first hypothesis is that K_{ATP} channels are directly modulated by ketone bodies. This phenomenon has been reported at hippocampal synapses (Kim do et al., 2015) and in the substantia nigra pars reticulata, where ketones inhibit neuronal firing by increasing K_{ATP}

open probability (Lutas et al., 2014; W. Ma, Berg, & Yellen, 2007). The second hypothesis is that glycolytic restriction deprives K_{ATP} channels of their favored ATP source – glycolysis at the plasma membrane – increasing their open probability by depleting the metabolite that promotes their closing (Dhar-Chowdhury et al., 2005; Dhar-Chowdhury et al., 2007; Hong et al., 2011; Yellen, 2008).

These two hypotheses are not mutually exclusive. In fact, considering them together may explain why we are able to manipulate K_{ATP} either positively or negatively and see a concordant change in seizure severity. Our data suggest that K_{ATP} is neuroprotective, as its loss makes seizures appear sooner, progress more quickly, and affect a higher percentage of flies (Figure 2A, 2B). Additionally, we are able to ameliorate seizures by opening K_{ATP} channels further with the KCO diazoxide, suggesting that their protective potential is not fully realized in the disease state. Thus, $ATP6^l$'s two known metabolic compensatory mechanisms may work in opposition at the K_{ATP} channel complex. Future studies will address whether K_{ATP} function or availability is compromised in ME disease and whether it is present in key brain areas where this could play a role in seizure pathogenesis.

The second promising therapeutic avenue we have discovered is the aforementioned KCO diazoxide, a drug which has been reported to act upon a number of intriguing cellular and mitochondrial targets in addition to ATP-sensitive K channels (Coetzee, 2013). These include ATPases like Complex II, succinate dehydrogenase (Dzeja et al., 2003), and even the ATP synthase complex itself (Comelli, Metelli, & Mavelli, 2007; Jasova et al., 2016). Upcoming studies will address whether diazoxide is acting via targets in addition to K_{ATP} , and whether any non- K_{ATP} effects are helpful or harmful to ME in the long-term. However, because diazoxide requires intact K_{ATP} to abrogate seizures (Figure 4) and quells excitability in the neurons of the diseased fly brain (Figures 5-7), we consider the most parsimonious explanation of its mechanism of action is through opening of K_{ATP} channels in the neuronal plasma membrane. While we have not demonstrated this definitively in our current electrophysiology experiments, future voltage clamp studies to observe K_{ATP} channel behavior directly may shed light on this outstanding question.

Whatever the mechanism, diazoxide is clearly suppressing excitability in the lateral ventral neurons in our ME model. While the effect on firing frequency in $ATP6^l$ and wild type neurons, (for which the values are only marginally different from each other) is small, especially when compared to the natural circadian variations in these neurons of 60% or more (Sheeba et al., 2008), we don't yet know if it is enough to have seizure or other behavioral implications, or whether the effect on firing frequency would be higher at other times of day, as these recordings were all performed between ZT2-6. It is possible that the K_{ATP} density in these neurons and/or their synaptic inputs is low and the effect on firing frequency would be higher in other populations. However, the effect of diazoxide on evoked excitability in these neurons is greater in magnitude, reducing the light response by 44.2% and the depolarization response by 63.8%; in the context of stress-evoked seizures, perhaps these are the more important parameters.

Conclusions

Overall, these studies are important initial steps that have revealed two promising avenues of treatment for the intractable seizures of ME, dietary modification and the drug diazoxide. Functional K_{ATP} is required to receive the benefits of these treatments in our model. Further work to elucidate the molecular basis of each therapy in an ME model will be crucial at a patient safety level due to the particular clinical concerns of metabolic dysfunction, and intriguing at a scientific level as the answers to our remaining questions may offer insights into the mechanism of both the treatment and the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ashcroft FM. The Walter B. Cannon Physiology in Perspective Lecture, 2007. ATP-sensitive K⁺ channels and disease: from molecule to malady. *Am J Physiol Endocrinol Metab.* 2007; 293(4):E880–889. DOI: 10.1152/ajpendo.00348.2007 [PubMed: 17652156]
- Ashcroft FM, Kakei M. ATP-sensitive K⁺ channels in rat pancreatic beta-cells: modulation by ATP and Mg²⁺ ions. *J Physiol.* 1989; 416:349–367. [PubMed: 2691645]
- Bellen HJ, Levis RW, He Y, Carlson JW, Evans-Holm M, Bae E, et al. Spradling AC. The Drosophila gene disruption project: progress using transposons with distinctive site specificities. *Genetics.* 2011; 188(3):731–743. DOI: 10.1534/genetics.111.126995 [PubMed: 21515576]
- Benzer S. From the gene to behavior. *JAMA.* 1971; 218(7):1015–1022. [PubMed: 4942064]
- Bough KJ, Wetherington J, Hassel B, Pare JF, Gawryluk JW, Greene JG, et al. Dingledine RJ. Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. *Ann Neurol.* 2006; 60(2):223–235. DOI: 10.1002/ana.20899 [PubMed: 16807920]
- Canafoglia L, Franceschetti S, Antozzi C, Carrara F, Farina L, Granata T, et al. Avanzini G. Epileptic phenotypes associated with mitochondrial disorders. *Neurology.* 2001; 56(10):1340–1346. [PubMed: 11376185]
- Celotto AM, Chiu WK, Van Voorhies W, Palladino MJ. Modes of metabolic compensation during mitochondrial disease using the Drosophila model of ATP6 dysfunction. *PLoS One.* 2011; 6(10):e25823.doi: 10.1371/journal.pone.0025823 [PubMed: 21991365]
- Celotto AM, Frank AC, McGrath SW, Fergestad T, Van Voorhies WA, Buttle KF, et al. Palladino MJ. Mitochondrial encephalomyopathy in Drosophila. *J Neurosci.* 2006; 26(3):810–820. DOI: 10.1523/JNEUROSCI.4162-05.2006 [PubMed: 16421301]
- Coetzee WA. Multiplicity of effectors of the cardioprotective agent, diazoxide. *Pharmacol Ther.* 2013; 140(2):167–175. DOI: 10.1016/j.pharmthera.2013.06.007 [PubMed: 23792087]
- Comelli M, Metelli G, Mavelli I. Downmodulation of mitochondrial F0F1 ATP synthase by diazoxide in cardiac myoblasts: a dual effect of the drug. *Am J Physiol Heart Circ Physiol.* 2007; 292(2):H820–829. DOI: 10.1152/ajpheart.00366.2006 [PubMed: 17287451]

- Danial NN, Hartman AL, Stafstrom CE, Thio LL. How does the ketogenic diet work? Four potential mechanisms. *J Child Neurol*. 2013; 28(8):1027–1033. DOI: 10.1177/0883073813487598 [PubMed: 23670253]
- Dhar-Chowdhury P, Harrell MD, Han SY, Jankowska D, Parachuru L, Morrissey A, et al. Coetzee WA. The glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, and pyruvate kinase are components of the K(ATP) channel macromolecular complex and regulate its function. *J Biol Chem*. 2005; 280(46):38464–38470. DOI: 10.1074/jbc.M508744200 [PubMed: 16170200]
- Dhar-Chowdhury P, Malester B, Rajacic P, Coetzee WA. The regulation of ion channels and transporters by glycolytically derived ATP. *Cell Mol Life Sci*. 2007; 64(23):3069–3083. DOI: 10.1007/s00018-007-7332-3 [PubMed: 17882378]
- Di Donato S. Multisystem manifestations of mitochondrial disorders. *J Neurol*. 2009; 256(5):693–710. DOI: 10.1007/s00415-009-5028-3 [PubMed: 19252802]
- DiMauro S, Schon EA. Mitochondrial respiratory-chain diseases. *N Engl J Med*. 2003; 348(26):2656–2668. DOI: 10.1056/NEJMra022567 [PubMed: 12826641]
- DiMauro S, Schon EA. Mitochondrial disorders in the nervous system. *Annu Rev Neurosci*. 2008; 31:91–123. DOI: 10.1146/annurev.neuro.30.051606.094302 [PubMed: 18333761]
- DiMauro S, Schon EA, Carelli V, Hirano M. The clinical maze of mitochondrial neurology. *Nat Rev Neurol*. 2013; 9(8):429–444. DOI: 10.1038/nrneurol.2013.126 [PubMed: 23835535]
- DiMauro S, Zeviani M, Moraes CT, Nakase H, Rizzuto R, Lombes A, et al. Schon EA. Mitochondrial encephalomyopathies. *Prog Clin Biol Res*. 1989; 306:117–128. [PubMed: 2544896]
- Dunne MJ, Cosgrove KE, Shepherd RM, Aynsley-Green A, Lindley KJ. Hyperinsulinism in infancy: from basic science to clinical disease. *Physiol Rev*. 2004; 84(1):239–275. DOI: 10.1152/physrev.00022.2003 [PubMed: 14715916]
- Dunne MJ, Petersen OH. Intracellular ADP activates K⁺ channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Lett*. 1986; 208(1):59–62. [PubMed: 2429868]
- Dzeja PP, Bast P, Ozcan C, Valverde A, Holmuhamedov EL, Van Wylen DG, Terzic A. Targeting nucleotide-requiring enzymes: implications for diazoxide-induced cardioprotection. *Am J Physiol Heart Circ Physiol*. 2003; 284(4):H1048–1056. [PubMed: 12666660]
- Elkins T, Ganetzky B. Conduction in the giant nerve fiber pathway in temperature-sensitive paralytic mutants of *Drosophila*. *J Neurogenet*. 1990; 6(4):207–219. [PubMed: 2231176]
- Engel JE, Wu CF. Interactions of membrane excitability mutations affecting potassium and sodium currents in the flight and giant fiber escape systems of *Drosophila*. *J Comp Physiol A*. 1992; 171(1):93–104. [PubMed: 1328625]
- Finsterer J. Mitochondriopathies. *Eur J Neurol*. 2004; 11(3):163–186. [PubMed: 15009163]
- Fogle KJ, Baik LS, Houl JH, Tran TT, Roberts L, Dahm NA, et al. Holmes TC. CRYPTOCHROME-mediated phototransduction by modulation of the potassium ion channel beta-subunit redox sensor. *Proc Natl Acad Sci U S A*. 2015; 112(7):2245–2250. DOI: 10.1073/pnas.1416586112 [PubMed: 25646452]
- Fogle KJ, Parson KG, Dahm NA, Holmes TC. CRYPTOCHROME is a blue-light sensor that regulates neuronal firing rate. *Science*. 2011; 331(6023):1409–1413. DOI: 10.1126/science.1199702 [PubMed: 21385718]
- Freeman JM, Vining EP. Ketogenic diet: a time-tested, effective, and safe method for treatment of intractable childhood epilepsy. *Epilepsia*. 1998; 39(4):450–451. [PubMed: 9578037]
- Ganetzky B, Wu CF. Indirect Suppression Involving Behavioral Mutants with Altered Nerve Excitability in *DROSOPHILA MELANOGASTER*. *Genetics*. 1982; 100(4):597–614. [PubMed: 17246073]
- Gano LB, Patel M, Rho JM. Ketogenic diets, mitochondria, and neurological diseases. *J Lipid Res*. 2014; 55(11):2211–2228. DOI: 10.1194/jlr.R048975 [PubMed: 24847102]
- Gasior M, French A, Joy MT, Tang RS, Hartman AL, Rogawski MA. The anticonvulsant activity of acetone, the major ketone body in the ketogenic diet, is not dependent on its metabolites acetol, 1,2-propanediol, methylglyoxal, or pyruvic acid. *Epilepsia*. 2007; 48(4):793–800. DOI: 10.1111/j.1528-1167.2007.01026.x [PubMed: 17386058]

- Greene RW. Adenosine: front and center in linking nutrition and metabolism to neuronal activity. *J Clin Invest.* 2011; 121(7):2548–2550. DOI: 10.1172/JCI58391 [PubMed: 21701073]
- Gribble FM, Proks P, Corkey BE, Ashcroft FM. Mechanism of cloned ATP-sensitive potassium channel activation by oleoyl-CoA. *J Biol Chem.* 1998; 273(41):26383–26387. [PubMed: 9756869]
- Gribble FM, Tucker SJ, Ashcroft FM. The essential role of the Walker A motifs of SUR1 in K-ATP channel activation by Mg-ADP and diazoxide. *EMBO J.* 1997; 16(6):1145–1152. DOI: 10.1093/emboj/16.6.1145 [PubMed: 9135131]
- Hartman AL, Gasior M, Vining EP, Rogawski MA. The neuropharmacology of the ketogenic diet. *Pediatr Neurol.* 2007; 36(5):281–292. DOI: 10.1016/j.pediatrneurol.2007.02.008 [PubMed: 17509459]
- Hibino H, Inanobe A, Furutani K, Murakami S, Findlay I, Kurachi Y. Inwardly rectifying potassium channels: their structure, function, and physiological roles. *Physiol Rev.* 2010; 90(1):291–366. DOI: 10.1152/physrev.00021.2009 [PubMed: 20086079]
- Hong M, Kefaloyianni E, Bao L, Malester B, Delaroché D, Neubert TA, Coetzee WA. Cardiac ATP-sensitive K⁺ channel associates with the glycolytic enzyme complex. *FASEB J.* 2011; 25(7):2456–2467. DOI: 10.1096/fj.10-176669 [PubMed: 21482559]
- Howlett IC, Rusan ZM, Parker L, Tanouye MA. Drosophila as a model for intractable epilepsy: gilgamesh suppresses seizures in para(bss1) heterozygote flies. *G3 (Bethesda).* 2013; 3(8):1399–1407. DOI: 10.1534/g3.113.006130 [PubMed: 23797108]
- Inagaki N, Gonoi T, Clement JPt, Namba N, Inazawa J, Gonzalez G, et al. Bryan J. Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. *Science.* 1995; 270(5239):1166–1170. [PubMed: 7502040]
- Inagaki N, Gonoi T, Seino S. Subunit stoichiometry of the pancreatic beta-cell ATP-sensitive K⁺ channel. *FEBS Lett.* 1997; 409(2):232–236. [PubMed: 9202152]
- Jarrett SG, Milder JB, Liang LP, Patel M. The ketogenic diet increases mitochondrial glutathione levels. *J Neurochem.* 2008; 106(3):1044–1051. DOI: 10.1111/j.1471-4159.2008.05460.x [PubMed: 18466343]
- Jasova M, Kancirova I, Murarikova M, Farkasova V, Waczulikova I, Ravingerova T, et al. Ferko M. Stimulation of mitochondrial ATP synthase activity - a new diazoxide-mediated mechanism of cardioprotection. *Physiol Res.* 2016; 65(1):S119–127. [PubMed: 27643934]
- Juge N, Gray JA, Omote H, Miyaji T, Inoue T, Hara C, et al. Moriyama Y. Metabolic control of vesicular glutamate transport and release. *Neuron.* 2010; 68(1):99–112. DOI: 10.1016/j.neuron.2010.09.002 [PubMed: 20920794]
- Jung J, Mauguier F, Clerc-Renaud P, Ollagnon E, Mousson de Camaret B, Ryvlin P. NARP mitochondrialriopathy: an unusual cause of progressive myoclonic epilepsy. *Neurology.* 2007; 68(17):1429–1430. DOI: 10.1212/01.wnl.0000264019.53959.10 [PubMed: 17452590]
- Kang HC, Lee YM, Kim HD, Lee JS, Slama A. Safe and effective use of the ketogenic diet in children with epilepsy and mitochondrial respiratory chain complex defects. *Epilepsia.* 2007; 48(1):82–88. DOI: 10.1111/j.1528-1167.2006.00906.x
- Kawamura M Jr, Ruskin DN, Geiger JD, Boison D, Masino SA. Ketogenic diet sensitizes glucose control of hippocampal excitability. *J Lipid Res.* 2014; 55(11):2254–2260. DOI: 10.1194/jlr.M046755 [PubMed: 25170119]
- Kim do Y, Abdelwahab MG, Lee SH, O'Neill D, Thompson RJ, Duff HJ, et al. Rho JM. Ketones prevent oxidative impairment of hippocampal synaptic integrity through KATP channels. *PLoS One.* 2015; 10(4):e0119316.doi: 10.1371/journal.pone.0119316 [PubMed: 25848768]
- Kossoff EH, Zupec-Kania BA, Amark PE, Ballaban-Gil KR, Christina Bergqvist AG, Blackford R, et al. International Ketogenic Diet Study, G. Optimal clinical management of children receiving the ketogenic diet: recommendations of the International Ketogenic Diet Study Group. *Epilepsia.* 2009; 50(2):304–317. DOI: 10.1111/j.1528-1167.2008.01765.x [PubMed: 18823325]
- Kuebler D, Tanouye MA. Modifications of seizure susceptibility in Drosophila. *J Neurophysiol.* 2000; 83(2):998–1009. [PubMed: 10669511]
- Larsson O, Deeney JT, Branstrom R, Berggren PO, Corkey BE. Activation of the ATP-sensitive K⁺ channel by long chain acyl-CoA. A role in modulation of pancreatic beta-cell glucose sensitivity. *J Biol Chem.* 1996; 271(18):10623–10626. [PubMed: 8631866]

- Lee J, Wu CF. Electroconvulsive seizure behavior in *Drosophila*: analysis of the physiological repertoire underlying a stereotyped action pattern in bang-sensitive mutants. *J Neurosci*. 2002; 22(24):11065–11079. [PubMed: 12486202]
- Leonard JV, Schapira AH. Mitochondrial respiratory chain disorders I: mitochondrial DNA defects. *Lancet*. 2000; 355(9200):299–304. [PubMed: 10675086]
- Lutas A, Birnbaumer L, Yellen G. Metabolism regulates the spontaneous firing of substantia nigra pars reticulata neurons via KATP and nonselective cation channels. *J Neurosci*. 2014; 34(49):16336–16347. DOI: 10.1523/JNEUROSCI.1357-14.2014 [PubMed: 25471572]
- Ma H, O'Farrell PH. Selections that isolate recombinant mitochondrial genomes in animals. *Elife*. 2015; 4doi: 10.7554/eLife.07247
- Ma H, O'Farrell PH. Selfish drive can trump function when animal mitochondrial genomes compete. *Nat Genet*. 2016; 48(7):798–802. DOI: 10.1038/ng.3587 [PubMed: 27270106]
- Ma W, Berg J, Yellen G. Ketogenic diet metabolites reduce firing in central neurons by opening K(ATP) channels. *J Neurosci*. 2007; 27(14):3618–3625. DOI: 10.1523/JNEUROSCI.0132-07.2007 [PubMed: 17409226]
- Masino SA, Kawamura M Jr, Ruskin DN. Adenosine receptors and epilepsy: current evidence and future potential. *Int Rev Neurobiol*. 2014; 119:233–255. DOI: 10.1016/B978-0-12-801022-8.00011-8 [PubMed: 25175969]
- Masino SA, Li T, Theofilas P, Sandau US, Ruskin DN, Fredholm BB, et al. Boison D. A ketogenic diet suppresses seizures in mice through adenosine A(1) receptors. *J Clin Invest*. 2011; 121(7):2679–2683. DOI: 10.1172/JCI57813 [PubMed: 21701065]
- McCarthy EV, Wu Y, Decarvalho T, Brandt C, Cao G, Nitabach MN. Synchronized bilateral synaptic inputs to *Drosophila melanogaster* neuropeptidergic rest/arousal neurons. *J Neurosci*. 2011; 31(22):8181–8193. DOI: 10.1523/JNEUROSCI.2017-10.2011 [PubMed: 21632940]
- Metaxakis A, Oehler S, Klinakis A, Savakis C. Minos as a genetic and genomic tool in *Drosophila melanogaster*. *Genetics*. 2005; 171(2):571–581. DOI: 10.1534/genetics.105.041848 [PubMed: 15972463]
- Neal EG, Chaffe H, Schwartz RH, Lawson MS, Edwards N, Fitzsimmons G, et al. Cross JH. The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. *Lancet Neurol*. 2008; 7(6):500–506. DOI: 10.1016/S1474-4422(08)70092-9 [PubMed: 18456557]
- Nichols CG, Shyng SL, Nestorowicz A, Glaser B, Clement JPt, Gonzalez G, et al. Bryan J. Adenosine diphosphate as an intracellular regulator of insulin secretion. *Science*. 1996; 272(5269):1785–1787. [PubMed: 8650576]
- Palladino MJ. Modeling mitochondrial encephalomyopathy in *Drosophila*. *Neurobiol Dis*. 2010; 40(1): 40–45. DOI: 10.1016/j.nbd.2010.05.009 [PubMed: 20472065]
- Parker L, Howlett IC, Rusan ZM, Tanouye MA. Seizure and epilepsy: studies of seizure disorders in *Drosophila*. *Int Rev Neurobiol*. 2011; 99:1–21. DOI: 10.1016/B978-0-12-387003-2.00001-X [PubMed: 21906534]
- Parker L, Padilla M, Du Y, Dong K, Tanouye MA. *Drosophila* as a model for epilepsy: bss is a gain-of-function mutation in the para sodium channel gene that leads to seizures. *Genetics*. 2011; 187(2): 523–534. DOI: 10.1534/genetics.110.123299 [PubMed: 21115970]
- Pavlidis P, Ramaswami M, Tanouye MA. The *Drosophila* easily shocked gene: a mutation in a phospholipid synthetic pathway causes seizure, neuronal failure, and paralysis. *Cell*. 1994; 79(1): 23–33. [PubMed: 7923374]
- Pavlidis P, Tanouye MA. Seizures and failures in the giant fiber pathway of *Drosophila* bang-sensitive paralytic mutants. *J Neurosci*. 1995; 15(8):5810–5819. [PubMed: 7643221]
- Sakura H, Ammala C, Smith PA, Gribble FM, Ashcroft FM. Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic beta-cells, brain, heart and skeletal muscle. *FEBS Lett*. 1995; 377(3):338–344. DOI: 10.1016/0014-5793(95)01369-5 [PubMed: 8549751]
- Santorelli FM, Tanji K, Shanske S, DiMauro S. Heterogeneous clinical presentation of the mtDNA NARP/T8993G mutation. *Neurology*. 1997; 49(1):270–273. [PubMed: 9222207]
- Schapira AH, Cock HR. Mitochondrial myopathies and encephalomyopathies. *Eur J Clin Invest*. 1999; 29(10):886–898. [PubMed: 10583431]

- Schutte RJ, Schutte SS, Algara J, Barragan EV, Gilligan J, Staber C, et al. O'Dowd DK. Knock-in model of Dravet syndrome reveals a constitutive and conditional reduction in sodium current. *J Neurophysiol.* 2014; 112(4):903–912. DOI: 10.1152/jn.00135.2014 [PubMed: 24805083]
- Seino S. ATP-sensitive potassium channels: a model of heteromultimeric potassium channel/receptor assemblies. *Annu Rev Physiol.* 1999; 61:337–362. DOI: 10.1146/annurev.physiol.61.1.337 [PubMed: 10099692]
- Sheeba V, Gu H, Sharma VK, O'Dowd DK, Holmes TC. Circadian- and light-dependent regulation of resting membrane potential and spontaneous action potential firing of *Drosophila* circadian pacemaker neurons. *J Neurophysiol.* 2008; 99(2):976–988. DOI: 10.1152/jn.00930.2007 [PubMed: 18077664]
- Shyng SL, Nichols CG. Membrane phospholipid control of nucleotide sensitivity of KATP channels. *Science.* 1998; 282(5391):1138–1141. [PubMed: 9804554]
- Stafstrom CE, Rho JM. The ketogenic diet as a treatment paradigm for diverse neurological disorders. *Front Pharmacol.* 2012; 3:59.doi: 10.3389/fphar.2012.00059 [PubMed: 22509165]
- Stone B, Evans L, Coleman J, Kuebler D. Genetic and pharmacological manipulations that alter metabolism suppress seizure-like activity in *Drosophila*. *Brain Res.* 2013; 1496:94–103. DOI: 10.1016/j.brainres.2012.12.007 [PubMed: 23247062]
- Sun HS, Feng ZP. Neuroprotective role of ATP-sensitive potassium channels in cerebral ischemia. *Acta Pharmacol Sin.* 2013; 34(1):24–32. DOI: 10.1038/aps.2012.138 [PubMed: 23123646]
- Sun HS, Feng ZP, Miki T, Seino S, French RJ. Enhanced neuronal damage after ischemic insults in mice lacking Kir6.2-containing ATP-sensitive K⁺ channels. *J Neurophysiol.* 2006; 95(4):2590–2601. DOI: 10.1152/jn.00970.2005 [PubMed: 16354731]
- Sun L, Gilligan J, Staber C, Schutte RJ, Nguyen V, O'Dowd DK, Reenan R. A knock-in model of human epilepsy in *Drosophila* reveals a novel cellular mechanism associated with heat-induced seizure. *J Neurosci.* 2012; 32(41):14145–14155. DOI: 10.1523/JNEUROSCI.2932-12.2012 [PubMed: 23055484]
- Tanouye MA, Wyman RJ. Motor outputs of giant nerve fiber in *Drosophila*. *J Neurophysiol.* 1980; 44(2):405–421. [PubMed: 6774064]
- Tinker A, Aziz Q, Thomas A. The role of ATP-sensitive potassium channels in cellular function and protection in the cardiovascular system. *Br J Pharmacol.* 2014; 171(1):12–23. DOI: 10.1111/bph.12407 [PubMed: 24102106]
- Trimarchi JR, Murphey RK. The shaking-B2 mutation disrupts electrical synapses in a flight circuit in adult *Drosophila*. *J Neurosci.* 1997; 17(12):4700–4710. [PubMed: 9169530]
- Tucker SJ, Gribble FM, Zhao C, Trapp S, Ashcroft FM. Truncation of Kir6.2 produces ATP-sensitive K⁺ channels in the absence of the sulphonylurea receptor. *Nature.* 1997; 387(6629):179–183. DOI: 10.1038/387179a0 [PubMed: 9144288]
- Vining EP, Freeman JM, Ballaban-Gil K, Camfield CS, Camfield PR, Holmes GL, et al. Wheless JW. A multicenter study of the efficacy of the ketogenic diet. *Arch Neurol.* 1998; 55(11):1433–1437. [PubMed: 9823827]
- Yang H, Guo R, Wu J, Peng Y, Xie D, Zheng W, et al. Song Z. The antiepileptic effect of the glycolytic inhibitor 2-deoxy-D-glucose is mediated by upregulation of K(ATP) channel subunits Kir6.1 and Kir6.2. *Neurochem Res.* 2013; 38(4):677–685. DOI: 10.1007/s11064-012-0958-z [PubMed: 23475455]
- Yellen G. Ketone bodies, glycolysis, and KATP channels in the mechanism of the ketogenic diet. *Epilepsia.* 2008; 49(8):80–82. DOI: 10.1111/j.1528-1167.2008.01843.x
- Zerangue N, Schwappach B, Jan YN, Jan LY. A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron.* 1999; 22(3):537–548. [PubMed: 10197533]

Biographies

Dr. Keri J. Fogle received a Ph.D. from the Dept. of Neurobiology and Behavior at Columbia University and postdoctoral training in the Dept. of Physiology and Biophysics at

the University of California, Irvine, and is currently a researcher in the Department of Pharmacology & Chemical Biology at the University of Pittsburgh School of Medicine.

James Ian Hertzler is an undergraduate student at the University of Pittsburgh majoring in neuroscience.

Joy H. Shon is an undergraduate student at the University of Pittsburgh majoring in neuroscience.

Dr. Michael J. Palladino received a PhD from the University of Connecticut School of Medicine, trained as a Jane Coffin Child's postdoctoral fellow with Dr. Barry Ganetzky at the University of Wisconsin, Madison, and is currently Professor and Vice-chair of academics in the Department of Pharmacology & Chemical Biology at the University of Pittsburgh School of Medicine.

Figure 1. Dietary modification reduces time to recovery (TTR) from seizures in the *ATP6^{IR}* model of mitochondrial encephalomyopathy

A. Progressive time course of *ATP6^{IR}* vs. wild type mitochondrial flies (w1118). At Day 15 ME flies begin to show measurable seizure activity ($4.92s \pm .49$, $n=113$) which progresses at day 18 ($26.9s \pm 3.3$, $n=87$) and day 21 ($77.4s \pm 3.6$, $n=111$). In contrast, nuclear genetic background-matched wild type mitochondrial flies do not show seizures at any time point (TTRs for Day 15= $0.30s \pm 0.1$, $n=94$; Day 18= $0.38s \pm .11$, $n=71$; Day 21= $0.24s \pm .07$, $n=92$. $p<.0001$ in each case).

B. Forced fasting for 24 hours reduces seizure in *ATP6^{IR}* flies at the intermediate time point (Red bars. Day 18= $12.87s \pm 2.0$, $n=41$, $p=.036$). Although time to recovery at day 15 ($3.24s \pm .07$, $n=43$) and day 21 ($64.75s \pm 7.3$, $n=40$) are trending lower, these values do not reach significance ($p=.11$ and $.18$, respectively). Reducing the sugar content of the flies' normal media was more beneficial to seizure recovery times. A 25% reduction (violet bars) was effective, reducing TTRs to $3.08s \pm .52$, $n=70$, $p=.013$; $8.86s \pm 1.32$, $n=63$, $p<.001$; and $59.12s \pm 4.5$, $n=68$, $p=.003$ at days 15, 18, and 21 respectively.

C. Offering flies a media with high fat content in most cases drastically abrogated time to recovery after seizure. At a 4:1 ratio of fat to carbohydrates (dark blue bars), TTR was reduced to $4.5s \pm .53$, $n=108$; $6.7s \pm .8$, $n=113$; and $50.6s \pm 6.9$, $n=47$ at days 15, 18, and 21; $p=0.91$, $<.001$, and $<.001$. vs. *ATP6^{IR}* fed normally. At a 3:1 ratio (intermediate blue), day 15= $2.0s \pm 0.3$, $n=61$, $p<.0001$; day 18 = $2.6s \pm 0.5$, $n=58$, $p<.001$; day 21 = $8.7s \pm 1.1$, $n=55$, $p<.001$ vs. *ATP6^{IR}* fed normally. At a 2:1 ratio (light blue bars), day 15 = $2.9s \pm 0.42$, $n=60$, $p=.032$; day 18 = $4.9s \pm .75$, $n=56$, $p<.001$; day 21 = $9.16s \pm .94$, $n=57$, $p<.001$.

D. The penetrance of the bang sensitive phenotype in *ATP6IR* under dietary modifications was calculated. On standard media (white bars), the penetrance was 80.5%, 88.5%, and 100% at days 15, 18, and 21. On KD 2:1 (light blue) it dropped to 53.3%, 64.4%, and 96.5%; KD 3:1 (medium blue) it dropped to 60.7%, 50.0%, and 90.9%; on KD 4:1 (dark blue) the values are 63.9%, 80.5%, and 100% penetrance. Caloric restriction by fasting (red) reduced day 15 penetrance to 48.8% but failed to positively affect the parameter at day 18 or 21, with 95.1% and 100% penetrance. Low nutrient media (violet) followed the same pattern, resulting in penetrance of 44.3%, 81%, and 100%.

Figure 2. The K_{ATP} channel complex is seizure-protective in ME and required for the benefits of dietary modification

A. *ATP6^{1R}* flies that lack functional K_{ATP} have earlier onset and more severe seizures. At day 12, *ATP6^{1R}* flies have not yet begun to exhibit a seizure phenotype ($1.64s \pm 0.3$, $n=58$). In contrast, *ATP6^{1R} K_{ATP}^{dr-1}* and *dr-2* double mutants already have measurable seizures (*dr-1*: $12.2s \pm 9$, $n=44$; *dr-2*: $20.7s \pm 1.14$, $n=60$; $p < .001$ vs. *ATP6^{1R}* for both). At days 15 and 18, both *ATP6^{1R} K_{ATP}* double mutants experience significantly worse seizures than *ATP6^{1R}* alone. (*dr-1*, day 15: $24.88s \pm 3.5$, $n=53$, $p < .001$ and day 18: $60.1s \pm 3.9$, $n=62$, $p < .001$; *dr-2*, day 15: $43.0s \pm 10.6$, $n=33$, $p < .001$ and day 18: $73.75s \pm 7.25$, $n=31$, $p < .001$). By advanced disease at day 21, *ATP6^{1R} K_{ATP}^{dr-1}* are still significantly worse than *ATP6^{1R}* (*dr-1*: $93.8s \pm 3.9$, $n=48$, $p=.018$; *dr-2*: $89.4s \pm 6.7$, $n=25$, $p=.266$).

B. Tolbutamide, a sulfonylurea drug which promotes K_{ATP} channel closing, worsens seizure phenotypes. After 24 hours of treatment (flies are 18 days old) tolbutamide-treated *ATP6^{1R}* flies take $33.19s \pm 3.96$ ($n=49$) to recover while vehicle-treated flies (ethanol) take $18.0s \pm 2.8$ ($n=49$), $p=.002$. After 72 hours of treatment (flies are 20 days old), tolbutamide-treated flies take $73.74s \pm 3.42$ ($n=49$) to recover while ethanol-treated flies take only $63.6s \pm 3.63$ ($n=49$), $p=.045$.

C. Dietary modification fails to reduce the TTR for the *ATP6^{1R}; K_{ATP}^{dr-1}* double mutant. At day 15, 24-hour fasting (noted as “CR” in the key; $37.54s \pm 10.6$, $n=8$) and the 3:1 ketogenic diet ($25.12s \pm 3.1$, $n=39$) do not differ from flies fed standard media ($p=.363$ by ANOVA).

At day 18, 24-hour fasting worsened the seizures of this double mutant ($101.74s \pm 8.6$, $n=7$, $p=.003$ compared to age-matched flies fed on standard media). The ketogenic diet had no effect at this age ($70.5s \pm 5.3$, $n=38$, $p=.242$), nor did fasting and the ketogenic diet at 21 days ($93.76s \pm 5.26$, $n=7$ for fasting and $105.13s \pm 5.96$, $n=36$ for the KD, $p=.222$ by ANOVA).

D. Similarly, dietary modification fails to improve or worsens the TTR for the *ATP6^{IR}*; *K_{ATPdr-2}* double mutant. At day 15, CR time to recovery is $41.46s \pm 4.9$, $n=42$; KD time to recovery is $41.88s \pm 4.3$, $n=54$, $p=0.981$ vs. flies fed on standard media. At day 18, CR worsens the TTR to $102.93s \pm 5.1$, $n=48$, $p=.001$ and the KD does not effect any change ($82.37s \pm 4.7$, $n=51$, $p=.543$). At day 21, both CR and the KD drastically worsen TTRs ($121.6s \pm 4.23$, $n=43$ for CR; $117.7s \pm 3.3$, $n=50$, $p<.001$ vs. standard media-fed flies).

Figure 3. Pharmacological treatment of flies with the potassium channel opener diazoxide improves seizure phenotypes in a dose-dependent manner

A. 17 day-old *ATP6^{IR}* flies treated with 900 μ M diazoxide, (dark green squares) show a significantly lower TTR than their time matched DMSO vehicle counterparts (gray squares) at 24 hours (center panel) (diazoxide = $33.7s \pm 2.2$, $n=103$; DMSO = $52.5s \pm 2.8$, $n=102$; $p < .001$) and at 72 hours (right panel) (diazoxide = $63.6s \pm 2.8$, $n=99$; DMSO = $80.96s \pm 2.86$, $n=98$; $p < .001$). At 675 μ M treatment for 72 hours reduces TTR significantly ($79.64s \pm 1.3$, $n=22$) vs. DMSO ($96.07s \pm 6.42$, $n=19$, $p=.01$) while at 24 hours this concentration reduces TTR slightly but does not reach significance (diazoxide = $61.4s \pm 5.19$, $n=22$; DMSO = $73.3s \pm 8.45$, $n=20$, $p=.228$). At 450 μ M, treatment with diazoxide does not significantly improve seizures at 24 or 72 hours, $p=.256$ and $.849$). Before treatment, flies that would go on to be treated with either diazoxide or DMSO did not differ for any concentration (left panel). In the three panels, all triangles, circles, and squares represent the same cohorts of flies as they aged/were treated, minus any incidental deaths during the course of the experiment.

B. At 24 hours, the change in TTR compared to time matched vehicle groups were -6.6%, -16.2%, and -35.8% for low, intermediate, and maximum concentrations, respectively. At 72 hours, the reductions were -1.1%, -17.1%, and -21.4%, respectively.

Figure 4. Seizure phenotype improvement with diazoxide is dependent upon functional K_{ATP}

A. $ATP6^{1R} K_{ATPdr-2}$ was treated with a 900 μ M concentration of diazoxide or a time matched treatment of DMSO. Before treatment, the groups (age 17 days) recovered at 82.07s $\pm$ 2.5 (n=15) for DMSO and 71.52s $\pm$ 7.9 (n=13, p=.16). After treatment, no difference was seen with diazoxide treatment (100.92s $\pm$ 5.0, n=18) as compared to DMSO treatment (102.37s $\pm$ 3.67, n=12, p=.835).

B. The percent change in TTR with maximum diazoxide treatment at 24 hours for $ATP6^{1R}$ (35.8%) vs. $ATP6^{1R} K_{ATPdr-2}$ (1.1%) is shown.

Figure 5. The K_{ATP} channel opener diazoxide slightly reduces spontaneous l-LNv firing frequency

A. Bath application of 100μM diazoxide to acutely dissected fly brain preps reduces firing frequency in the large lateral ventral neurons (age 22d *ATP6^l; sesB^l/sesB⁺; CRYGAL4-UAS-NC1-GFP* fly shown).

B. Quantified reduction of FF. In wild type flies (*ATP6⁺; sesB^l/sesB⁺; CRYGAL4-UAS-NC1-GFP*) ages 21-24 days, diazoxide application reduced firing frequency from 5.18Hz ± 0.24 (n=27) to 4.54Hz ± 0.47 (n=19, p=0.19, 12.4% reduction). In age-matched *ATP6^l; sesB^l/sesB⁺; CRYGAL4-UAS-NC1-GFP* neurons, firing frequency was lowered to borderline significance, from 4.5Hz ± 0.17 (n=39) to 4.0Hz ± 0.22 (n=30, p=.046, 12.5% reduction). The sampling of firing frequencies before and after diazoxide application (n values) were taken from 7 *ATP6^l* flies and 6 wild type flies.

Figure 6. The K_{ATP} channel opener diazoxide reduces the acute response to light in the large lateral ventral neurons

A. The control response of the LNV to bright light (top) is reduced in $ATP6^l; sesB^l/sesB^+; CRYGAL4-UAS-NC1-GFP$ neurons after 3-10 minutes of DZ exposure (bottom; same cell). Dark bar over the first four seconds of the trace represents dark conditions whereas the white bar over the second four seconds represents the bright white light exposure that increases the firing frequency of the large lateral ventral neurons.

B. Quantified reduction of the light response. The light response (firing frequency on / firing frequency off) of $ATP6^+; sesB^l/sesB^+; CRYGAL4-UAS-NC1-GFP$ (wild type) flies is 1.25 ± 0.11 (n=3) before diazoxide treatment and 1.32 ± 0.14 (n=7) after diazoxide treatment (p=.728), a 28% increase. $ATP6^l; sesB^l/sesB^+; CRYGAL4-UAS-NC1-GFP$ flies exhibit a significantly reduced light response after diazoxide exposure, seen as a decrease in FF on / FF off ratio, from 1.43 ± 0.05 (n=5) to 1.24 ± 0.04 (n=14), p=.017, a 44.2% decrease. 3 wild type flies and 5 $ATP6^l$ flies were used to obtain this data.

Figure 7. The K_{ATP} channel opener diazoxide reduces the evoked excitability in response to depolarizing current injections

A. Voltage traces of $ATP6^l; sesB^l/sesB^{+}; CRYGAL4-UAS-NC1-GFP$ neurons in the absence (top) and presence (bottom) of diazoxide, given current injections of ± 20 and 25 pA. The evoked response (increase in FF over baseline) appears more extreme in the absence of DZ than with DZ present.

B. Quantification of the effect of DZ on evoked depolarization. In $ATP6^{+/+}; sesB^l/sesB^{+}; CRYGAL4-UAS-NC1-GFP$ (wild type) flies, the evoked depolarization normalized firing frequency response is 7.5 ± 1.9 ($n=9$) in the absence of diazoxide and 1.9 ± 0.2 ($n=6$) in the presence of diazoxide ($p=.036$), a 74.7% decrease. In $ATP6^l; sesB^l/sesB^{+}; CRYGAL4-UAS-NC1-GFP$ flies, the evoked response decreases from 16.3 ± 3.9 ($n=12$) to 5.9 ± 1.2 ($n=9$) after diazoxide treatment ($p=.037$), a 63.8% decrease. This data was obtained from 4 wild type flies in the absence of diazoxide, 3 wild type flies in the presence of diazoxide, 5 $ATP6^l$ flies in the absence of diazoxide, and 4 $ATP6^l$ flies in the presence of diazoxide.