

RESEARCH ARTICLE

Contribution of a natural polymorphism in protein kinase G modulates electroconvulsive seizure recovery in *Drosophila melanogaster*

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ABSTRACT

Drosophila melanogaster is a well-characterized model for neurological disorders and is widely used for investigating causes of altered neuronal excitability leading to seizure-like behavior. One method used to analyze behavioral output of neuronal perturbation is recording the time to locomotor recovery from an electroconvulsive shock. Based on this behavior, we sought to quantify seizure susceptibility in larval *D. melanogaster* with differences in the enzymatic activity levels of a major protein, cGMP-dependent protein kinase (PKG). PKG, encoded by *foraging*, has two natural allelic variants and has previously been implicated in several important physiological characteristics including: foraging patterns, learning and memory, and environmental stress tolerance. The well-established NO/cGMP/PKG signaling pathway found in the fly, which potentially targets downstream K⁺ channel(s), ultimately impacts membrane excitability, leading to our hypothesis: altering PKG enzymatic activity modulates time to recovery from an electroconvulsive seizure. Our results show that by both genetically and pharmacologically increasing PKG enzymatic activity, we can decrease the locomotor recovery time from an electroconvulsive seizure in larval *D. melanogaster*.

KEY WORDS: Seizure, Epilepsy, PKG, Protein kinase G, Neuronal excitability

INTRODUCTION

A seizure is a physical manifestation of altered cellular excitability. These events are relatively common, occurring in about 1% of the population, and can be caused by a number of scenarios including: head trauma, fever, lack of sleep and certain medications (England et al., 2012). It is possible, however, that sufferers are more susceptible based on genetic predisposition, most commonly neuronal channelopathies (Parker et al., 2011b; Kaplan et al., 2016). While the individual mechanisms that induce a seizure are unknown in most patients, reoccurring seizures can be treated with the use of antiepileptic drugs, many of which have been discovered using rodent models of epilepsy (White et al., 1998). However, while mammalian models of epilepsy have been successfully utilized for novel anti-epileptic drug (AED) discovery in the past, there are drawbacks such as genetic variation between strains, high

expense and the need for invasive procedures to analyze seizure mechanisms (Cunliffe et al., 2015). Consequently, there may be benefits to the further development of invertebrate seizure models which exhibit seizure-like behaviors in similar ways to rodent models (Parker et al., 2011a; Song and Tanouye, 2008; Tickoo and Russell, 2002; Marley and Baines, 2011). Additionally, conservation of many genes and proteins involved in seizure susceptibility across species enables simpler and less costly investigation into drug screening and epileptic mechanisms.

The fruit fly, *Drosophila melanogaster*, exhibits high homology to mammalian nervous systems in terms of membrane make-up and ion channel functioning and its genome contains functional homologs of approximately 75% of human disease genes (Lloyd and Taylor, 2010). This enables straightforward behavioral analysis of complex behaviors and physiological functioning that can be compared with that of both rodents and humans (Renger et al., 1999; Barrese et al., 2010). In addition, robust and publicly available genetic manipulative techniques have made flies an attractive seizure model for several decades, nominating *D. melanogaster* as an ideal model to study a major protein that may play a role in seizure susceptibility.

cGMP-dependent protein kinase (protein kinase G, PKG), encoded by the human *PRKG1* gene, has been associated with a number of behavioral and physiological processes in both insects and humans (Ørstavik et al., 1997; Sokolowski et al., 2017). In *D. melanogaster*, PKG exists as a naturally occurring polymorphism encoded by the *foraging* (*for*) gene and has been attributed to neuronal protection during anoxic, oxidative and hyperthermic stress in flies (Caplan et al., 2013; Dawson-Scully et al., 2010; Anreiter et al., 2017; Osborne et al., 1997). *for* encodes different levels of PKG enzymatic activity in flies, where *for^R* (rover) flies have higher PKG enzymatic activity than *for^s* (sitter) flies (Renger et al., 1999). Drugs, along with genetics, have been shown to alter the endogenous activity levels of cellular PKG, consequently altering synaptic transmission and membrane excitability by modulating a downstream K⁺ channel(s) via the NO (nitric oxide)/cGMP/PKG signaling pathway in regard to oxidative stress (Caplan et al., 2013). Based on these findings, we hypothesized that an increase in PKG enzymatic activity would decrease the time to recovery from an electroconvulsive seizure in larval *D. melanogaster*. Using a previously established larval electroconvulsive seizure assay (Marley and Baines, 2011), we have successfully demonstrated that behavioral recovery from an electroconvulsive seizure is reduced when PKG activity is increased both genetically and pharmacologically.

MATERIALS AND METHODS

Fly stocks

All flies were reared on 50 ml of standard Bloomington Fly Food (Indiana University, Bloomington, IN, USA) at 25°C on a 12 h:12 h

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light:dark cycle with lights on at 07:00 h. Fly populations were kept to approximately 50 flies per bottle. Adult flies were transferred to new bottles on the 5th and 6th days to allow collection of wandering third-instar larvae. The *foraging* gene underlies a naturally occurring polymorphism whereby rover (*for^R*) and sitter (*for^S*) strains display different levels of PKG enzymatic activity. The rover and sitter strains have a common first and third chromosome and differ on the isogenized second chromosomes where *foraging* is located. As a control, the *for^{S2}* strain was used, which is a sitter *for* mutant generated in a *for^R* genetic background (Osborne et al., 1997). Additionally, genetic mutants with modified expression of the *foraging* gene were tested: the *foraging* null mutant (*for⁰*; *ie1*; *for⁰/cyo-GFP;ie4*) expresses extremely low levels of PKG enzymatic activity as a result of deletion of the *foraging* gene, the *for^{rescue}* (*ie1;for⁰;BAC;wt*) contains a transgenic copy of the *foraging* locus inserted into a null background, and the *foraging* overexpressor mutant (*for^{overex.}*; *ie1;ie4;BAC;wt*) has a duplication of the *foraging* allele on a sitter background (Allen et al., 2017). *for⁰* was selected according to GFP expression using a fluorescence microscope. All fly strains were a gift from Marla Sokolowski (Department of Ecology and Evolutionary Biology, University of Toronto, ON, Canada).

Larval electroconvulsive seizure assay

The larval electroconvulsive seizure assay was adapted and modified from Marley and Baines (2011). Briefly, an individual larva from each strain was placed on an agar plate coated with a thin layer of Spectra 360^R electrode gel (Parker Laboratories, Fairfield, NJ, USA). Two metal insect pins were clipped to the positive and negative electrodes of a Grass S88 stimulator (Grass-Telefactor, West Warwick, RI, USA) and applied to the lateral anterior sides of the larval central nervous system (CNS) to deliver an electrical pulse to induce a seizure (Fig. 1). Applied voltage was checked using a Hantek DSO5072P digital storage oscilloscope (Qingdao Hantek Electronic Co., Ltd, Qingdao City, Shandong Province, China).

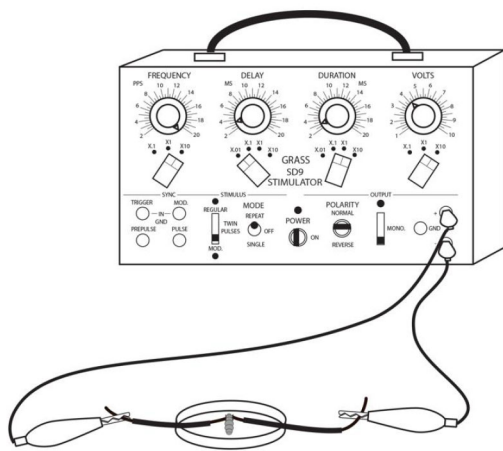


Fig. 1. An electroconvulsive seizure assay adapted for *Drosophila melanogaster*. A larva is carefully transferred to an agar plate and allowed to resume normal locomotion. Stainless steel pins attached to a stimulator are then placed on either side of the central nervous system and an electric shock is delivered according to the stimulus protocol. Time to recovery is recorded when normal locomotion (as defined by two consecutive peristaltic movements forward) resumes.

Larvae were shocked for 3 s at different voltages to determine the optimum voltage protocol (Figs S1 and S2). Time to recovery was determined as the time from the end of the applied shock to the time when muscle contractions ceased and normal locomotion was regained. During normal forward locomotion, muscles contract sequentially from the posterior to the anterior segments (Kohsaka et al., 2017). Perturbations in the CNS are reflected in abnormal motor output, which is demonstrated by abnormal and unilateral body contractions. We defined normal locomotion as two forward peristaltic movements, and most larvae continued to move forward normally. The experimenter was blind to genotype for all trials and other factors like circadian rhythms, lighting, temperature, humidity and time to acclimate were precisely controlled.

Pharmacology

Bloomington fly food 50 ml food bottles were microwaved for approximately 2 min with lids on until the food was liquefied, then left for a few minutes to cool down with the lid off. The drug or sham control (deionized H₂O) was added into the food and shaken by hand for around 20 s. Final drug concentrations were: 100 $\mu\text{mol l}^{-1}$ and 1 mmol l^{-1} 8-bromo-cGMP; 10 and 100 $\mu\text{mol l}^{-1}$ sildenafil citrate. Approximately 50 *for^S* flies were transferred into the prepared bottles to seed them. When larvae reached the wandering third-instar stage of development, they were tested following the regular protocol.

Statistics

Induction of electroconvulsive seizure was conducted for a total of 30 larvae per strain and treatment. Data were analyzed using a Kruskal–Wallis one-way ANOVA on ranks followed by a *post hoc* multiple comparisons test [Student–Newman–Keuls (SNK), Tukey test or Dunn’s test] where indicated. All statistical tests were performed using SigmaPlot 11.0 (San Jose, CA, USA).

RESULTS

Increased PKG enzymatic activity decreases time to recovery from an electroconvulsive seizure

To determine whether PKG enzymatic activity levels affect time to recovery from an electroconvulsive shock, we used the two naturally occurring *foraging* strains along with the transgenic control *for^{S2}* which contains a sitter allele in a rover genetic background (Osborne et al., 1997). *Drosophila melanogaster* wandering third-instar larvae were stimulated at 15 V in the larval electroconvulsive seizure assay. We found that rover larvae, with a high level of PKG enzymatic activity, recovered in less time (averaging 34 s to recovery) than either of the low-PKG sitter strains or *for^{S2}*, which recovered at approximately 86 and 84 s, respectively (one-way ANOVA $F_{2,90}=10.955$, $P<0.001$; SNK $P<0.001$; Fig. 2).

Decreased enzymatic activity in PKG mutants increases time to recovery from an electroconvulsive seizure

Genetically altering PKG activity in various PKG mutants increased time to seizure recovery (Fig. 3). Individual *for^S* third-instar larvae were stimulated at 12 V in the larval electroconvulsive seizure assay. *for^S* larvae took less time to recover from a seizure when compared with *for⁰* mutants, which do not express PKG, averaging around 86 and 201 s, respectively (one-way ANOVA on ranks, $F_{3,84}=21.236$, $P<0.001$; Tukey, $P<0.05$). As expected, the recovery time of the rescue and overexpressor lines, which both express similar low levels of PKG to *for^S* larvae, was not significantly different from that of sitter larvae.

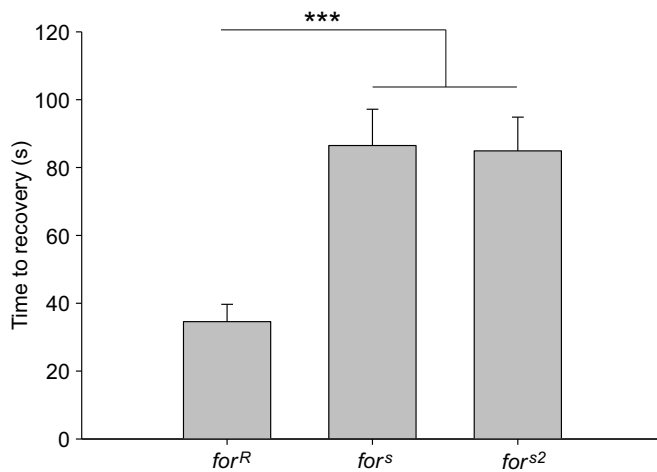


Fig. 2. Increased cGMP-dependent protein kinase (PKG) activity decreases time to recovery. Time to recovery in *D. melanogaster* third-instar larvae ($n \geq 30$) stimulated at 15 V in the larval electroconvulsive seizure assay. Rover larvae (*for^R*, high PKG) took less time to recover from a seizure when compared with sitter larvae (*for^S* and *for^{S2}*, low PKG). Bars represent means \pm s.e.m. and asterisks denote a significant difference ($***P \leq 0.001$). Significance was determined using a one-way ANOVA followed by Student–Newman–Keuls' *post hoc* multiple comparisons test.

Pharmacological activation of the cGMP/PKG pathway decreases time to recovery from an electroconvulsive seizure

Drosophila melanogaster wandering third-instar sitter larvae were treated with two drugs that increase PKG enzymatic activity and tested at 15 V (Fig. 4). 8-Bromo-cGMP is a cGMP analog and sildenafil citrate is a phosphodiesterase-5 inhibitor, both of which activate the PKG pathway. Larvae treated with 1 mmol l⁻¹ 8-bromo-cGMP showed a decrease in recovery time from approximately 119 s to approximately 75 s (one-way ANOVA on ranks, $F_{2,95}=7.321$, $P=0.026$; Dunn, $P<0.005$; Fig. 4A). Larvae treated with 10 μ mol l⁻¹ sildenafil citrate decreased the time to recovery from approximately 111 s to approximately 62 s (one-way ANOVA on ranks, $F_{2,93}=12.558$, $P=0.002$; Dunn, $P<0.05$; Fig. 4B). There was no observable phenotypic difference in body size or

normal locomotion between control flies versus drug-treated flies without a shock.

Pharmacological inhibition of the cGMP/PKG pathway does not increase time to recovery from an electroconvulsive seizure

Drosophila melanogaster wandering third-instar sitter larvae were treated with the PKG inhibitor Rp-8-bromo-cGMP and stimulated at 12 V in the larval electroconvulsive seizure assay (Fig. 5). Two doses of the PKG inhibitor (10 and 100 μ mol l⁻¹) were used to determine whether the time to recovery could be increased. Although the higher dose of Rp-8-bromo-cGMP increased the time to recovery slightly, there was no significant difference between the sham control and drug treatments.

DISCUSSION

PKG in *D. melanogaster* has been linked to several phenotypes including variations in foraging behavior, learning and memory, and, interestingly, anoxia tolerance (Renger et al., 1999; Boccia et al., 2011; Kohn et al., 2013; Mery et al., 2007). There are two natural allelic variants of *foraging*: *for^R*, the rover strain, with a high PKG enzymatic activity, and *for^S*, the sitter strain, with lower PKG enzymatic activity. Past evidence indicates that when *for^R* and *for^S* adult flies are exposed to anoxic conditions for 6 h, the *for^R* strain has a significantly higher probability of survival (Dawson-Scully et al., 2010). A possible explanation for the increase in survival rates relates to the endogenous increase in PKG enzymatic activity, which we predict might consequently increase K⁺ conductance, reducing neuronal excitability. This, in turn, reduces the chance of neuronal depolarization, decreasing the potential for homeostasis loss and cellular damage. As we predicted based on previous evidence, our initial results utilizing the natural allelic variants showed that increasing PKG activity correlates with a shorter time to recovery from an electroconvulsive seizure.

Next, we investigated whether genetic manipulation recreates the wild-type seizure. *for⁰*, which has a deletion at the *for* locus, showed an increase in time to recovery compared with other low-PKG activity mutants and the *for^S* strain. *for⁰* larvae showed a large increase in time to recovery, most likely due to the dramatic decrease in levels of PKG protein expression. It should be noted that deletion of the *foraging* locus also led to the arrest of the *D. melanogaster* life

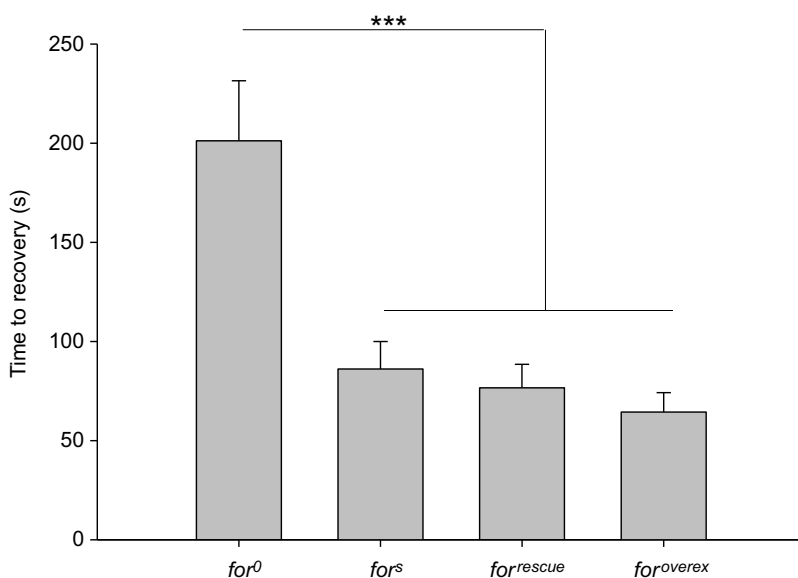


Fig. 3. Decreased enzymatic activity in PKG mutants increases time to recovery from an electroshock. *Drosophila melanogaster* third-instar larvae ($n \geq 30$) were stimulated at 12 V in the larval electroconvulsive seizure assay. Sitter larvae took less time to recover from a seizure when compared with the *foraging* null mutant (*for⁰*, no PKG) whereas the recovery time of the null rescue (*for^{rescue}*) and overexpressor (*for^{overex}*) lines, which express low levels of PKG, was not significantly different from that of the sitter larvae. Bars represent means \pm s.e.m. and asterisks denote a significant difference ($***P \leq 0.001$). Significance was determined using a one-way ANOVA on ranks followed by Tukey's *post hoc* multiple comparisons test.

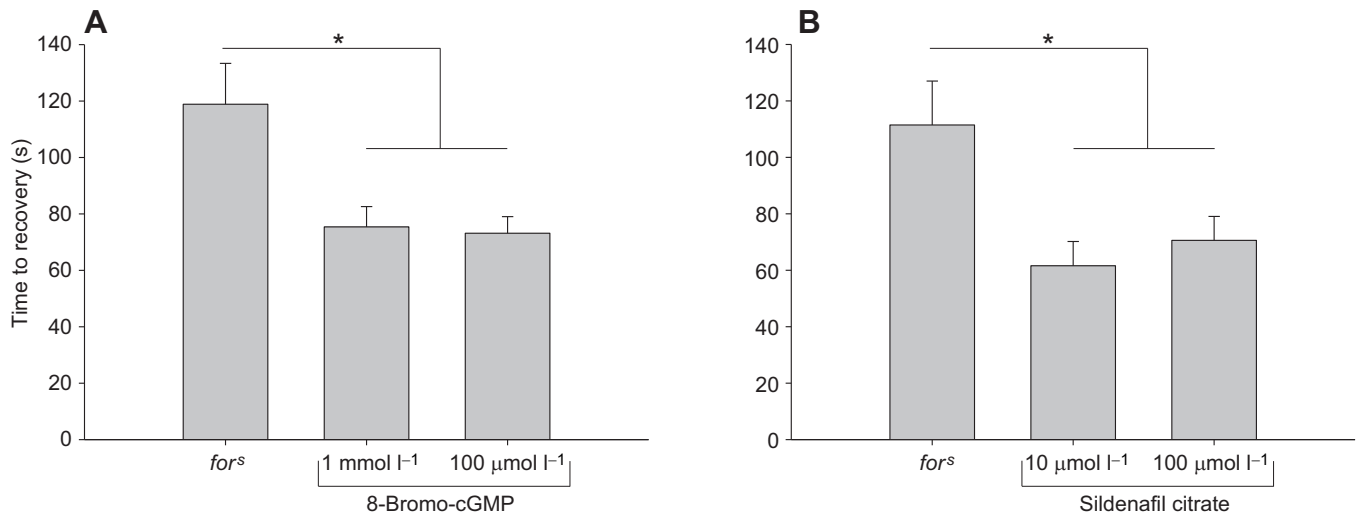


Fig. 4. Pharmacological activation of the cGMP/PKG pathway decreases time to recovery from an electroshock. *Drosophila melanogaster* third-instar sitter larvae ($n \geq 30$) were tested at 15 V with two PKG activators: (A) 8-bromo-cGMP and (B) sildenafil citrate. For both compounds, a 100 $\mu\text{mol l}^{-1}$ dose was sufficient to decrease recovery time. Bars represent means \pm s.e.m. and asterisks denote a significant difference ($*P < 0.05$). Significance was determined using a one-way ANOVA followed by Dunn's multiple comparisons test.

cycle at pupariation; consequently, none of the *for⁰* larvae survived to adulthood. It is well documented that the cGMP/PKG pathway plays a major role in the activation of various biological targets through phosphorylation, such as memory acquisition, retention processes and glucose homeostasis. Therefore, deletion of this gene most likely resulted in the loss of vital targets necessary to allow flies to reach adulthood (Dawson-Scully et al., 2010; Kaun et al., 2007).

We then sought to decrease the time to recovery by pharmacologically activating the PKG pathway. We treated larvae with the PKG activator 8-bromo-cGMP, which has successfully been used in previous studies published by our laboratory to increase PKG enzymatic activity (Dawson-Scully et al., 2010). In our study, treatment with 8-bromo-cGMP led to a significant decrease in time to recovery from an electroconvulsive seizure. The

same results were seen when the larvae were treated with sildenafil citrate, a PDE-5 inhibitor, which also activates the cGMP/PKG pathway (Wallis, 1999). These findings confirm results from both the *for^R* and *for^s* natural allelic variants and genetic modification of the *foraging* gene. While these two drugs successfully decreased time to recovery, the PKG inhibitor Rp-8-bromo-cGMP did not have a significant effect on seizure recovery. Previous work using this inhibitor has shown that it alters synaptic transmission in larval *Drosophila*; however, it is possible that as the *for^s* strain already has low endogenous protein expression levels of PKG, there is a floor effect of the drugs' usage (Caplan et al., 2013; Dawson-Scully et al., 2010).

K⁺ channels have been established as the downstream target regarding thermotolerance and anoxia, but the specific type(s) of K⁺ channels has yet to be elucidated. Multiple studies confirm the downstream channels are expressed in neurons and that PKG is expressed neuronally in *D. melanogaster* (Renger et al., 1999;

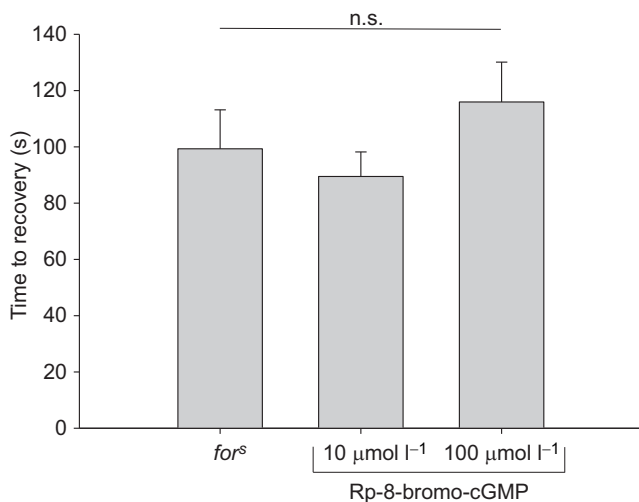


Fig. 5. Pharmacological inhibition of the cGMP/PKG pathway does not increase time to recovery from an electroshock. *Drosophila melanogaster* third-instar sitter larvae ($n \geq 30$) were tested at 12 V with the PKG inhibitor Rp-8-bromo-cGMP. A 100 $\mu\text{mol l}^{-1}$ dose slightly increased the time to recovery but not significantly (n.s.). Bars represent means \pm s.e.m.

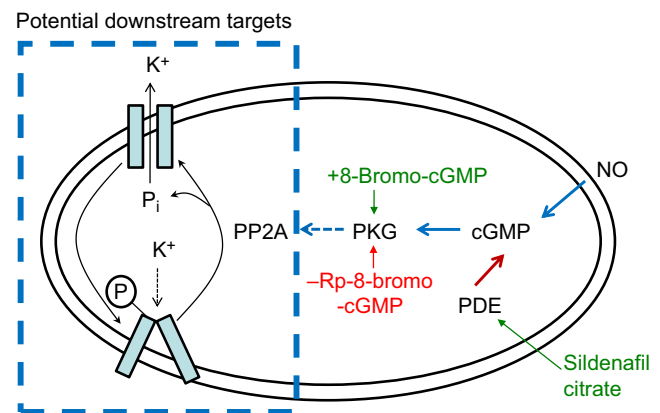


Fig. 6. The cGMP/PKG signaling pathway. This diagram represents the different components of the PKG pathway: PDE5 (phosphodiesterase 5), NO (nitric oxide), cGMP (cyclic guanosine monophosphate), PKG (protein kinase G) and PP2A (protein phosphatase 2A). Pharmacological reagents that activate the pathway (shown in green) include 8-bromo-cGMP and sildenafil citrate. The reagent used to inhibit the pathway (shown in red) is Rp-8-bromo-cGMP. Modified from Dawson-Scully et al. (2010).

Dawson-Scully et al., 2007; Krill and Dawson-Scully, 2016). Convincing evidence exists that links multiple families of K^+ channels that reduce neuronal excitability when K^+ conductance is increased in invertebrate and mammalian models (Kuebler et al., 2001; Li et al., 2017; Wang et al., 2015; Boddum et al., 2017; Gooshe et al., 2017; Whitmire et al., 2017). One study investigated the role of a K_{ATP} channel, Kir6.2, in mice lacking BAD, a protein involved in glucose metabolism. BAD $^{-/-}$ mice showed increased K_{ATP} channel activity and protection from seizures induced by chemical convulsants (Foley et al., 2017). These results are promising and provide convincing evidence that K^+ channels are a potential downstream target to further investigate, and support our proposed signaling pathway (Fig. 6).

The PKG pathway, which has been previously implicated in neuroprotection in *D. melanogaster*, appears to alter recovery time from an electroconvulsive seizure. Higher PKG activity levels, produced either genetically or pharmacologically, decreased the recovery time from an electroconvulsive seizure. Further downstream in the PKG signaling pathway is PP2A, one of many serine/threonine phosphatases that has a large effect on several cellular functions including the cell cycle, cell growth and apoptosis. PP2A inactivation and dysregulation has also been implicated in a number of diseases including cancer, cardiovascular disease and neurodegenerative diseases (O'Connor et al., 2018). While PP2A has several targets, it has also been shown to affect K^+ conductance, leading to hyperpolarization of the membrane potential. This hyperpolarization may be effective in restoring neuronal function after an electroconvulsive seizure. We aim in future studies to develop an electrophysiological preparation using sharp electrode recording at the larval *Drosophila* neuromuscular junction to determine the extent of muscular and neuronal damage as well as to investigate the possible effects of PP2A and K^+ channels on seizure susceptibility.

Acknowledgements

We would like to thank Marla Sokolowski (Department of Ecology and Evolutionary Biology, University of Toronto, ON, Canada) for providing the fly lines and Julieta Di Mase for the schematic drawing of Fig. 1.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.P.K., M.G.R., K.D.-S.; Methodology: S.P.K., M.G.R., L.E.M.; Software: S.P.K., M.G.R.; Formal analysis: S.P.K., M.G.R., L.E.M.; Investigation: L.E.M.; Writing - original draft: S.P.K., M.G.R.; Writing - review & editing: S.P.K., M.G.R.; Visualization: K.D.-S.; Supervision: K.D.-S.; Project administration: K.D.-S.

Funding

This research was supported by a CTP (Compound Transfer Program) grant from Pfizer (W1225058 to K.D.-S.).

Data availability

All relevant data are freely available upon request from the corresponding author.

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.179747.supplemental>

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Supplemental information

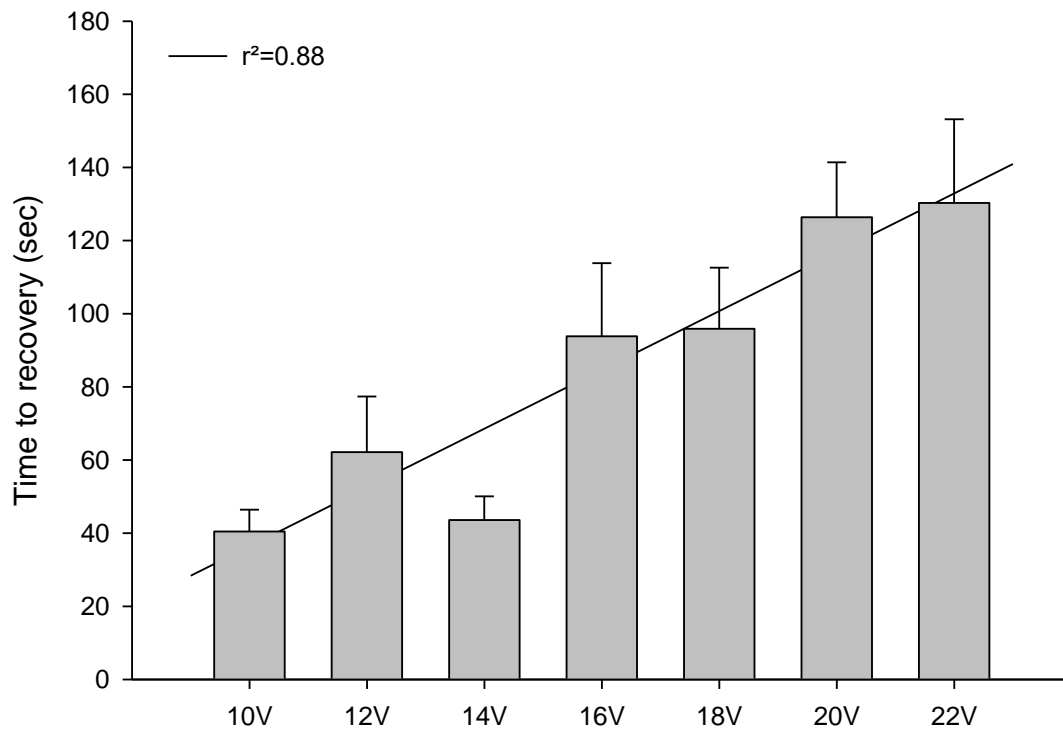


Figure S1. **Voltage curve in sitter to determine optimal voltage protocol.**

Sitters were shocked at increasing voltage increments to find the half maximal recovery time. The voltage curve was paired with the percentage of flies that recovered at each voltage to determine the optimal voltage for experiments to maximize the data collection. $n \geq 15$ for all voltages. All bar graphs represent mean \pm s.e.m.

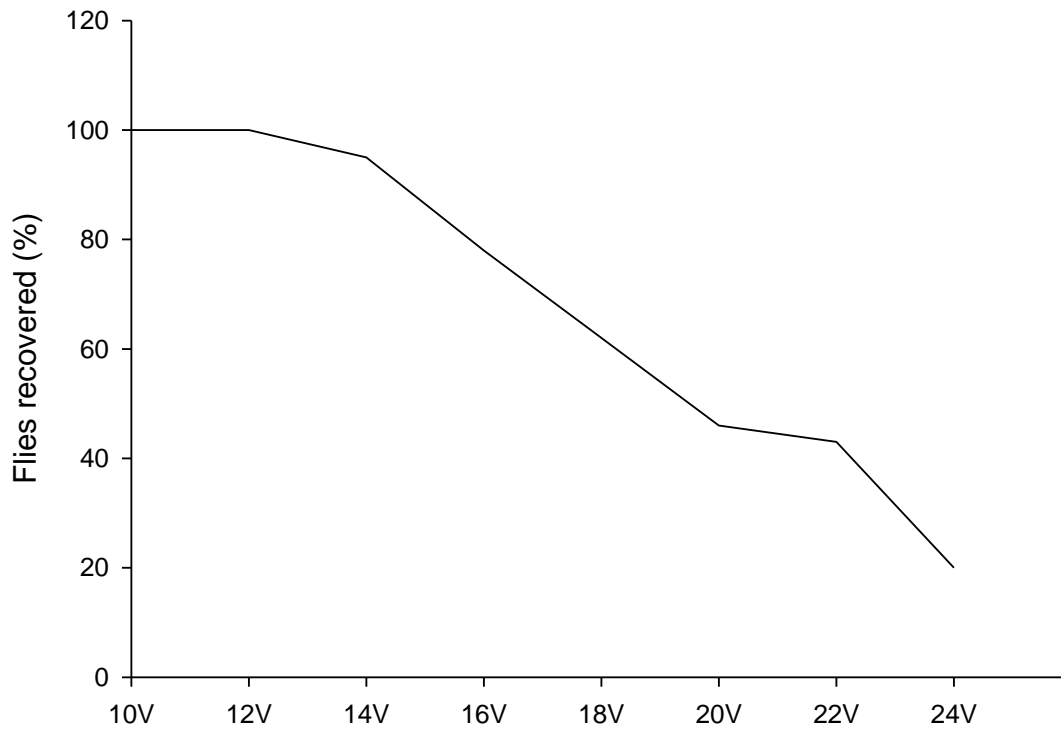


Fig S2. Percentage of sitters that recovered from the electroshock during voltage curve testing.

Sitters were shocked at increasing voltage increments to find the half maximal recovery time. The voltage curve was paired with the percentage of flies that recovered at each voltage to determine the optimal voltage for experiments to maximize the data collection. $n \geq 15$ for all voltages.