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RESEARCH ARTICLE

Natural polymorphism in protein kinase G modulates functional senescence in *Drosophila melanogaster*

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ABSTRACT

The common fruit fly, *Drosophila melanogaster*, is a well-characterized model for neurological disorders and is widely used to investigate the biology of aging, stress tolerance and pleiotropy. The *foraging* (*for*) gene encodes a cGMP-dependent protein kinase (PKG), which has been implicated in several behavioral phenotypes including feeding, sleep, learning and memory, and environmental stress tolerance. We used the well-established *Drosophila* activity monitor (DAM) to investigate the effects of the conserved NO/cGMP/PKG signaling pathway on functional senescence. Our results show that the polymorphic *for* gene confers protection during low oxygen stress at the expense of longevity and a decline in locomotor activity with age in *D. melanogaster*, which suggests a novel role for the PKG pathway in healthy aging and senescence.

KEY WORDS: PKG, Aging, Stress tolerance, Antagonistic pleiotropy, Functional senescence, Hypoxia

INTRODUCTION

Aging is characterized by damage and degeneration of cells, leading to loss of function, vulnerability to disease and eventual death (Jacobson et al., 2010; Kirkwood, 2005; Vijg and Campisi, 2008). A hallmark of the aging process is the progressive decline of the nervous system, including both motor and cognitive functions (Mahoney et al., 2014). To complicate matters, natural variation in aging populations makes it difficult to study contributing factors of behavioral decline. As the aging process is also typically slow, biomarkers can be difficult to elucidate, and the ability to predict differences between individuals in time to death has proven elusive (Warner, 2004). Functional senescence, defined here as inherent age-related decline in physiological and behavioral function, may be affected by pleiotropic genes (Le Cunff et al., 2014; Rodriguez et al., 2017; Grotewiel et al., 2005). Pleiotropic genes control more than one trait; antagonistic pleiotropy occurs when one trait is beneficial to an organism's fitness and the other is detrimental. Because genetic conflicts may arise from a number of physiological, developmental or behavioral sources, pleiotropy is more commonly referred to as 'trade-offs' between early- and late-life performance in animal models of aging such as worms, fruit flies and mice (Ewald et al., 2018; Miquel et al., 1976; Jobson et al., 2015; Fleming et al., 1992; Bartke, 2005; Chubb, 1987; Miskin and Masos, 1997).

In 1957, George Williams proposed the antagonistic pleiotropy theory of aging (Williams, 1957), suggesting that pleiotropic genes are beneficial to an organism's fitness early in life, but cause functional decline and aging phenotypes in adulthood. An example of this was demonstrated in a tight correlation between early fecundity and short lifespan, where late fecundity resulted in a long lifespan in *Drosophila melanogaster* (Zwaan et al., 1995). In 2003, Phelan et al. also demonstrated a positive correlation between stress resistance and lifespan in *D. melanogaster*, also showing that the correlation between two fitness traits can change over time. With a rapid lifespan, easily manipulated genetics, low-cost maintenance and quantifiable behaviors, fruit flies are ideally suited for research into the basic molecular mechanisms underlying cell function. Approximately 75% of human disease genes have homologous counterparts in the fly genome, with many cellular functions and pathways conserved across phyla (Frank et al., 2013; Chien et al., 2002); thus, flies are a powerful model to further our understanding of how senescence affects neuronal function and survival.

In *D. melanogaster*, the naturally occurring polymorphism of the *foraging* (*for*) gene encodes a cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent protein kinase (protein kinase G, PKG) that catalyzes the phosphorylation of serine/threonine residues of proteins and contributes to various cellular and physiological processes including axonal guidance (Nishiyama et al., 2003), smooth muscle function (Ono and Trautwein, 1991) and regulation of the circadian clock (Tischkau et al., 2004). PKG, therefore, affects several behaviors, such as metabolism (Kaun et al., 2007), feeding behavior (Sokolowski, 1980; Shaver et al., 1998), and learning and memory (Mery et al., 2007; Wang et al., 2008; Kohn et al., 2013). PKG has also been credited with neuroprotection during anoxia, oxidative stress and hyperthermia in flies (Armstrong et al., 2010; Caplan et al., 2013; Chen et al., 2011; Dawson-Scully et al., 2010; Anreiter et al., 2017; Osborne et al., 1997).

This natural polymorphism affects not only larval feeding behavior but also tolerance to stress conditions. In a natural population under normal temperate conditions, ∼70% of flies demonstrate higher PKG activity and express the rover allele of the *for* gene, whereas ∼30% have lower PKG activity and express the sitter allele (Dawson-Scully et al., 2010). As larvae, rovers (+PKG) move more and feed less in the presence of food compared with sitters (−PKG); however, there is no difference in locomotion in the absence of food (Sokolowski, 2001). Food deprivation causes rovers to behave like sitters (Kent et al., 2009; de Belle et al., 1989, 1993; Kaun et al., 2007). While irregular food sources and high population densities provide a selective advantage for rovers (+PKG), more regularly distributed food and low population densities lend an advantage to sitters (−PKG) (Ben-Shahar et al., 2002). It has been shown that under prolonged hypoxia, rover embryos and larvae (+PKG) survive significantly longer than sitters (−PKG) (Wingrove and O'Farrell, 1999), and this correlates with adults (Dawson-Scully et al., 2010). In 2014, Urquhart-Cronish and Sokolowski also showed that low larval nutrition coupled with high adult nutrition resulted in better stress buffering for the sitter variation of the *for* gene.

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PKG also plays a role in a pathway that affects K^+ channel conductance. cGMP activates PKG, which activates protein phosphatase 2A, dephosphorylating a K^+ channel, causing it to assume an open state and thereby increasing K^+ conductance (Zhou et al., 1996) (see below). Modulation of K^+ channel conductance plays a role in neuroprotection in *Drosophila* under hypoxic stress, and it is proposed that changes in neuroprotection are a result of changes to cell membrane excitability and maintenance of homeostasis (Dawson-Scully et al., 2010). Oxidative stress and alterations in neuronal excitability have been considered some of the culprits in the aging process (Kelly, 1978; Landfield et al., 1978; Hori et al., 1992); therefore, we sought to determine whether or not the stress tolerance conferred by modulating K^+ channels in young flies has the same effect in senescent flies. Genetic polymorphism shows that while expression of *for* may confer protection or resilience in one context, it may lead to susceptibility in other situations like sleep or memory storage (Donlea et al., 2012). *for*, while not typically known as a 'longevity' gene, is associated with stress tolerance and, therefore, we hypothesize that it may also influence healthy aging and lifespan.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster Meigen adults were reared on 50 ml of standard Bloomington fly food (Indiana University) and maintained at 25°C on a 12 h:12 h light:dark cycle. The *for* gene is located on the second chromosome and underlies a naturally occurring polymorphism whereby rover (for^R) flies express high levels of PKG enzymatic activity and sitter (for^s) flies express low levels. The rover and sitter strains have common first and third chromosomes and differ on the isogenized second chromosomes. To control for incidental genetic background, the for^{s2} strain, where the sitter allele is generated on a for^R genetic background, was also used (Osborne et al., 1997). for^{s2} expresses low levels of PKG enzymatic activity like the sitter strain, so differences between rover (for^R) and both of the low PKG strains (for^s and for^{s2}) indicate effects due to *for* (Wang and Sokolowski, 2017). Approximately 100 age-matched flies (~70 females and ~30 males) were added to bottles with 100 ml of standard Bloomington fly food for 24 h. Flies that eclosed on the first day were discarded, flies that eclosed on the second day were transferred to vials of ~5 ml fresh food as age-matched adults and allowed to mate for 48 h. The flies were then lightly anesthetized with CO_2 and males were quickly transferred to fresh food vials. This ensured that the flies were not subjected to CO_2 in the 24 h prior to experimentation (Linford et al., 2013). As the food remained fresh for over a week, flies were flipped into fresh food vials once a week instead of every 3 days to minimize the number of flies escaping during the flipping process (Miquel et al., 1976). The position of vials in the incubators was also randomized to avoid any micro-climate effects (Ja et al., 2009). Only male flies were used at the ages of 5–9 days old (young), 20–30 days old (midlife) and 40–50 days old (old/senescent) in all experiments to avoid differences in lifespan, reproduction and feeding behaviors (Ormerod et al., 2017). All fly strains were a generous gift from Marla Sokolowski, Department of Ecology and Evolutionary Biology, University of Toronto, ON, Canada.

Lifespan assay

Ten vials of ~20 adult males per genotype were collected 1 day after eclosion and aged in vials with ~5 ml of food. Flies were transferred to fresh food once a week (Miquel et al., 1976). The number of deaths was recorded every day and a Kaplan–Meier survival curve was performed using SigmaPlot (San Jose, CA, USA).

Drosophila activity monitor (DAM) system

The DAM is most commonly used to determine circadian rhythms in normoxic conditions (Konopka and Benzer, 1971; Zordan et al., 2007). Previous studies on locomotor behavior in *Drosophila* have typically assayed either negative geotaxis, a behavioral response in which flies are tapped to the bottom of a container and climb the walls in a specified amount of time, or geotaxis, in which flies are not mechanically stimulated but simply allowed to climb bifurcated tubes towards a light cue (Gargano et al., 2005; Simon et al., 2006; Le Bourg and Lints, 1992). To avoid animal fatigue from consecutive negative geotaxis trials, especially in senescent flies, we used the DAM system to automate average activity rates (crosses per minute) during normoxic conditions and hypoxic stress, or time to failure as determined by 0 crosses min^{-1} for 1 continuous hour (Zordan et al., 2007; Hendricks et al., 2000; Shaw et al., 2000; Slocumb et al., 2015). Adult flies were individually aspirated into each of the 32 DAM tubes and randomly loaded into a DAM system (Trikinetics, Waltham, MA, USA). Each system was placed into either normoxic (21% O_2) or hypoxic (3% or 1% O_2) conditions. Argon gas was used to displace the oxygen in a C-Shuttle Glove Box (BioSpherix, NY, USA) that was pre-set to the desired hypoxic conditions (Fig. 1). The two normoxic DAM systems were placed just outside of the plastic window of the glove box and connected to the computer. The two hypoxic DAM systems were placed into the outer chamber of the glove box with the inner door closed. Once the oxygen level was restored to the pre-set level, the inner door was opened, the two DAM systems were placed next to the plastic window and connected to the computer, and hypoxic gas was flushed through the gas manifold using a 60 ml Monoject luer-lock tip syringe (Covidien, Walpole, MA, USA). As the flies move back and forth in the tubes, an infra-red beam in the middle of the tube is broken and the computer counts it as a cross. The computer automatically records the number of crosses for every specified interval, resulting in a measure of activity over time. N=6 trials per condition and n=48 animals total.

Statistics

All data were analyzed using Student's *t*-test, Mann–Whitney rank sum test, Kaplan–Meier log-rank survival curve followed by Holm–Šidák all pairwise multiple comparisons test, or Kruskal–Wallis one-way ANOVA on ranks followed by a *post hoc* multiple comparisons test (Tukey's test, Holm–Šidák or Dunn's test) where indicated. All graphs represent means±s.e.m. and asterisks denote significance (*P≤0.05, **P≤0.01, ***P≤0.001; ns, not significant). For the failure and longevity assays, the significance between curves was determined using the Kaplan–Meier log-rank test (*P≤0.05, **P≤0.01, ***P≤0.001 indicated on the graph and/or legend; ns, not significant). All statistical tests were performed using SigmaPlot 11.0. All relevant data are freely available upon request from the corresponding author (ken.dawson-scully@fau.edu).

RESULTS

Drosophila melanogaster behavioral response to stress in hypoxia chamber

Our data suggest that PKG levels determine time to failure in 1% O_2. Adult flies (20–30 days old) were placed in the DAM system and subjected to 6 h of 1% O_2 hypoxic stress in the chamber. Flies expressing high levels of PKG (for^R) averaged 100 min to failure and showed a significantly shorter time to failure (Fig. 2B) compared with both strains expressing low PKG levels (for^s and for^{s2}) (Fig. 2A), which failed at approximately 247.5 and 232.5 min,

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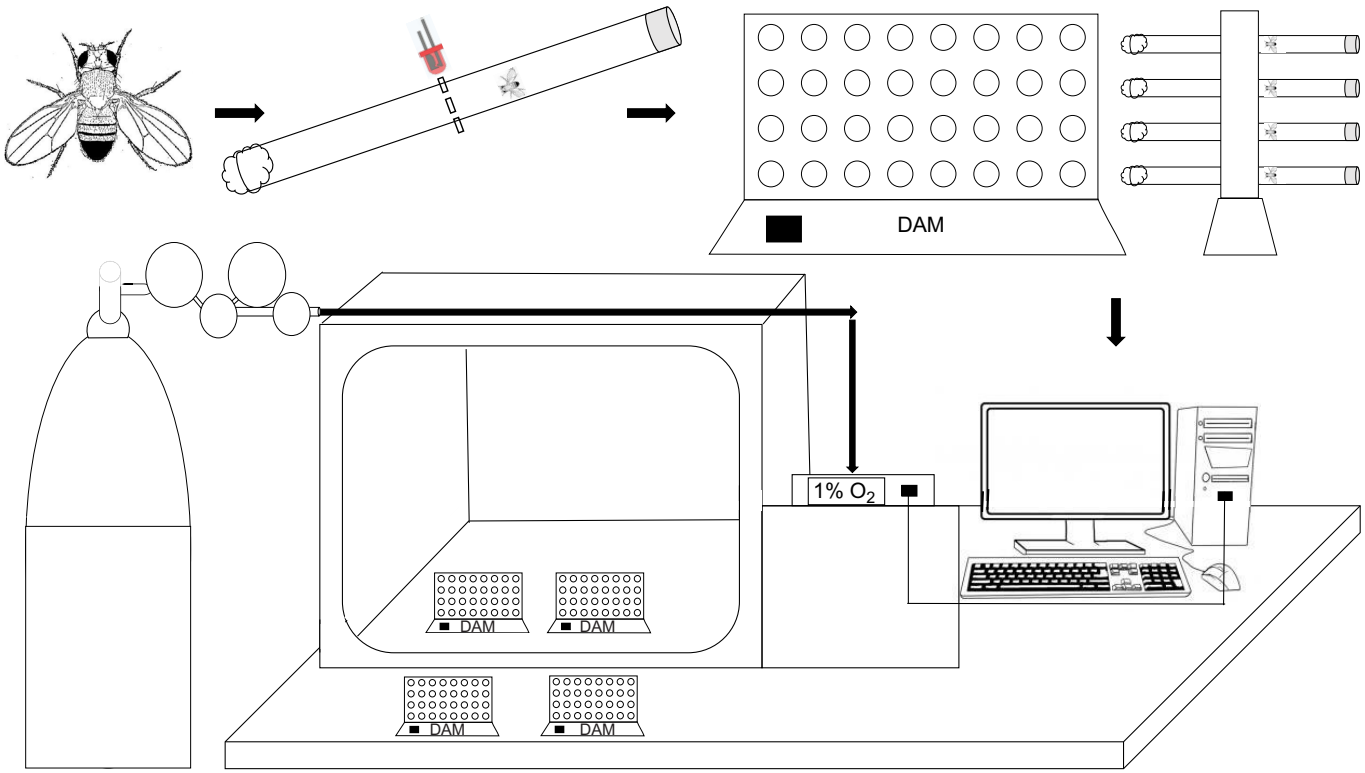

Fig. 1. Schematic diagram of the experimental design. An individual fly is aspirated into a single tube and the tube is plugged. Thirty-two tubes are evenly loaded into one *Drosophila* activity monitor (DAM) system and the DAM systems are put either into or just outside of a C-Shuttle Glove Box for hypoxic and normoxic conditions, respectively. For clarity, the gloves in the chamber are not depicted in the drawing. The flies are continuously monitored for movement as they walk back and forth through the tube. Each time the fly breaks an infra-red beam in the DAM, it is counted as a cross and the computer records the number of crosses for a specified interval of time.

respectively (one-way ANOVA on ranks, H=9.065, 2 d.f., P=0.011). These results recapitulate the previous data showing anoxic coma onset from Dawson-Scully et al. (2010), demonstrating the effectiveness of using the DAM system in the hypoxia chamber. Significance was determined using a one-way ANOVA on ranks followed by Tukey's *post hoc* multiple comparisons test.

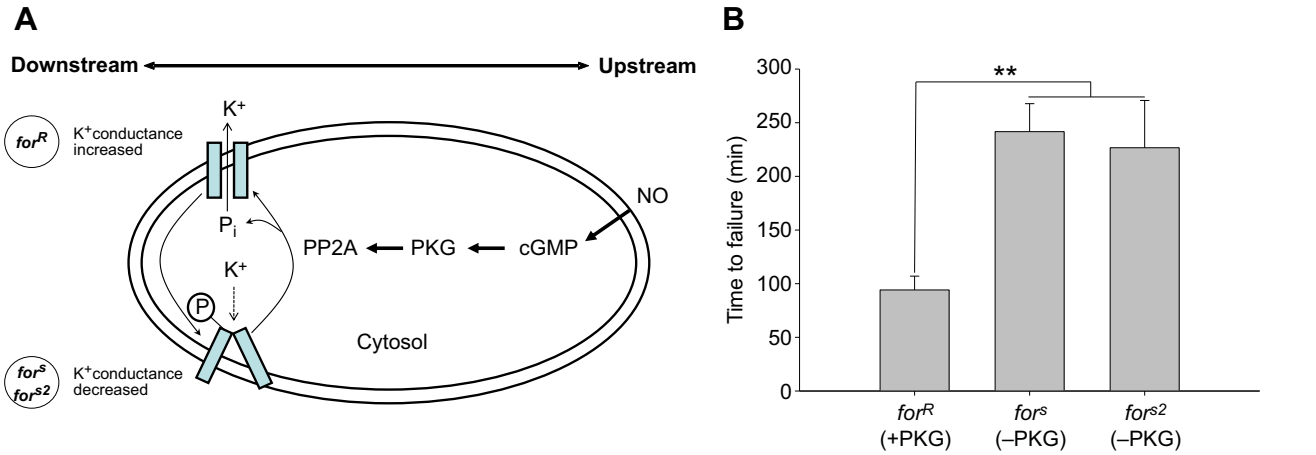

Fig. 2. Schematic diagram of the NO/cGMP/PKG pathway: PKG levels determine time to onset of anoxic coma. (A) Nitric oxide (NO) increases synthesis of cyclic guanosine monophosphate (cGMP). cGMP activates the second messenger protein kinase G (PKG)-signaling pathway which activates protein phosphatase 2A (PP2A). Activation of PP2A dephosphorylates K^+ channels, increasing K^+ conductance and decreasing neuronal excitability. *Drosophila melanogaster* has a naturally occurring polymorphism in the *foraging* (*for*) gene, resulting in the for^R (rover) and for^s (sitter) strains, named for foraging patterns in the presence of food. Rovers naturally express higher levels of PKG than sitters or for^{s2}. The for^{s2} strain is a genetic control created by inserting the sitter allele into a rover genetic background. Adapted from Dawson-Scully et al. (2010). (B) PKG levels determine time to failure in 1% O_2. Adult flies were placed in the DAM system and subjected to 6 h of 1% O_2 hypoxic stress in the chamber. Time to failure was determined by 0 crosses min^{-1} for 1 continuous hour. Flies expressing high levels of PKG (for^R) showed a significant decrease in time to failure (averaging 100 min to failure) than both strains expressing low PKG levels (for^s and for^{s2}), which failed at approximately 247.5 and 232.5 min, respectively (one-way ANOVA on ranks, H=9.065, 2 d.f., P=0.011). The results recapitulate previous data from Dawson-Scully et al. (2010), demonstrating the effectiveness of this assay. All bar graphs represent means±s.e.m. and asterisks denote significance between bars (**P≤0.01; ns, not significant). Significance was determined by using a one-way ANOVA on ranks followed by Tukey's *post hoc* multiple comparisons test.

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Activity during stress is not affected by foraging behavior regardless of age

To ensure that activity levels were not affected by differences in foraging behavior, both young (5–9 days old) and old (40–50 days old) flies were subjected to 4 h of hypoxia (1% O_2) with either food or agar. There was no significant difference between mean (±s.d.) activity with food (0.319±0.225) and mean activity with agar (0.375 ±0.248) in young rovers (*for*^{R}, high PKG; t_{10}=−0.412, P=0.689; Fig. 3A) or between mean activity with food (median 0.161, range 0.0591–1.336) and mean activity with agar (0.171, 0.0805–1.253) in young sitters (*for*^{s}, low PKG; U=17.000, P=0.937). Similar results were found with old age. There was no significant difference between mean activity with food (0.0424, 0.00194–0.186; Fig. 3B) and mean activity with agar (0.0469, 0.0212–0.160) in old rovers (*for*^{R}, high PKG; U=14.000, P=0.589) or between mean activity with food (0.0214, 0.00129–0.510) and mean activity with agar (0.0987, 0.0582–0.286) in old sitters (*for*^{s}, low PKG; U=12.000, P=0.394). The lack of a difference between activity on food and activity on agar indicates that foraging behavior and desiccation are not factors in this assay and the differences between genotypes are not due to feeding behavior. Significance was determined by using a *t*-test (Shapiro–Wilk for equal variance and Mann–Whitney rank sum test where normality failed).

for affects locomotor activity in old flies during hypoxia

To investigate the effect of age on locomotor activity in both young and old flies, male flies were aged to the appropriate young (5–9 days old) or old (40–50 days old) age range and average activity was measured over the course of 250 min in both normoxic (21% O_2) and hypoxic (3% O_2) conditions. Data were averaged into 5 min bins. We found that there were significant differences in activity between all three strains at a young age in both 21% O_2 (H=81.252, 2 d.f., P≤0.001; Fig. 4A) and 3% O_2 (H=106.563, 2 d.f., P≤0.001; Fig. 4C), indicating that the *for* gene is not involved in this phenotype at a young age. Age had a significant effect on activity in both normoxic and hypoxic conditions. Rovers (*for*^{R}) with high levels of PKG were significantly different from both low PKG strains (*for*^{s} and *for*^{s2}) in 21% O_2 (H=11.721, 2 d.f., P=0.003; Fig. 4B) and in 3% O_2 (H=99.558, 2 d.f., P≤0.001; Fig. 4D). In normoxic conditions, old rovers tended to have slightly higher locomotor activity at the start of the experiment, which then lowered to the same level as that of both low PKG variants, both of which slightly increased their activity, indicating a small PKG effect. In response to low oxygen stress, however, old rovers drastically reduced their locomotor activity whereas both sitters and *for*^{s2} increased their locomotor activity, suggesting that enzymatic levels of PKG enhance the stress response with age. Significance was determined using a one-way ANOVA on ranks followed by Tukey's *post hoc* multiple comparisons test (P≤0.05 for all comparisons).

Differing genetic levels of PKG alter response to low oxygen stress and age

Aged male *Drosophila* were then exposed to even greater hypoxic stress levels (1% O_2) to determine whether PKG affects activity during severe anoxic stress. Like the results in normoxia and hypoxia (3% O_2), there was no PKG effect on young flies in 21% O_2 (H=98.069, 2 d.f., P≤0.001; Fig. 5A). There was a PKG effect with age in 21% O_2, with a significant difference between old rovers and both sitters and *for*^{s2} (H=142.051, 2 d.f., P≤0.001; Fig. 5B). There was also a PKG effect during 1% hypoxic stress between rovers and both sitters and *for*^{s2} in young flies (H=59.923, 2 d.f., P≤0.001; Fig. 5C) and old flies (H=69.422, 2 d.f., P≤0.001; Fig. 5D). Significance was determined using a one-way ANOVA on ranks followed by Tukey's *post hoc* multiple comparisons test (P≤0.05 for all comparisons). Young rovers failed at approximately 100 min into the 250 min of stress while both sitters and *for*^{s2} maintained a low but consistent pattern of activity throughout the length of the assay (Fig. 5C). This pattern was more pronounced in the senescent flies, with rovers failing at approximately 45 min while the others maintained low activity rates (Fig. 5D).

Reduced levels of PKG suggest increased longevity

To assess the effects of PKG activity levels on longevity, 20 male flies per vial were raised on standard Bloomington food and deaths were scored every day until all flies were dead. Both low PKG strains (*for*^{s} and *for*^{s2}) had a longer lifespan (mean 39 days) than the

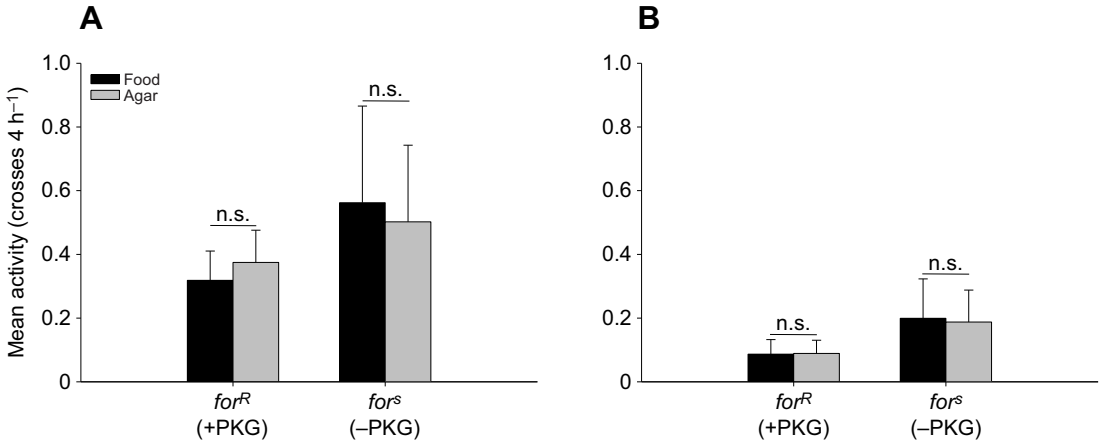

Fig. 3. Activity during stress is not affected by foraging behavior regardless of age. (A) There was no significant difference between mean (±s.d.) activity with food (0.319±0.225) and mean activity with agar (0.375±0.248) in the young (5–9 days old) rover (*for*^{R}, high PKG) strain (t_{10}=−0.412, P=0.689). There was also no significant difference between mean activity with food (0.161, range 0.0591–1.336) and mean activity with agar (0.171, 0.0805–1.253) in the young sitter (*for*^{s}, low PKG) strain (U=17.000, P=0.937). (B) The same results were found with old age (40+ days old). There was no significant difference between mean (±s.d.) activity with food (0.0424, 0.00194–0.186) and mean activity with agar (0.0469, 0.0212–0.160) in the old rover (*for*^{R}, high PKG) strain (U=14.000, P=0.589). There was also no significant difference between mean activity with food (0.0214, 0.00129–0.510) and mean activity with agar (0.0987, 0.0582–0.286) in the old sitter (*for*^{s}, low PKG) strain (U=12.000, P=0.394). All bar graphs represent means±s.e.m. (n.s., not significant). Significance was determined using a *t*-test (Shapiro–Wilk for equal variance and Mann–Whitney rank sum test where normality failed).

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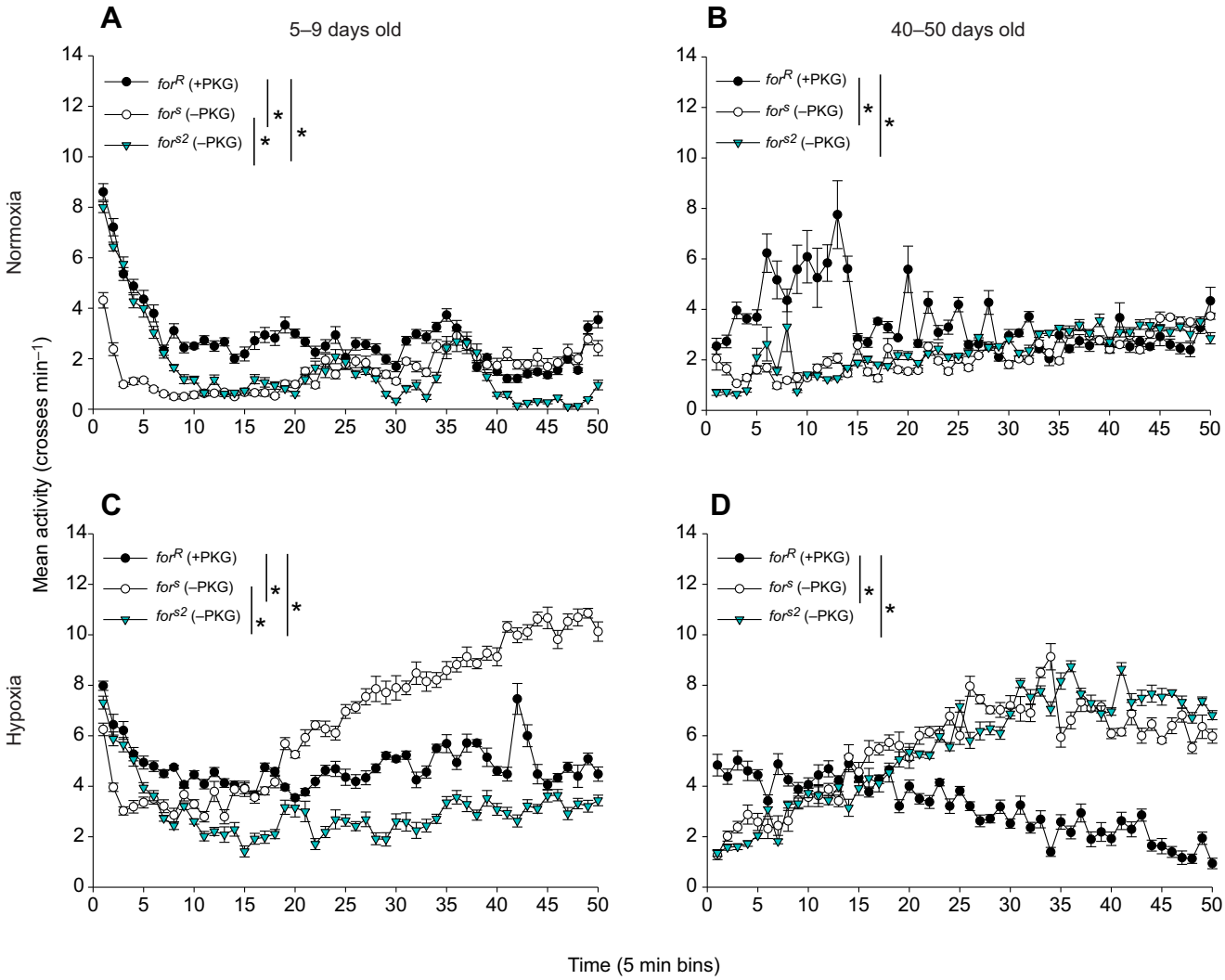

Fig. 4. Effect of age on average activity measured during both normoxia (21% O_2) and hypoxia (3% O_2). Male flies were aged to the appropriate young (5–9 days old) or old (40–50 days old) age range and average activity was measured over the course of 250 min. Data were averaged into 5 min bins, *N*=6 trials and *n*=48 animals per genotype, age and condition. There were significant differences between all three strains at a young age in both (A) 21% O_2 (*H*=81.252, 2 d.f., $P \leq 0.001$) and (C) 3% O_2 (*H*=106.563, 2 d.f., $P \leq 0.001$). Age had a significant effect on activity in both normoxic and hypoxic conditions. Activity of rovers (for^R) with high levels of PKG was significantly different from that of both low PKG strains (for^s and for^{s2}) in (B) 21% O_2 (*H*=11.721, 2 d.f., *P*=0.003) and (D) 3% O_2 (*H*=99.558, 2 d.f., $P \leq 0.001$). All bar graphs represent means±s.e.m. and asterisks denote significance between bars (*$P \leq 0.05$). Significance was determined using a one-way ANOVA on ranks followed by Tukey's *post hoc* multiple comparisons test ($P \leq 0.05$ for all comparisons).

high PKG strain (for^R) (mean 35 days) under normal conditions (Fig. 6). Significant differences were found using a Kaplan–Meier log-rank survival curve (χ^2=43.203, 2 d.f., $P<0.001$) between all strains, followed by a Holm–Šidák all pairwise multiple comparison test (*$P<0.05$, ***$P<0.001$ for all strains; *N*=10, *n*=200 per genotype). A slight reduction in lifespan in rovers may be a trade-off for early-life stress tolerance.

DISCUSSION

The *for* gene or one of its homologs is conserved across many species. This study allowed us to use the behavior of intact aged animals to investigate the effects of PKG on age and stress tolerance. We show that there is no PKG effect on behavior in young animals in the absence of food (Fig. 3) or hypoxic stress (Figs 4A and 5A). While previous studies showed that reduction of locomotion during acute hypoxia was associated with inhibition of the PKG pathway, there was a trade-off of a decrease in survival after acute anoxia (Renger et al., 1999; Dawson-Scully et al., 2010). We have shown here that PKG influences stress tolerance in hypoxic conditions (3% O_2) in old flies (Fig. 4D). Under more severe anoxic stress (1% O_2), there was a PKG effect in both young and old flies (Fig. 5C,D). Interestingly, we found a significant PKG effect with age in normoxic (21% O_2) conditions (Figs 4B and 5B). While the results shown in Fig. 5B are more pronounced than those in Fig. 4B, they show the same trends and have the same statistical significance, with rovers decreasing their activity and both low PKG strains increasing their activity. To our knowledge, this is the first report that PKG levels affect senescence with a robust behavioral component. This study shows that this genetic polymorphism trades higher stress tolerance for decreased locomotion in senescence and longevity.

In nature, rovers have the advantage in an environment with patchy food while sitters have the advantage when there are continuous food sources (Sokolowski et al., 1997). The strains have been found at constant frequencies of 70% rovers to 30% sitters when sampled in the wild, suggesting that fitness is correlated with foraging behavior (Sokolowski, 1980). In the laboratory, there are

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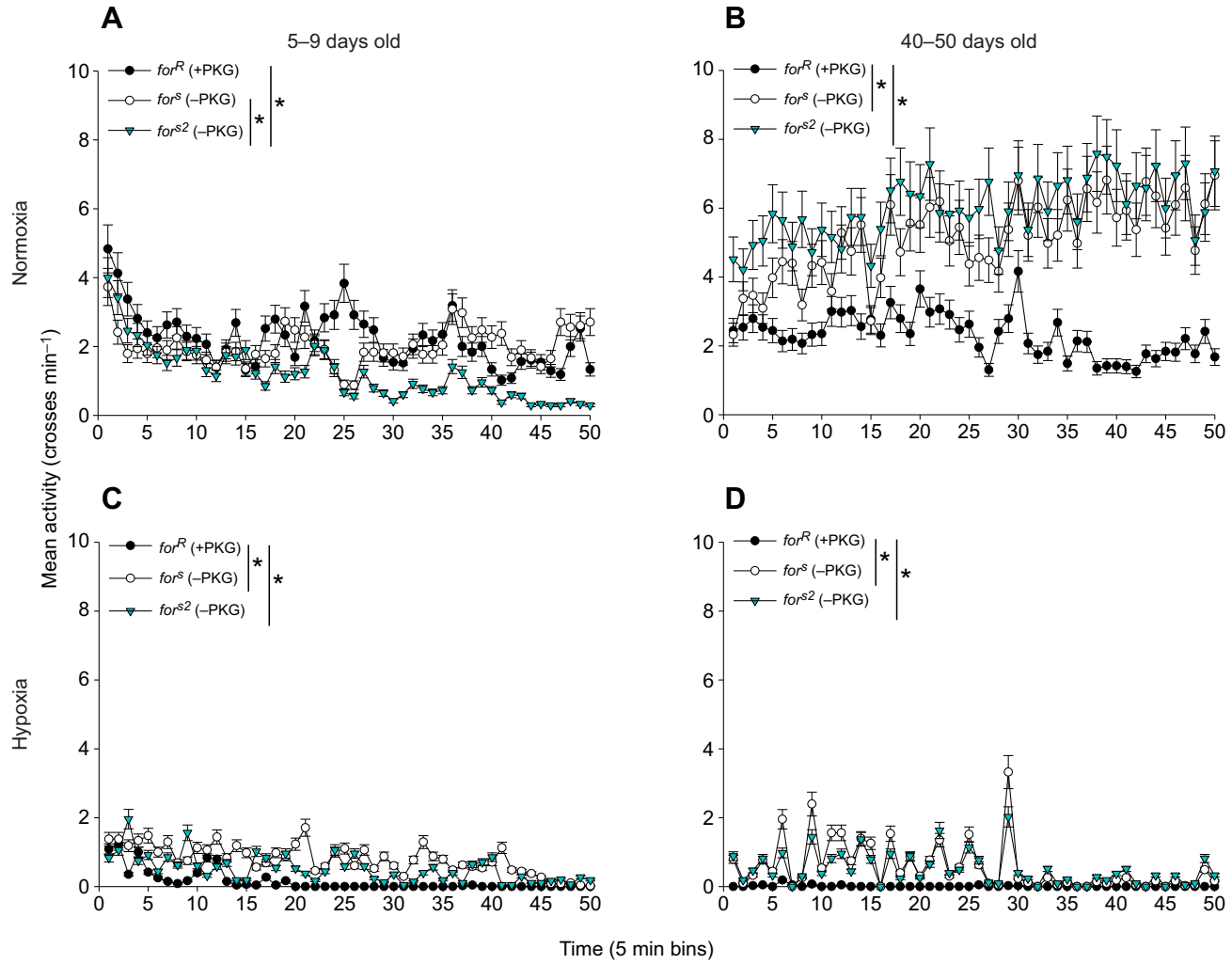

Fig. 5. Genetically altering PKG levels affects the stress response to low oxygen and age. Male flies were aged to the appropriate young (5–9 days old) or old (40–50 days old) age range and average activity was measured in both normoxic (21% O_2) and hypoxic (1% O_2) conditions over the course of 250 min. There was not a PKG effect on young flies in 21% O_2 as rovers and sitters were not different from each other but were significantly different from for^{s2} (H=98.069, 2 d.f., P≤0.001). A PKG effect with age is seen with a significant difference between old rovers and both old sitters and for^{s2} (H=142.051, 2 d.f., P≤0.001). There was also a PKG effect during 1% hypoxic stress between rovers and both sitters and for^{s2} in young flies (H=59.923, 2 d.f., P=<0.001) and old flies (H=69.422, 2 d.f., P≤0.001). Significance was determined using a one-way ANOVA on ranks followed by Tukey's *post hoc* multiple comparisons test (P≤0.05 for all comparisons).

no obvious differences in fertility, fecundity and survivorship (Sokolowski, 1982). The slightly shorter lifespan in the laboratory suggests that higher stress tolerance in rovers may be a trade-off to increase their early-life fitness.

Previous work done with flies on modulating anoxic tolerance demonstrated that the level of PKG affected the stress response in *for* strains. Rovers (+PKG) failed faster than sitters (−PKG) but had a higher survival rate 24 h after the hypoxic insult (Dawson-Scully et al., 2010). One of the characteristics of the sitter variant is reduced voltage-dependent K^+ conductance, which results in membrane hyperexcitability (Renger et al., 1999). Other studies suggest that inhibition of the PKG pathway prolongs neural function during acute hypoxia (Dawson-Scully et al., 2010; Bollinger et al., 2018). This resistance to acute oxidative stress may be the reason that sitters (−PKG) maintain higher levels of locomotor activity as they age (Fig. 4D) and live longer than rovers (+PKG) (Fig. 6); therefore, modulating the levels of $[K^+]_o$ could have a significant neuroprotective effect on aged or damaged brains.

Neuronal membrane excitability is not the only change associated with aging in the brain. Alterations in the intrinsic cellular properties of neurons may be responsible for cognitive deficits in normal aging. The oxidative stress hypothesis posits that reactive oxygen species, mostly free radicals from the electron transport chain in the mitochondria, cause oxidative damage leading to cell damage and apoptosis. Energy dysfunction of the Na^+/K^+-ATPase is a consequence of many pathological insults, including low-oxygen stress. A lack of oxygen leads to a failure of the mitochondrial ATP pumps (Djamgoz et al., 1998; Yi et al., 2004), which affects the Na^+/K^+-ATPase, causing cell depolarization and an influx of Ca^{2+}. Subsequently, excessive amounts of excitotoxic neurotransmitters, including glutamate and dopamine, are released into the synaptic cleft, triggering a series of cellular events leading to damage or cell death (Reed et al., 2010; Milton and Dawson-Scully, 2013; Van Voorhies, 2009). Constant changes in Ca^{2+} influx are a hallmark of glutamate-induced excitotoxicity associated with neurodegeneration, cardiovascular disease and ischemia (Gonzalez-Forero et al., 2007; Whitaker et al., 2013; Haddad, 2000; Teshima et al., 2003). NO/cGMP/PKG is one of the many intracellular pathways that can regulate Na^+/K^+-ATPase activity (Davies et al., 1997; Day et al., 2005). Basal age-related decline of Na^+/K^+-ATPase may be a

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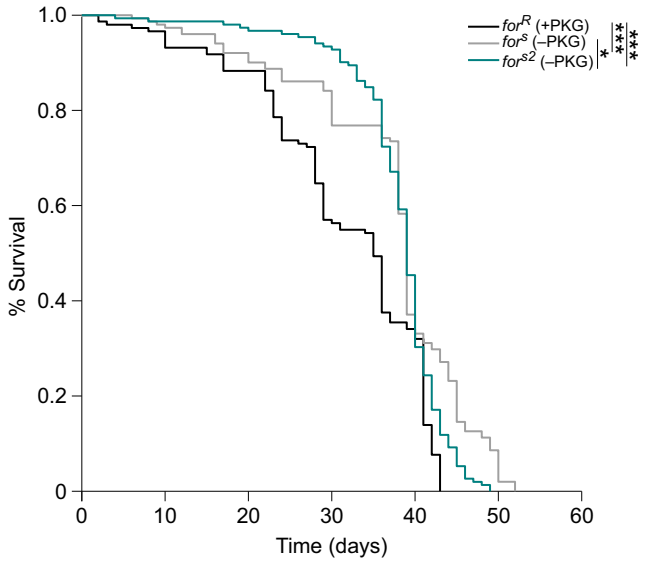

Fig. 6. Lower levels of PKG indicate increased longevity. To assess the effects of genetic levels of PKG on longevity, 20 male flies per vial were raised on standard Bloomington food and deaths were scored every day until all flies were dead. Both low PKG strains (for^{s} and for^{s2}) had a longer lifespan than the high PKG strain (for^{R}) under normal conditions. Kaplan–Meier log-rank survival curve (P<0.001) between all strains followed by a Holm–Šidák all pairwise multiple comparison test where *P<0.05 for all strains (N=10, n=200 per genotype).

consequence of decreased cGMP/PKG (Scavone et al., 2005), indicating that this pathway may be a powerful potential target for age-related neurological diseases, such as Alzheimer's, Huntington's and Parkinson's diseases (Teich et al., 2016).

Regardless of whether this study is indicative of behavior and fitness consequences in nature, our results make the fruit fly a clear choice for investigating potential diseases involving energy dysfunction. We have demonstrated that the PKG pathway, which is regulated by a naturally occurring polymorphic gene, plays an important role in healthy aging and stress tolerance in *D. melanogaster*. In these animals, higher tolerance to low-oxygen stress increases fitness early in life but comes at a cost to lifespan and locomotor activity later in life; however, such trade-offs may only be present in laboratory conditions. Because adult flies are considered post-mitotic (Bozcuk, 1972; Ito and Hotta, 1992), changes in functional senescence are more likely to be the result of a failure to maintain cellular homeostasis (Helfand and Rogina, 2003). Homologous patterns of aging between flies and humans may point to underlying mechanisms of aging diseases like stroke and neurodegeneration (Lim et al., 2012; Zhao et al., 2012; Williams et al., 2006; Day et al., 2005). By understanding functional decline, we will be able to find biomarkers of aging and identify therapeutic interventions to counteract the deleterious effects of aging. With increasing human lifespan comes a need to understand how we can also age well.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.P.K., K.D.-S.; Methodology: S.P.K., K.D.-S.; Software: S.P.K., K.D.-S.; Validation: S.P.K., K.D.-S.; Formal analysis: S.P.K., K.D.-S.; Investigation: S.P.K., K.D.-S.; Resources: S.P.K., K.D.-S.; Data curation: S.P.K., K.D.-S.; Writing - original draft: S.P.K., K.D.-S.; Writing - review & editing: S.P.K., K.D.-S.; Visualization: S.P.K., K.D.-S.; Supervision: K.D.-S.; Project administration: S.P.K., K.D.-S.; Funding acquisition: K.D.-S.

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