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Polyphenols as treatment at the intersection of environmental and genetic causes of Parkinson's disease in a *LRRK2* model.

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Abstract

Parkinson's disease (PD) is a chronic, neurodegenerative disorder that affects between 4 and 5 million people worldwide. The etiology of PD is both environmental and genetic. Thus far, research has shown that the reduction of environmentally triggered reactive oxygen species (ROS) levels can reduce parkinsonian symptoms in several animal models (Bonilla-Ramirez *et al.*, 2011). Furthermore, several genetic mutations including *LRRK2* (Leucine-rich repeat kinase 2) that lead to impaired mitochondrial function have been shown to impact Parkinson disease onset (Guo, 2012). We have used a fly *LRRK2* knockout model to study the role of environmental factors in PD development. *LRRK2* knockout flies were fed an antioxidant solution of polyphenols (propyl gallate, epicatechin, gallic acid, and epigallocatechin gallate) every 5 days. Polyphenol-fed flies and controls were examined using several measures of parkinsonian symptoms. Survival numbers, climbing ability, and dopamine immunohistochemistry were performed on flies at several times post-eclosion. *LRRK2* knockout flies fed with polyphenols did not show an altered lifespan, but showed a decrease in motor impairments. Treatment with polyphenols also decreased dopaminergic neuron degeneration as compared to control. It may be that polyphenols can effectively combat increased ROS due to impaired mitochondrial function in *LRRK2* mutants.

Introduction

Parkinson's disease (PD) is a chronic, neurodegenerative disorder that affects motor movement due to death of dopaminergic neurons in the substantia nigra pars compacta. Approximately 50,000 people in the U.S. are diagnosed with PD annually, and between 4 and 5 million people suffer from Parkinson's disease worldwide (Michael J. Fox Foundation, 2012). The major symptoms of PD include tremors, trembling, rigidity of limbs, slowed movement, poor posture, and issues with balance. There is no known diagnostic test or a cure for PD.

PD has both environmental and genetic origins and has been associated with defects in mitochondrial function. Defective mitochondria produce more reactive oxygen species (ROS), which are associated with aging, age-related disease, and neurodegeneration. Certain industrial environmental agents and agricultural chemicals, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or rotenone, produce PD-like symptoms by damaging mitochondria, which then produce increased levels of ROS (Jimenez-Del-Rio *et al.*, 2010). Heavy metals and paraquat (PQ) generate ROS and, in turn, parkinsonian symptoms (Jimenez-Del-Rio

et al., 2010). A study by Bonilla-Ramirez *et al.* (2011) showed that flies exposed to iron (Fe), manganese (Mn), and Copper (Cu) had reduced lifespan and locomotor activity, as well as degeneration in DA clusters due to oxidative damage. This study also showed that antioxidants (polyphenols: propyl gallate, epicatechin, gallic acid, and epigallocatechin gallate) can reduce parkinsonian symptoms caused by these ROS generators. Polyphenols exhibit neuroprotective effects based on their ability to lessen oxidative damage or by activating hormetic pathways that protect against cell toxicity (Vanzour, 2012). Therefore, ROS levels correlate with parkinsonian symptoms, and high ROS levels can be reduced using environmental antioxidants.

There is only one known gene that causes PD. Mutations in the *α-synuclein* (SNCA) gene cause an early-onset form of PD. Late-onset, or sporadic Parkinson's disease, which makes up about 90-95% of cases, is linked to the genes *Parkin*, *DJ-1* (*PARK7*), *PINK1*, and *LRRK* (*PARK8* or *dardarin*), all of which have been shown to cause to mitochondrial dysfunction (Guo, 2012). *LRRK2* is the most commonly associated gene for late-onset PD. *LRRK2* is a nuclear gene that encodes a cytoplasmic protein necessary for mitochondrial function (Smith *et al.,* 2005). The specific function of *LRRK2* is unknown, although the protein has both GTPase and kinase domains (Yue and Lachenmayer, 2011). Mutations of *LRRK2* primarily affect PD onset and can cause both familial and sporadic PD. However, the interaction is complex and the exact role of *LRRK2* in generating the disease is still unknown (Liu *et al.*, 2011).

One study by Ng *et al.* (2012) showed that the *Drosophila LRRK2* G2019S mutant (a gain of function mutation) exhibits swollen mitochondria in flight muscles as well as degeneration of dopaminergic neurons in the brain. Interestingly, the study also shows that EGCG protects against DA degeneration. Given that mitochondrial dysfunction and increased ROS production is central to the development of PD, these experiments support the notion that environmental antioxidants can be used to manipulate ROS levels in a *LRRK2* model.

The present study examined the effects of antioxidants on lifespan, motor ability, and DA degeneration in *LRRK2* knockout flies rather than the gain of function mutant used in Ng *et al.* (2012). We added a combination of polyphenols to the diet of *LRRK2* knockout flies, including propyl gallate (PG), epicatechin (EC), gallic acid (GA), and epigallocatechin gallate (EGCG). *LRRK2* knockout flies were generated using an antisense construct expressed in dopaminergic neurons in the brain. Our expectation is that the antioxidants would increase lifespan, decrease motor impairments, and decrease dopaminergic neuron degeneration, as compared to flies not fed with the polyphenol treatment. The antioxidants had a clear effect on dopaminergic degeneration and motor impairment, and no effect on lifespan.

Experimental Procedure

Fly Stock

Stocks were obtained from the Bloomington stock center. All flies raised for these experiments were fed on the standard cornmeal, molasses food and raised at 25^{o}C. We crossed *Ddc*-GAL4 flies with UAS-anti-*LRRK2* flies to generate a tissue specific LRRK knockout model. In our PD model, the F1 generation has the *LRRK2* knockout in only dopaminergic neurons (Guo, 2012).

Antioxidant Feeding

Two-three day old *Ddc*-Gal4/UAS-anti-*LRRK2* flies were administered 0.1 mM polyphenol solution in yeast paste spread on the surface of standard food in a fly bottle. The polyphenol solution consisted of a 0.1 mM solution of propyl gallate (PG), epicatechin (EC), gallic acid (GA), and epigallocatechin gallate (EGCG) in distilled water (Ortega-Arellano *et al.,* 2011). In all experiments, flies fed with the polyphenol treatment were compared to control flies that were administered yeast paste without polyphenols.

Lifespan

Eighty flies were placed into bottles spread with yeast paste with or without the polyphenol solution. Three replicates of each condition were performed. The number of live male and female flies in the vials were counted every 5 days and transferred into fresh bottles with yeast paste until no live flies remained (Bonilla-Ramirez *et al.,* 2013). The proportion of live flies was compared between flies fed with polyphenols and controls.

Climbing Assay

Two replicates of polyphenol fed and control fly bottles containing 50 flies each were used for climbing assays, which tested for locomotor impairments. Flies were placed into bottles with yeast paste and transferred every five days into fresh bottles until they were the appropriate age for testing. To perform the climbing assay, ten flies were placed in five separate, empty, clear vials with a line drawn at five centimeters. After a ten-minute rest period, the flies were gently tapped to the bottom of the vial. The number of flies that were able to climb above the five cm line in six seconds was recorded. The tests were performed three times with 1-minute intervals in between trials (Jimenez-Del-Rio *et al.*, 2010). Climbing assays were performed on flies at ages 5, 15, 20, 30, and 40 days.

Immunohistochemistry and TUNEL labeling

Dopaminergic degeneration was measured using immunohistochemistry staining on fly brains at ages 5, 15, 30, and 50 days. Flies were etherized and the heads and proboscises removed surgically. The heads were placed into 4% paraformaldehyde (PFA) to fix and then the brains were dissected under a dissecting microscope. The brains were fixed in PFA for 15 minutes and were then washed with 1× PBS. The PBS was then removed, and the brains underwent an ethanol dehydration series. The brains were then stored in 70% ethanol in the freezer. Ten replicate brains were dissected and stained for each condition.

Once removed from the freezer, the brain were rehydrated and incubated for 10 minutes at room temperature in 0.2 N HCl, then rinsed with 1× PBS. The brains were then blocked with a solution of PBS and 3% goat serum for 1 hour at room temperature. The primary antibody, 1000 fold dilution of rabbit tyrosine hydroxylase in PBGS (0.2% goat serum solution, Sigma-Aldrich, St. Louis Missouri) was added to the brains and incubated overnight. The brains were then washed extensively with PBS with 0.03% Triton X (PBTx). The secondary antibody, 1000 fold dilution anti-rabbit IgG conjugated with fluorescein in PBGS (Sigma-Aldrich, St. Louis Missouri), was added and the brains were incubated for 1 hour. The brains were then washed with PBTx. The TUNEL reaction mixture was then added and incubated at 37°C for 1 hour. The brains were then rinsed extensively in 1× PBS, mounted on a clean slide in Vectastain solution, and viewed under confocal microscopy (Feany and Bender, 2000). The number of DA clusters existing were counted and compared between polyphenol treated flies and control flies (Yang *et al.*, 2012).

Statistical Analyses

Analysis of variance (ANOVA) was used to compare groups of flies fed with polyphenols to control groups over time for lifespan, climbing assays, and dopaminergic degeneration. T-tests were preformed to compare dopaminergic death overall and degradation within specific clusters.

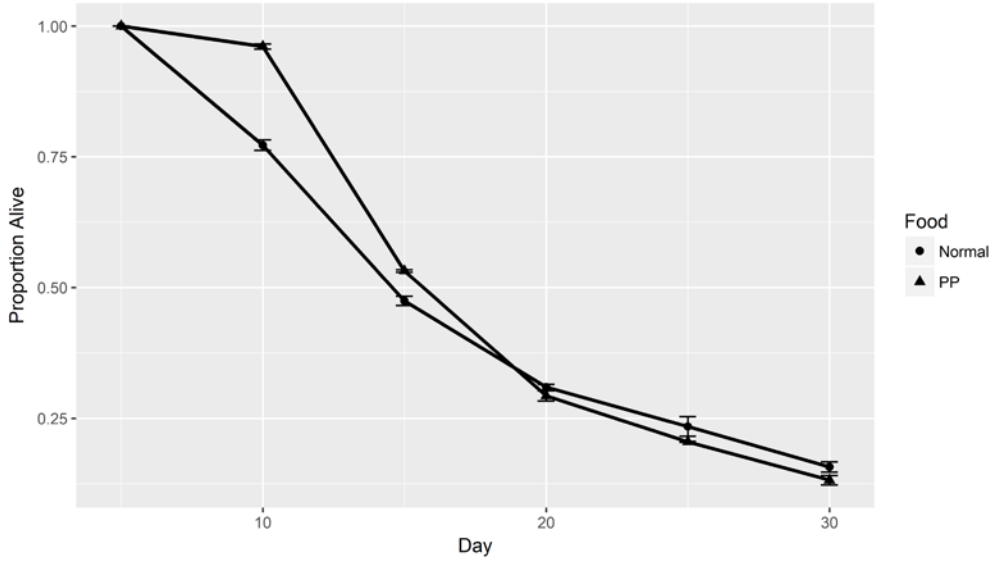

Figure 1. Polyphenol feeding did not have an impact on lifespan of *LRRK2* flies. Three replicate populations were assessed for treatment and control groups starting with 80 flies in each population and error bars are standard error of the mean.

Results

Polyphenols do not significantly increase lifespan

LRRK2 flies have a substantially reduced lifespan from those observed with wildtype flies (data not shown). Feeding a polyphenol solution to *LRKK2* knockout flies did not have an impact on lifespan of the flies (Figure 1). An analysis of the survival did not show a significant interaction between treatment and age on survival (F value = 1.66, P value = 0.21). As expected age had significant effect on lifespan ($P < 0.0001$), but treatment had no effect ($P = 0.27$).

Polyphenols significantly improve motor behavior

Polyphenols improve the climbing ability of *LRRK2* knockout flies (Figure 2). While there was no significant interaction between treatment and age and climbing (F value F = 0.089, P = 0.76), there was a significant effect of treatment on climbing ($P = 0.0005$) and age on climbing ($P < 0.0001$). Therefore, polyphenols appear to mitigate the negative impact of mitochondrial function in the LRKK2 knockout mutant.

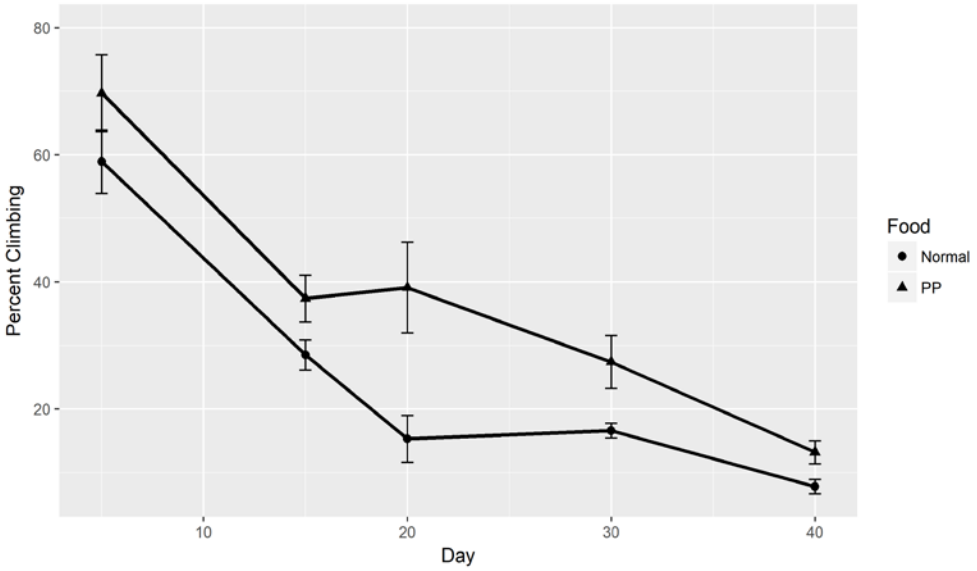

Figure 2. Polyphenols significantly improve climbing in *LRRK2* mutants. The tests were performed three times with 1 minute intervals in between trials and the average proportion of flies that climbed above the 5 cm line in 6 seconds at each age group was recorded for control and polyphenol fed flies. Data were compiled from 50 replicate flies and error bars are standard error of the mean.

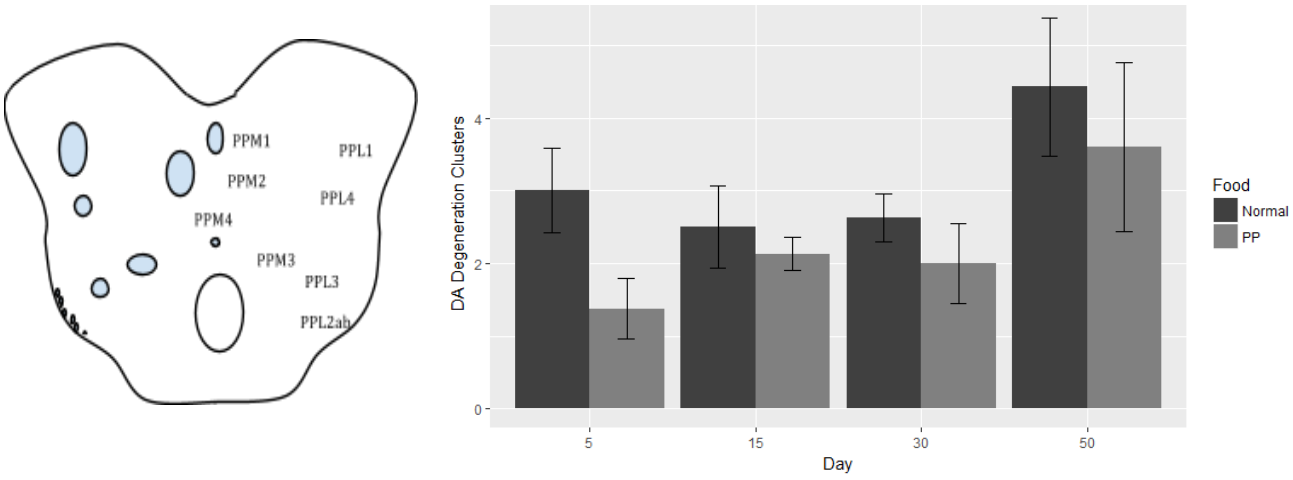

Figure 3. Impact of polyphenol treatment on *LRRK2* knockout flies over lifespan. The presence of DA and apoptotic cells was recorded, according to a map of 8 major DA clusters in *Drosophila* (3a). There was not a significant effect of polyphenols on DA degeneration over time (3b). Error bars are standard error of the mean.

Polyphenol treatment shows a small impact on dopaminergic degeneration

Brains from both treatment and control *LRRK2* knockout groups were observed for tyrosine hydroxylase staining (a dopamine marker) and for apoptotic degeneration using TUNEL staining. The expected pattern of dopaminergic neurons in the fly brain is shown in Figure 3a. Dopaminergic neurons undergoing degeneration will be obvious since they will appear yellow as compared to green dopaminergic neurons and red degenerating neurons. Both degenerating and nondegenerating dopaminergic neurons were observed; however, almost no neurons were degenerating that were not dopaminergic.

We found these data challenging to interpret. At any given time point (Figure 3b), there were no significant interactions between treatment and age on dopaminergic degeneration ($F = 0.0069$, $P = 0.934$), and there was no significant effect of the treatment on DA degeneration ($P = 0.127$), although as expected there was a significant effect of age ($P = 0.0008$). However, the average number of neurons per brain is significantly different (3.5 control and 2 for treatment, $P = 0.015$), and total overall number of degenerating neurons for an equivalent number of brains (80 *vs*. 50 averaged over three replicates, $P = 0.017$) is significantly different between control and treatment groups.

We also saw differential impacts on different clusters, although the small number of neurons in any given cluster evaluated here prevents statistical comparisons. There were many fewer degenerating neurons in cluster PPL1, PPL3, and PPL4 in the treatment groups. This differential effect on clusters has been reported before (Ng *et al.,* 2012). The time frame analysis is also problematic, since a neuron in a given brain may have degenerated before the time where the observation is done, and so it is not counted, although we did see a light increase in neurodegeneration with age. Therefore, we feel it is likely that the treatment had an impact on dopaminergic degeneration as suggested by our data and likely based on the impact of treatment we observed on climbing behavior.

Discussion

The purpose of this study was to determine if polyphenols could protect against parkinsonian phenotypes in a *LRRK2* knockout *Drosophila* model of Parkinson's disease. We examined the effect of treatment with polyphenols using a *Ddc*-Gal4/UAS-anti-*LRRK2* knockout. We hypothesized that flies fed with a solution containing multiple polyphenols (EC, PG, EGCG, GA) would have increased lifespan, decreased motor impairments, and decreased dopaminergic degeneration, as compared to flies fed without the polyphenol treatment. Polyphenol treatment did not significantly protect the lifespan of *LRRK2* flies although previous studies such as Yang *et al.* (2012) show an increase in lifespan with curcumin. The discrepancy may be a result of using the *LRRK2* knockout model versus a *LRRK2* mutation. Both gain and loss of function show Parkinson-like phenotypes. The knockout of *LRRK2* may be too debilitating beyond midlife for antioxidants to protect flies from death any longer. We did see a significant improvement in climbing assays in polyphenol fed flies versus control flies. These data is consistent with certain aspects of previous studies. Bonilla-Ramirez *et al.* (2013) showed that propyl gallate (PG) alone did not improve locomotor activity in knock-down *parkin* flies although EGCG was found to improve locomotion in both parkin and LRRK dominant mutant flies in Ng *et al.* (2012). Finally, although statistically inconclusive, we did find evidence that treatment was neuroprotective consistent with previous reports.

Even though polyphenols have exhibited neuroprotective effects, the underlying mechanisms remain unclear. Polyphenols are known to donate electrons or hydrogens to neutralize free radicals. These compounds may also induce other antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase. Some literature highlights the potential role of protein and lipid kinase signaling in polyphenol function as opposed to the traditional antioxidant role as electron donors (Tsao, 2010). The lack of clarity behind polyphenols' mechanisms suggests that other factors, such as the induction of mild cell stress (hormesis), may supplement or even be necessary for their benefits. Implications of the current study and future related studies include the possible use of polyphenols as a treatment to target the intersection of genetic and environmental causes of PD. PD has multiple causes, yet no existing cure. Clarification of the mechanisms of the disease and the relationships between both causes will help develop potential dietary and pharmacological applications for polyphenols.

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RNAi effects on the alpha glycerophosphate dehydrogenase, the alpha glycerophosphate oxidase and the arginine kinase paralogs of *Drosophila melanogaster.*

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Introduction

Alpha glycerophosphate dehydrogenase (GPDH) and alpha glycerophosphate oxidase (GPO) cooperate in the adult thoracic flight muscles to drive the alpha glycerophosphate cycle producing the ATP necessary for continual flight (Sacktor, 1965; and see Figure 1 in Davis and MacIntyre, 1988). Null mutants in either enzyme, as homozygotes or hemizygotes, cannot fly. The structural genes for the flight muscle variants for the two enzymes were mapped in the 1970's and 1980's (Grell, 1967; O'Brien and MacIntyre, 1972; O'Brien and Gethman, 1973; Davis and MacIntyre, 1988). When the genome of *Drosophila melanogaster* was sequenced, two additional paralogs were discovered. Carmon and MacIntyre (2010) compared the sequences and the exon/intron structures of the three forms of GPDH (GPDH-1, 2, and 3) and GPO (GPO-1, 2, and 3). GPDH-1 and GPO-1 encode the flight muscle specific forms mentioned above, whereas GPDH-2 and 3 and GPO-2 and 3 are expressed only in the testis.

Arginine kinase (AK), like GPDH and GPO, is particularly abundant in indirect flight muscle (Lang *et al*., 1980), although low levels are also present in other tissues (James and Collier, 1988). The structural gene for arginine kinase is located 66F (Fu and Collier, 1983; Munneke and Collier, 1988) and is responsible for four alternative protein products, all of which share a common catalytic domain. The single EMS-induced null is due to an amino acid substitution (L182Q) in the common domain. It is an embryonic lethal which precludes assessing its role in flight muscle energetics. There are also two additional paralogs of AK that are only expressed in the testis.

The relevant information on each of the nine paralogs is shown in Table 1. All forms of each enzyme are evolving under purifying selection indicating they are functionally important in the fly.

To further assess the functional roles of the paralogs for GPDH, GPO, and AK, we have inactivated them with RNAi's from the Vienna collection. These RNA lines are also listed in Table 1. To drive the expression of the RNAi's, we have used two Gal4 constructs, one with a tubulin promoter (tub-gal4) and one with a promoter from the *bag of marbles* gene (bam-gal4) (M^{c}Kearin and Spradling, 1990). The former should drive the RNAi's in most if not all cells, whereas the latter should do so in the male germ line. We have assessed three different phenotypes reflecting the actual and possible roles of GPDH and GPO during