Genetic Modifiers of Neurotoxicity in a *Drosophila* Model of Parkinson's Disease

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In partial fulfillment of the requirements for graduation with the Health Science Honors Degree in Neuroscience

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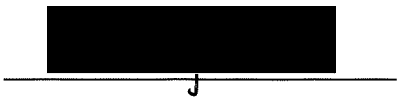

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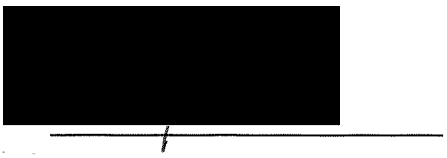

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Abstract

Parkinson's Disease (PD) is a neurological motor disorder that stems from the death of dopaminergic neurons in the substantia nigra, a region of the midbrain. In the United States, it is prevalent in 1% of people over 60 years of age. Rotenone is a compound that is known to induce PD-like symptoms via direct inhibition of Complex 1 in the mitochondria, leading to the formation of reactive oxygen species (ROS) and cell death in dopaminergic neurons. In *Drosophila melanogaster*, exposure to rotenone leads to locomotor impairments like those found in PD and has previously been used to model PD in flies. In this study, we used rotenone to induce PD-like symptoms in a subset of the *Drosophila* Genetic Reference Panel (DGRP) lines, which have known, unique polymorphisms. We assessed the effects of rotenone using a locomotive behavioral assay and compared DGRP lines to identify genetic variants that contribute to rotenone sensitivity. This means that a difference in assay performance can be attributed to the different genotype, so we can compare the polymorphisms between the fly lines and identify which mutation(s) may be responsible. We found 30 single nucleotide polymorphisms that are significantly associated with differential rotenone sensitivity, potentially providing insight into genetic factors associated with PD.

Introduction

Parkinson's Disease (PD) is a neurodegenerative disease that involves the progressive death of dopaminergic (DA) neurons in the substantia nigra pars compacta, a region located in the basal ganglia. Without these neurons, there is a lack of dopamine production in the brain. The loss of DA neurons affects 5 major pathways that involve the basal ganglia, which include the motor, oculo-motor, associative, limbic, and orbitofrontal circuits (Obeso et al., 2008). The impairment of these pathways gives rise to PD symptoms including tremors, bradykinesia (slowness of movement), gait disturbances (abnormal walking), and postural instability (Dauer and Przedborski, 2003).

There are nearly 1 million people living in the United States with PD, 10 million worldwide. While levodopa (l-DOPA), the current treatment for PD, temporarily alleviates symptoms, there is no definitive cure for the disease. This treatment has its own limitations. Since most drugs are unable to cross the blood brain barrier (BBB), it is impossible to simply inject pure dopamine into the brain. l-DOPA is a molecule that can cross the BBB and once it does, dopamine decarboxylase (DDC) converts l-DOPA to dopamine. Once DDC gets saturated, increases in l-DOPA administration will no longer lead to increases in DA, making the success of the drug only temporary. Repeated use of l-DOPA also causes levodopa-induced dyskinesia because l-DOPA causes widespread changes to striatal DNA methylation, which further emphasizes the limitations of this treatment (Figge et al., 2016).

While some instances of PD are inherited, known as familial PD, the cause in most cases of the disease is unknown. Some extremely strong toxins, such as MPTP, have been observed to cause PD in humans. The consumption of MPTP induced PD symptoms within 3 days in a student. An autopsy confirmed it to be PD when they found degenerated DA neurons in his substantia nigra (Fahn, 1996). Since then, the contribution of other environmental toxins to PD has been studied. While many toxins have been found to contribute to DA neurodegeneration, the exact cause of PD is still unknown. It has been suggested that PD results from both genetic and environmental factors working together (Samii et al., 2004).

Drosophila melanogaster, the fruit fly, has been used to model many human diseases *in vivo*. These animals share many of the same genes as humans and are very easy to maintain and reproduce. Along with the observation that orthologs of 75% of all human disease genes are found within the *Drosophila* genome, these factors make them an efficient organism in which to study human disease. Over the last several years, *Drosophila* PD models have been created, both using genetic knockouts and treatment with neurotoxins. Some of the most notable genetic models include the LRRK2 model, where dLRRK, *Drosophila's* human LRRK2 homolog, is knocked out (Xiong and Yu, 2018), and the α-Syn transgenic models, where mutants of human α-Syn are expressed using GAL4/UAS system (Feany and Bender, 2000). The neurotoxin-induced models include MPTP, 6-OHDA, Rotenone, and Paraquat models (Hisahara and Shimohama, 2010).

This study utilizes the rotenone model of PD in *Drosophila*. Rotenone is a direct Complex 1 inhibitor in the mitochondria, causing the formation of reactive oxygen species (ROS). Increased ROS leads to an increased expression of α-synuclein, a protein with currently unknown function. When α-synuclein aggregates in the brain, they form structures called Lewy bodies. While the direct relationship between these Lewy bodies and PD is unestablished, Lewy bodies are found the autopsy of PD patients and is a pathological hallmark of the disease (Davie, 2008). The drug has been shown to lead to age-related DA neuron degeneration and induce symptoms of PD in flies (Coulom and Birman, 2004). We chose to use rotenone because it was moderately safe to handle compared with MPTP. The impact of genetic variation on neurotoxin sensitivity has not been previously studied in *Drosophila*. Here, we tested the effects of various genetic backgrounds on rotenone sensitivity by analyzing motor performance in *Drosophila* to identify specific polymorphisms that could influence PD.

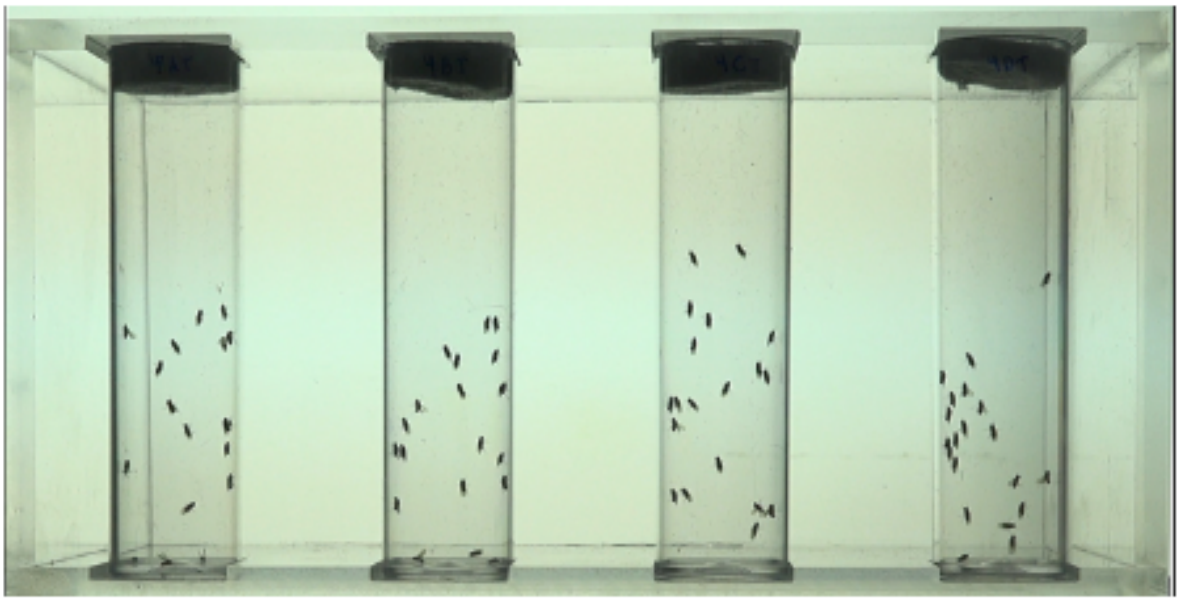

Figure 1. RING Assay Set-Up. Flies of the same genotype and the same condition group (treated in this example) are placed side-by-side in a clear, acrylic box. This processed image shows the final vertical position of each fly at 4 seconds. Vertical positions of the flies in each vial are averaged. Afterwards, those averages are further averaged across all vials of the same genotype and condition.

The rapid iterative negative geotaxis (RING) assay was developed to assess age-related locomotor decline in *Drosophila* (Gargano et al., 2005). As shown in **Figure 1**, vials of flies are placed in an apparatus, side by side, that is tapped, causing the flies to fall to the bottom. The flies will climb back up due to a motor reflex, and their motor performance is recorded using a camera. Vertical distance at a certain time point is usually used as a measure of locomotor performance of the fly. Climbing velocity and path taken can also be analyzed for other studies. Since PD causes motor deficiencies, the RING assay has been used to assess many different studies done on *Drosophila* models of PD (Barone and Bohmann, 2013). Our study will use this to determine the

sensitivity of the different strains to rotenone by analyzing locomotor performance of treated flies against the untreated flies.

The *Drosophila* Genetic Reference Panel (DGRP) is a panel of 205 fly lines each with known, unique polymorphisms that have been inbred over many generations (Mackay et al., 2012, Huang et al., 2014). Inbreeding over many generations gave rise to fly lines that are homozygous for their respective unique polymorphisms. This group of flies have been used to examine the impact of genetic background variation on many phenotypes related to different diseases, e.g. retinitis pigmentosa (Wang et al., 2017). Using the DGRP to conduct a genome wide association study (GWAS) is a fairly novel approach. Most recently, the DGRP has even been used to identify candidate modifier genes for the LRRK2 G2019S mutation in PD (Lavoy et al., 2018). We extend this approach to our study. Using a small number (30) of these DGRP lines, we assess the effects of genetic variation on the severity of rotenone effect as it concerns motor behavior and identify with new candidate genes that could possibly contribute to the progression of PD.

Materials and Methods

Animals and Rotenone Treatment

The 30 DGRP lines used in this study were obtained from the Bloomington *Drosophila* Stock Center. Flies were maintained at 25°C on a standard cornmeal-agar diet under a day/night cycle LD 12:12 (12 h of light and 12 h of darkness). Adult females (1-4 days post eclosion) were transferred into a vial containing Formula 4-24 instant *Drosophila* medium (Carolina Biological Supply, Burlington, NC) hydrated with a water/DMSO solution (3.5 g medium, 9.95 mL water, 50 µL DMSO) for the untreated group and water/DMSO/rotenone solution (3.5 g medium, 9.95 mL water, 47.5 µL DMSO, 2.5 µL 100 mM rotenone) for the treated group. Rotenone was obtained from Sigma-Aldrich and dissolved in a solution of DMSO to produce a 100 mM rotenone solution. The final concentration of rotenone in the food was 25 µM. This concentration was decided upon after many pilot experiments testing the survival rate of Canton-S flies after treatment of rotenone. We found that a concentration of 50 µM or higher kills almost all flies in under one week, which is surprising considering previous studies had a higher survival rate with much higher concentrations (Coulom and Birman, 2004). The results of this pilot experiment are shown in Figure 2. The rotenone solution was kept in the -20°C freezer. Food was made fresh whenever required. Treated flies were kept on a rotenone diet for one week, then they were moved onto the untreated diet for the second week. Flies were transferred into freshly made vials of food twice a week, every 3-4 days.

Rotenone Treatment (μM)	Survival at 1 Week (# of Flies Alive/Death)	Survival at 2 Weeks (# of Flies Alive/Death)
0	20/0	19/1
10	18/2	16/2
25	19/1	19/0
50	12/8	8/4
100	1/19	1/0
250	2/18	2/0
500	0/20	0/0

Figure 2. Results of Rotenone Survival Pilot Experiment. Each group started with 20 flies and were treated with different concentrations of rotenone (shown above). The first number in the survival column is the total number of flies that survived after a given time point and the second number represents how many flies have died since the last time point. For example, at 2 weeks, the second number represents how many flies have died between 1 week and 2 weeks. Method of rotenone treatment is the same as described in the materials and methods sections, but the concentration of rotenone was adjusted for the tested concentrations.

Geotaxis Assay

Flies were assayed at one week and two weeks after treatment. For the assay, flies were transferred into empty, clear vials, which were then inserted into a clear apparatus that was created for us by The University of Texas Mechanical Engineering Shop. The apparatus holds four vials (**Figure 1**). This apparatus was adapted from the original apparatus designed in Gargano et al., 2005. During the tests, the apparatus was tapped five times 9.25 inches in front of a light box (2 15 W bulbs) shining uniform white light. Assays were done in a dark room to minimize the effect of phototaxis on the flies' climbing performance. The light box, however, must be used for us to visualize the flies in the dark room. Climbing performance of the flies were then recorded using a camera (SONY Handycam FDR-AX100, obtained from Amazon). This was repeated 5

times for each vial. As done in previous studies that used the RING assay, one minute of rest was given for the flies between each trial.

Processing Data

Using Adobe Premiere, videos were paused at 4 seconds and that frame was isolated. That frame was further cropped and scaled using Adobe Photoshop. Using a custom-made macro, written by John Aldrich, for ImageJ Fiji, horizontal and vertical positions were calculated. The macro analyzed the processed images and determined an XY coordinate for each fly, which were then imported to Microsoft Excel. Microsoft Excel was then used to identify which vial each fly was in due to horizontal position. From there, the vials were then identified for each image and matched with their genotype. Vertical position was used as an indication of motor performance.

Genome Wide Association Study

Vertical position was averaged across the five trials and further averaged between all replicates of the same genotype. This final number was termed the position number. For each genotype, there was a position number for the treated group and untreated group. The ratio of position numbers for treated to untreated groups of the same genotype was calculated to use for a genome wide association study (GWAS), which was performed through a website that had all the DGRP genotypes: http://dgrp.gnets.ncsu.edu/ (Mackay et al., 2012, Huang et al., 2014). Candidate associations were determined based on a p-value threshold of $p < 10^{-5}$. The website has a database of the sequenced

Drosophila genome and a map of the SNPs in the genome. Using this, the website gives us genes related to the SNP possibly associated with the phenotype (sensitivity to rotenone).

Results

GWAS Reveals Candidate Modifiers of Rotenone Effect on Motor Performance

Geotaxis performance, quantitatively measured by vertical distance, was observed to vary across the different DGRP backgrounds, which suggests that genetic variation plays a role in rotenone-induced motor impairments.

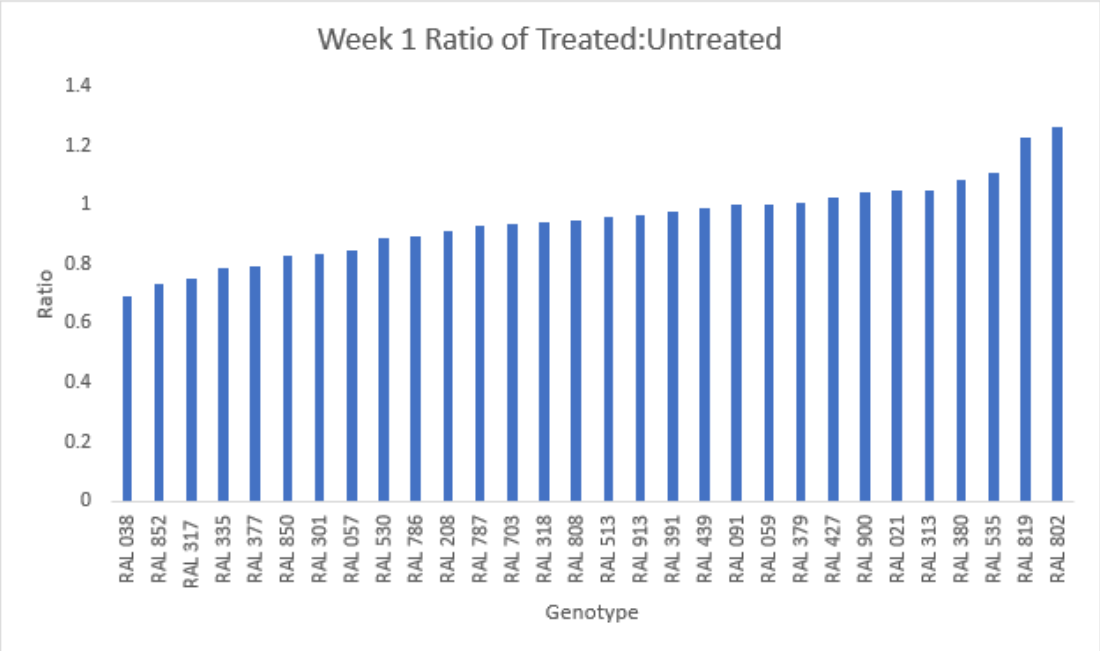

Figure 3. Distribution of Ratio Treated:Untreated Week 1. The ratio of the vertical distance of the treated group to the untreated group was calculated across each DGRP line. This data was obtained from the RING Assay performed 1 week after treatment. The graph shows the ratios for each genotype. Average = 1.03, Standard Deviation = 0.21

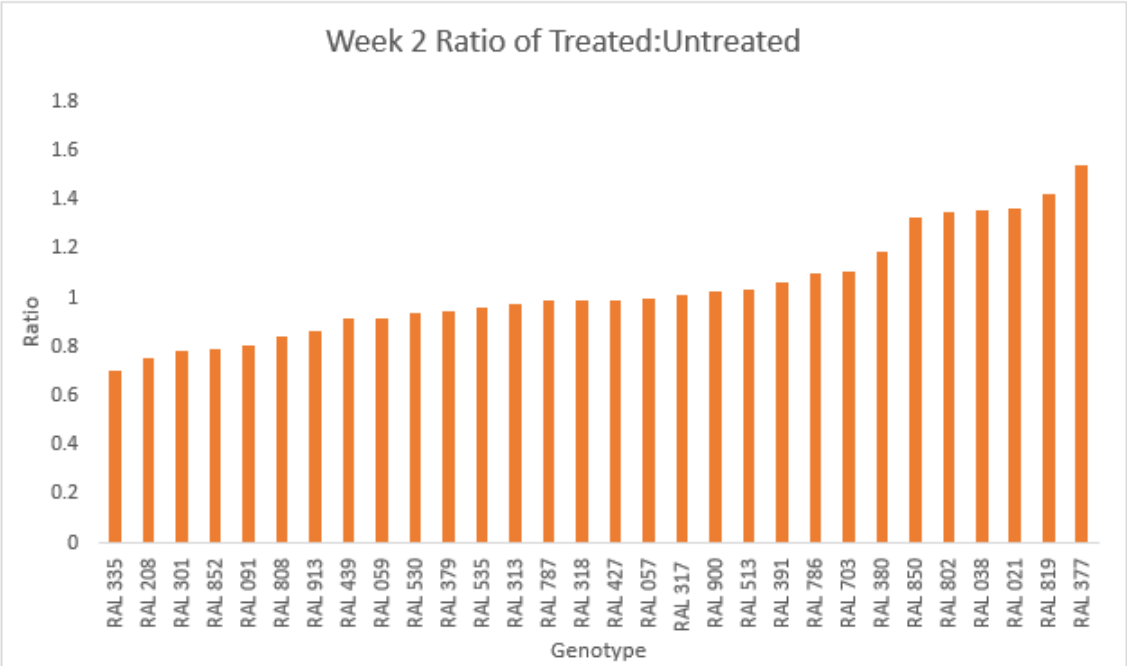

Figure 4. Distribution of Ratio Treated:Untreated Week 2. The ratio of the vertical distance of the treated group to the untreated group was calculated across each DGRP line. This data was obtained from the RING Assay performed 2 weeks after treatment. The graph shows the ratios for each genotype. Average = 0.95, Standard Deviation = 0.13

We calculated the ratio of vertical distance achieved in the geotaxis assay treated to untreated of each genotype and used those numbers to run a genome-wide association study testing for associated single nucleotide polymorphisms (SNPs). **Figure 3** and **Figure 4** show the distribution of these ratios for Week 1 (Avg = 1.03, Std. Dev = 0.21) and Week 2 (Avg = 0.95, Std. Dev = 0.13) assays, respectively. A ratio less than 1 means that the treated flies performed worse than the untreated flies, and a ratio more than 1 means that the treated flies performed better than the treated flies. The ratios from Week 2 Assays were used in the GWAS. 264,627 SNPs were tested over 30 DGRP lines. This approach allowed us to identify candidate genes that potentially contribute to the variation in geotaxis performance, but further testing, described in the discussion, must be done to make this association definitive.

Rank	SNP[a]	Candidate Gene(s)	p-value	Human Ortholog
1	3L_2593568_SNP	*dos*	$1.95E^{-6}$	*GAB1/GAB2/GAB4/GAB3*
2	3L_2593595_SNP	*dos*	$1.95E^{-6}$	*GAB1/GAB2/GAB4/GAB3*
3	2R_11149502_SNP	*alphaPS4*	$3.91E^{-6}$	*ITGA4/ITGA9*
4	2R_13617484_SNP	*CG4975*	$8.26E^{-6}$	*ATXN10*

[a]SNP with most statistically significant association for the candidate gene

Table 1. Top association candidate genes. This table presents the top associated ($p < 10^{-6}$) candidate SNPs, their corresponding candidate genes, p-value, and human orthologs. They are ranked from most associated to least associated (smallest to largest p-value). The GWAS reported the SNPs, candidate genes, and p-values. We matched the candidate genes to human orthologs using FlyBase.

A total of 18 candidate genes with 30 SNPs, collectively, were found to be associated with the variation in rotenone-induced geotaxis performance at a p-value threshold of $p < 10^{-5}$. 3 of these genes (with 4 associated SNPs) fall under the more restrictive p-value threshold of $p < 10^{-6}$, so they are considered top candidates by statistical association (**Table 1**). Each of these 3 genes have human orthologs listed in **Table 1**. The *dos* gene encodes a protein involved in SH2 domain binding, which is essential for signaling different receptor tyrosine kinases (Allard et al., 1998). This process is involved in photoreceptor cell and wing development for the fly (Herbst et al., 1996, Luschnig et al., 2000, Raabe et al., 1996). The *alphaPS4* gene is a protein coding gene that plays a role in cell adhesion (Brown, 2000, Hynes and Zhao, 2000). The final top associated candidate gene (*CG4975*) has a human ortholog (ATXN10) that is the causative mutated gene in spinocerebellar ataxia type 10. Expansions of ATXN10 with no repeat interruption motifs have also been found to be associated with PD in humans (Schüle et al., 2017). **Table 2** lists the top 10 candidate genes, after the top associated

candidate genes. Of these 10, 6 genes have obvious human orthologs. Of all 30 candidate SNP associations, 14 of them have human analogs.

Rank	SNP[a]	Candidate Gene(s)	p-value	Human Ortholog
1	3R_2958489_SNP	*CG31493*	$1.85E^{-5}$	-
2	2R_16481581_SNP	*bl*	$2.05E^{-5}$	-
3	2R_16980850_SNP	*CG34396*	$2.29E^{-5}$	*KCNK18*
4	3L_7624224_SNP	*CG33275*	$2.29E^{-5}$	*PLEKHG4*
5	3L_7739884_SNP	*CG32373*	$2.94E^{-5}$	*SCUBE2/SCUBE3*
6	3L_7740540_SNP	*CG13685*	$2.94E^{-5}$	-
7	3L_3270454_SNP	*CG14960*	$3.66E^{-5}$	-
8	X_13873994_SNP	*mamo*	$4.79E^{-5}$	*SP1*
9	3L_1600019_SNP	*CG13917*	$5.79E^{-5}$	*BTBD8*
10	3L_2593483_SNP	*dos*	$6.42E^{-5}$	*GAB1/GAB2/GAB4/GAB3*

[a]SNP with most statistically significant association for the candidate gene

Table 2. Top 10 candidate genes associated with variability. This table presents the next 10 statistically significant associated ($p < 10^{-5}$) candidate SNPs after the top association candidates, their corresponding candidate genes, p-value, and human orthologs. They are ranked from most associated to least associated (smallest to largest p-value). The GWAS reported the SNPs, candidate genes, and p-values. We matched the candidate genes to human orthologs using FlyBase.

Discussion and Future Directions

The results of the geotaxis assay revealed that after one week of treatment, 18 of the 30 lines (60%) performed worse after being treated with rotenone. When the assay was done at two weeks after treatment, 22 of the 30 lines (73.3%) performed worse after being treated with rotenone. This suggests the progressive nature of DA neurodegeneration induced by rotenone. We expect if we ran the assay at a later time point, almost all the treated lines will perform worse. Surprisingly, 3 of the 18 lines (16.7%) that performed worse (ratio < 1) in Week 1 performed better (ratio > 1) in Week

2. It is possible the genetic variation in these lines contributed to a recovery effect, but additional studies would need to be done to investigate this effect. We chose to use the ratios from Week 2 for the GWAS because at that time point, the effect of rotenone was prominent in more flies (Week 1 Average = 1.03, Week 2 Average = 0.95), better reflecting the results of the drug.

The aim of this study was to discover novel genes that could contribute to PD. With this in mind, our approach only resulted in candidate genes that could potentially affect our geotaxis phenotype, which was measured by vertical distance climbed by the fly after a certain timepoint. A gene ontology enrichment analysis is yet to be done to reveal the functional groups of those candidate genes. This analysis may provide insight on the pathways involved in rotenone metabolism. For example, an overrepresentation of a particular biochemical pathway might suggest the importance of that pathway in rotenone metabolism or uptake.

Furthermore, this study was designed as a pilot study for future experiments, which is why only 30 of the 205 DGRP lines were used. Extending this study to include all 205 DGRP lines will provide us with more candidate genes, and it is something we are planning to do next. Adding more lines to the experiment will also increase the statistical robustness of the study. If a certain gene or association consistently shows up across the added DGRP lines, it will make that association more statistically significant by decreasing the p-value.

As described in Lavoy et al., 2018, in future studies to validate candidate SNPs, we will knock-down candidate genes to analyze the impact on rotenone-induced neurodegeneration. RNAi lines will be constructed for each candidate, starting with the top-associated genes. Throughout this process, we can also visualize the process of neurodegeneration at any timepoint using Tyrosine-Hydroxylase (TH) Immunostaining. This technique was used in many earlier PD studies done in *Drosophila* to confirm neurodegeneration of DA neurons, thus replicating the pathophysiology of PD (Barone and Bohmann, 2013). TH is an enzyme that is necessary for dopamine production and exists in DA neurons so this type of staining will allow us to examine the progress of DA neuron death.

While the cause of PD is unknown, we know that the onset and progression of the disease is affected by genetic and environmental factors. The candidate genes that we reported, if validated through further testing, could provide insight on the interaction between these genetic and environmental factors. Our approach to discover how genetic and environmental factors affect each other could help drive future studies in PD by testing other environmental factors that contribute to the disease. This approach to use the DGRP to test the influence of environmental effects against different genetic backgrounds and running a GWAS with the data to discover more candidate associations can also extend to other diseases beyond PD.

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