PHARMACOLOGICAL RESCUE OF PARKINSON’S DISEASE SYMPTOMS

WITH DROSOPHILA LARVAE

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Abstract

Parkinson’s disease (PD) is a neurodegenerative disorder that is caused by the loss of dopaminergic neurons in the brain. Despite the fact that PD has wide-ranging impacts, both economically and on the quality of life of its sufferers, treatments for the disorder, including the “gold standard” drug L-DOPA, remain inadequate. In order to advance PD treatments and understanding of the disorder itself, it is necessary to develop animal models that are fast, versatile, and sufficiently mimic the pathology of human PD. Prior studies have demonstrated Drosophila larvae to be a useful model of PD, showing two key features: locomotion deficits and loss of dopamine neurons. In this study, I have extended this model to screen and better understand PD treatments. Both toxin and genetic PD models were used to quantify the degree of symptomatic improvement in locomotion speed, turning rate, and pause time after treatment with potential PD drugs. First, the drug L-DOPA was tested to determine whether PD-model Drosophila larvae experience a similar improvement to human patients in response to the drug. Second, nobiletin, a citrus flavonoid, was tested to determine its potential as a PD drug, and its potential mechanisms were studied using transgenic lines. Both L-DOPA and nobiletin were effective in treating PD-model Drosophila, with nobiletin found to act via dopamine D2 autoreceptors. These findings set the stage for a fast and reliable way to discover and study PD treatments in the future.
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Introduction

Background of Parkinson’s Disease

Parkinson’s disease (PD) is a neurodegenerative disorder affecting over four million people worldwide. It is the second most common neurodegenerative disorder behind Alzheimer’s disease, with far-reaching public health and economic effects. In the United States, PD was found to be the fourteenth leading cause of death in 2010 (Murphy et al., 2013). Its economic impact in the United States alone exceeds $10 billion annually, which includes the cost of prescription drugs and nursing home care (Chen, 2010). Much of the cost burden to PD patients also includes the loss of income due to the early retirement as the disease progresses (Keränen et al., 2003). PD seems to be exceptional in its impact on the quality of life of both sufferers and caregivers. In a study of over 800,000 veterans from the database of the US Veterans Health Administration, it was found that those subjects who suffered from PD had lower physical and mental quality of life scores than those who suffered from “angina/coronary heart disease, arthritis, chronic low back pain, congestive heart failure, diabetes, and stroke” (Gage et al., 2003). Besides the health of those who suffer from PD, it has been found that the disease decreases the quality of life of those who care for its sufferers. In one study, it was found that caregivers, 75% of whom are the spouses of patients, were more likely to have higher anxiety and depression scores and lower health-related quality of life scores than the general population, which was found to magnify with the progression of the disease (Martinez-Martin et al., 2008). Unfortunately, despite its huge negative impacts on patients, caregivers, and society as
a whole, PD is still poorly understood in many ways, and the standard treatments for the disease remain inadequate.

PD manifests itself via a wide variety of symptoms in the patient. It is most notable for the motor effects that it produces in the patient, which are abbreviated by the mnemonic TRAP. This acronym encompasses tremor, specifically a 3-6 Hz resting tremor, rigidity and stiffness of muscles, akinesia, the difficulty in initiating and maintaining movement, and postural instability, which includes problems with balance and gait (Frank et al., 2006). As Xia and Mao (2012) explain, PD is heterogeneous in that some sufferers experience mainly problems with rigidity and akinesia while others are mostly affected by tremors. PD involves progressive degeneration of the central nervous system, which causes the motor capabilities of patients to deteriorate over time. During the course of the disease, bradykinesia (slowness of movement), rigidity, and gait problems increase as the disease progresses. However, motor function deteriorates most quickly at the early stages of the disease and then decreases more slowly in the latter stages. Interestingly, the severity of tremors seems to be relatively stable even as other motor symptoms worsen (Xia & Mao, 2012). The symptoms included in TRAP are the most recognizable hallmarks of the disorder, but it has more recently become clear that PD is not just restricted to motor symptoms. Although James Parkinson described the motor symptoms of PD in the 1800s, the non-motor symptoms of the disease have received significantly less attention since that time. These symptoms span a wide variety of conditions affecting many areas of the body, including dementia, depression, anxiety,
sleep disturbances, gastrointestinal symptoms, and problems with olfaction. As with the motor symptoms, many of these symptoms increase with severity as the disease progresses (Chaudhuri et al., 2006). In this study, the primary focus will be on motor symptoms and how they can be treated.

The pathology of PD, especially the development of motor symptoms, is fairly well-understood. As Shulman et al. (2011) explain, the primary cause of PD is the loss of neurons that release the neurotransmitter dopamine, also known as dopaminergic or DA neurons, in the region of the brain known as the substantia nigra pars compacta (SNpc). The SNpc is a region of the midbrain that contains DA neurons with a high concentration of neuromelanin, which gives them a dark color (Figure 1). This region plays several roles in the nervous system, including reward and movement, and includes two major parts: the pars reticulata and the pars compacta. The DA neurons of the SNpc have axons that project to the striatum, which regulates movement via its indirect connections to the cerebral cortex. Normally, the output of the DA neurons in the SNpc excites the striatum, which leads to inhibition of the globus pallidus interna, a region which then inhibits the thalamus (Figure 2a). The thalamus normally acts to excite the motor cortex and initiate movement. However, as the neurons in the SNpc die, the dopamine output of this region is decreased, which causes greater inhibitory input to the thalamus (Figure 2b). This decreases the ability of the thalamus to excite the motor cortex, which explains the motor features of PD such as akinesia (Shulman et al., 2011). In summary, the degeneration of DA neurons in the SNpc and the subsequent reduced dopaminergic input to the striatum causes the development of
motor symptoms in PD, and the continued degeneration of these neurons explains why the disease progresses over time.

Figure 1: Illustration of the location of the substantia nigra in the midbrain as well as other nearby brain regions (Blausen.com staff, 2014). As seen in the sections on the right, PD results in the lightening of the substantia nigra due to the degeneration of DA neurons containing the pigment neuromelanin.
Figure 2: (a) The normal connection between the dopamine output of the SNpc and the motor cortex in the brain. (b) The degeneration of DA neurons in PD causes a decrease in dopamine input to the striatum. The loss of inhibitory output to the globus pallidus interna causes excessive inhibition of the thalamus, leading to motor dysfunction.
One obstacle in PD treatment is that the most visible symptoms appear only after large amounts of neurodegeneration have already occurred in the brain. By the time PD is generally diagnosed, 60% of DA neurons in the SNpc have already died, which causes an 80% decrease in dopamine input to the striatum, further underscoring the need for better criteria for early diagnosis (Shulman et al., 2011). Because of this, it is important to explore more options for early detection and neuroprotection in PD.

A predominant morphological feature of PD is the presence of structures called Lewy bodies in affected neurons. Lewy bodies are filamentous aggregates containing the protein alpha-synuclein (Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998). Although Lewy bodies are useful in post-mortem diagnosis of PD (as well as Lewy body-related dementia), it is not yet fully understood what role they play in the pathogenesis of PD. Several mutations of the alpha-synuclein gene, including A53T and A30P, are associated with an increased risk of PD, and both of these mutated forms of alpha-synuclein have a greater propensity to aggregate into Lewy bodies (Narhi et al., 1999). However, the exact mechanism of neurotoxicity is unknown because Lewy bodies also contain many other components besides alpha-synuclein, and it has been suggested that they may even represent an effort by the neuron to protect itself from further damage during the course of PD progression (Beyer, Domingo-Sàbat, & Ariza, 2009). Despite being a ubiquitous feature of PD, Lewy bodies remain poorly understood in their role in the development of the disorder.

The reasons for the development of PD are also not as well understood as the pathology itself. Besides alpha-synuclein mutations like A53T and A30P, there are
several other genes associated with the development of PD, such as LRRK2 and parkin (Shulman et al., 2011). However, very few cases can be linked to specific genes, although 10-30% of PD patients have PD in their family history, and those with first-degree relatives with PD have an increased risk of developing the disorder (Shulman et al., 2011). According to de Lau and Breteler (2006), most cases of PD are considered sporadic, in that they cannot be linked to a specific genetic mutation. A few risk factors have been identified, including exposure to the pesticides rotenone and paraquat, both of which interfere with electron transport, which is necessary for the generation of ATP in the mitochondria, as well as cause the increase of reactive oxygen species, which are known to be harmful to cell components (Blesa et al., 2012; de Lau & Breteler, 2006; Shulman et al., 2011). More research into the causes of PD development will be vital in helping researchers create better PD models, identify risk factors for individuals, and develop better treatments in the future.

**Advances in PD Treatments**

According to Brooks (2008), the discovery of L-DOPA, also known as levodopa, as an effective treatment for PD in the 1960s was the first major breakthrough in treatment of the disorder. In fact, the drug has proven so effective that, to this day, it is still considered the “gold standard treatment for PD” (Brooks, 2008). With its introduction, patients finally had a way to dramatically increase their quality of life and mobility after a long history of drugs that were both ineffective and had a high incidence of side effects.
Given what is now known about the pathogenesis of PD, it is clear why L-DOPA is such an effective treatment for the disorder. As discussed earlier, PD occurs due to the degeneration of DA neurons in the SNpc. To remedy this situation, one possible treatment is to directly administer dopamine to replace the reduced levels of the neurotransmitter. Unfortunately, dopamine itself cannot cross the blood-brain barrier (BBB) and thus treatment with dopamine is ineffective at affecting its level in the brain. On the other hand, L-DOPA, a precursor to dopamine, can pass from the circulation into the brain, where it is then converted to dopamine by the enzyme DOPA decarboxylase (DDC). This effectively increases the level of dopamine in the brain, which has been shown to effectively remedy the motor symptoms of PD (Connolly & Lang, 2014). Other drugs are usually prescribed in concert with L-DOPA to increase its effectiveness. For example, carbidopa is a DDC inhibitor that is commonly found in formulations of L-DOPA (Connolly & Lang, 2014). Although it may seem counterintuitive to inhibit the enzyme that catalyzes the conversion of L-DOPA to dopamine, the goal is actually to prevent this conversion from occurring in the peripheral circulation, which would prevent the drug from being able to cross the BBB. Therefore, formulations such as levodopa-carbidopa work the increase the availability of levodopa in the brain, producing a more profound therapeutic effect in PD (Connolly & Lang, 2014).

Despite its high efficacy in treating the motor symptoms of PD, L-DOPA treatment is far from a perfect drug. Long-term L-DOPA treatment is often associated with motor side effects such as dyskinesia, which involves involuntary muscle
movements. In some cases, the reduction in quality of life caused by these side effects can outweigh the benefits of treatment with the drug (Brooks, 2008; Sethi, 2008). In addition, L-DOPA is not equally effective against all the symptoms of PD. In terms of motor symptoms, L-DOPA is most effective against bradykinesia and rigidity, effective against tremor in a smaller subset of patients, and mostly ineffective against postural instability, freezing of gait, and falling, which can be severely debilitating in PD patients (Sethi, 2008). Non-motor symptoms of PD, such as depression and sleep problems, are also generally unresponsive to L-DOPA treatment (Sethi, 2008). In addition, even when the drug is effective, its effects “wear off” between doses, which produces periods during the day when the patient’s symptoms return (Brooks, 2008). Finally, there is a concern that L-DOPA may be neurotoxic in the brain because its metabolites include reactive oxygen species, which has the potential to accelerate neurodegeneration. Although this neurotoxicity has not been established *in vivo*, this may still provide an incentive to pursue other forms of treatment that carry less risk (Münchau & Bhatia, 2000). Although it is currently a “gold-standard” drug, L-DOPA’s inadequacies have led to research into other types of drugs to combat PD symptoms.

One common example of these newer drugs is the category known as the dopamine agonists. These drugs, rather than directly replacing dopamine in the brain, mimic the neurotransmitter’s structure in nigrostriatal dopamine receptors. Activating these receptors provides an alternative method to replace the reduced dopamine levels in the brain. Dopamine agonists, representing a wide variety of drugs with different
effects and targets, can provide several advantages over L-DOPA treatment. Prominent members of this group include pramipexole and ropinirole, which both preferentially target D2 and D3 dopamine receptors (Münchau & Bhatia, 2000). These drugs are often less likely to produce motor side effects, such as dyskinesia, which can be beneficial for patients in whom these effects are debilitating (Brooks, 2008; Münchau & Bhatia, 2000). When used in combination with L-DOPA, dopamine agonists can reduce the necessary dose of L-DOPA, which both reduces the risk of side effects as well as the potential for generation of reactive oxygen species (Münchau & Bhatia, 2000). However, L-DOPA remains the “gold standard” treatment because the known DA agonists are less effective against PD symptoms as the disorder progresses, and L-DOPA is generally prescribed at some point for most PD patients, even if other drugs are used at the same time (Brooks, 2008).

Due to the shortcomings of all existing PD drugs, it is vital to explore new options that have the potential be more efficacious or produce fewer side effects.

**Animal Models of PD**

In order to better understand the disorder, the factors that cause it, and its potential treatments, researchers have utilized a variety of animal models. These include a diversity of animals, including monkeys, rodents (rats and mice), fruit flies (*Drosophila melanogaster*), and *C. elegans*, as well as many different treatments to model the disease itself, including chemical and genetic options (Blandini & Armentero, 2012).
According to Blesa et al. (2012), the chemical models include the previously mentioned pesticides rotenone and paraquat, which have been implicated in human PD, as well as MPTP, a structural analog to paraquat, which all act via generating oxidative stress in the target cells. The rotenone model is particularly promising because it has been shown to replicate the full spectrum of PD symptoms in model organisms, including changes in locomotion, the formation of Lewy bodies due to alpha-synuclein aggregation, and even non-motor symptoms like gastrointestinal problems (Blesa et al., 2012).

Genetic models attempts to use the few genes known to be associated with human PD to reliably produce the condition in the model animal. Some of the common models are mutations in the gene for alpha-synuclein, such as A53T or A30P, as well as the other aforementioned genes such as LRRK2 and parkin. The A53T model in rodents has been shown to reliably induce motor deficits and to produce Lewy bodies in DA neurons, but has been less successful in inducing degeneration of DA neurons, a hallmark symptom of PD (Blesa et al., 2012). *Drosophila melanogaster*, which normally do not produce alpha-synuclein at all, can be made to express the gene for mutated human alpha-synuclein (A53T), which reliably produces locomotor deficits, Lewy body-like aggregations, and the degeneration of DA neurons (Auluck et al., 2002; Feany & Bender, 2000; Varga et al., 2014).

With such a wide variety of both animals and treatments to model PD, the next step is to select a model that is fast, versatile, and useful for modeling human PD.
**Drosophila Larvae as a PD Model**

The model chosen for this study was larval *Drosophila*. *Drosophila* provide a useful system for studying PD for several reasons. As described by Varga et al. (2014), models that produce PD-like symptoms in other animals, including A53T and rotenone, are also successful in larval fruit flies, inducing locomotor deficits and neurodegeneration. In addition, A53T and rotenone have both been shown to have time-dependent effects on locomotion and neurodegeneration in fruit flies, similarly to human PD progression. Although adult *Drosophila* can produce the symptoms described above in response to expression of human alpha-synuclein, they have been somewhat inconsistent, with some studies showing no DA neuron degeneration or locomotion deficits (Auluck et al., 2002; Feany & Bender, 2000). On the other hand, larval *Drosophila* consistently show both locomotion deficits and degeneration of DA neurons (Varga et al., 2014). *Drosophila* larvae provide a consistent way to model the hallmark PD symptoms, which is necessary to accurately modeling the disorder.

*Drosophila* is known as an important model organism because of its wide variety of genetic tools, and researchers can take advantage of them to better understand PD and its treatment. One of the most useful, which has been utilized in this study, is the UAS/GAL4 system. As Brand and Perrimon (1993) explain, this binary genetic system allows the targeting of the expression of certain genes to a specific subset of cells. To do this, the GAL4 protein, which is a transcriptional activator originally found in yeast, is placed downstream of a promoter in the *Drosophila* genome, so that it is expressed whenever the promoter’s gene is
transcribed. In turn, the GAL4 protein activates the transcription of genes containing a GAL4 binding site, also known as an upstream activation sequence (UAS). In order to direct the expression of genes in certain cells, the GAL4 is only placed downstream of promoters of genes that are uniquely expressed in that cell type. For example, in order to cause the expression of a certain gene in dopaminergic neurons, GAL4 can be placed under the promoter for the gene for tyrosine hydroxylase (TH), an enzyme necessary for the production of dopamine. However, because the UAS/GAL4 is a binary system, if a fly line has the genotype TH-GAL4 but has no UAS, the GAL4 protein will not activate the transcription of any genes. On the other hand, a different fly line can then have a UAS attached to any given gene (for example, A53T), but A53T will not be expressed if the line has no corresponding GAL4 protein. If the two lines, TH-GAL4 and UAS-A53T, mate, both elements of the binary system will be present, and the offspring will express A53T in cells that express TH, specifically DA neurons (Brand & Perrimon, 1993). This powerful genetic tool allows targeting of gene expression and the studying of the precise spatial mechanisms of different therapies.

Another important advantage of the Drosophila larval model is that it can reliably generate PD-like symptoms in four days, which means that treatments can be tested extremely quickly compared to the slow generation times of mammalian models and even the relatively slower adult Drosophila models (Varga et al., 2014). This is a very important factor because it can allow more treatments to be tested in a given period of time, potentially leading to the discovery of more drugs to combat PD.
Due to all of these advantages, *Drosophila* larvae have been shown to be a fast and versatile model of PD, and the goal of this study is the further development of this model.

**Experimental Goals**

Despite its usefulness, the *Drosophila* larval model has not been used to study PD treatments, despite the fact that these various advantages would be useful in quickly screening drugs as well as probing their mechanisms. In order to determine whether the larval PD-like condition was related to human PD, it needed to respond to standard treatment. Establishing this relationship would provide more evidence that novel treatments discovered using this model have the potential to be beneficial for human patients.

Therefore, there were three goals for this study. *First, a more comprehensive locomotion assay for identification of PD-like symptoms was developed.* In the past, locomotion dysfunction in the *Drosophila* larval PD-like condition was measured using only the locomotion speed (Varga et al., 2014). However, as described above, slow movement (bradykinesia) is only one of the motor symptoms of PD, and drugs like L-DOPA do not work equally well in treating all symptoms. Therefore, a more nuanced model would take other measures of locomotion into account besides locomotion speed. In order to expand the model, a protocol for determining two additional parameters that have been observed to suffer in the *Drosophila* PD-like condition, turning rate and pause time, was developed. *Second, the larvae were*
exposed to L-DOPA, the “gold standard” drug for treating PD in humans, to determine whether they showed a similar symptomatic improvement. The degree of improvement was measured using the locomotion speed, turning rate, and pause time. Third, with this therapeutic effect established, the goal was demonstrate how the model could be used to test previously understudied treatments or screen new drugs. For this purpose, the citrus flavonoid nobiletin was chosen for its previously demonstrated potential therapeutic benefit in various disorders of the central nervous system, including PD, Alzheimer’s disease, and brain ischemia (Yabuki et al., 2014). To demonstrate the uses of Drosophila genetic tools in understanding the mechanisms of drug candidates, a chosen gene (DD2R) was down-regulated in target cells using the UAS/GAL4 system to demonstrate its role in mediating the therapeutic effects of nobiletin.
Materials and Methods

Fly Culture Maintenance

*Drosophila melanogaster* fruit flies were raised in media prepared according to the following guidelines:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Gram/liter ddH₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry yeast</td>
<td>21.00</td>
</tr>
<tr>
<td>Agar</td>
<td>9.00</td>
</tr>
<tr>
<td>Dextrose</td>
<td>48.75</td>
</tr>
<tr>
<td>Sucrose</td>
<td>21.00</td>
</tr>
<tr>
<td>Cornmeal</td>
<td>60.00</td>
</tr>
</tbody>
</table>

The media also contained 0.4% propionic acid as a preservative.

The flies were grown on a 12-hour light/dark cycle at 20-25°C and were transferred to new bottles on a weekly basis.

Collection of Larvae for Testing

Fruit fly media was removed from an empty bottle and was melted while stirring vigorously. The media (5mL) was poured into the lid of a CytoOne 35mm culture dish and was topped with a small amount of yeast paste after being allowed to cool to room temperature. Adult flies were placed in an empty plastic bottle topped with the food plate, which was then inverted to allow for egg-laying. The bottle was placed in a dark area for three hours, after which the egg-laying plate was removed.
and incubated for 90 hours to obtain third-instar larvae. During testing, larvae were removed from the food using double-distilled water (ddH2O).

Administration of Chemical Agents

Three chemicals were administered to larvae in this study: rotenone, L-DOPA, and nobiletin. All of the chemicals were added to the melted food in the egg-laying plates before solidifying and larvae were incubated in this food for the entire 90-hour period before testing. All stock solutions were prepared monthly and kept at 4°C to ensure freshness.

Rotenone was kept in a DMSO stock solution and administered to flies at a concentration of 10μm, a dose that has been found to significantly impair larval locomotion while still ensuring a 72.5% third-instar survival rate (Varga et al., 2014). L-DOPA was administered in media in the presence of 25mg/100mL ascorbic acid to prevent oxidation (Chen et al., 2014). Nobiletin was administered in a DMSO stock solution.

All plates (including controls) contained 0.1% DMSO, and all L-DOPA controls contained matching amounts of ascorbic acid.
Fly Strains

The following strains were used in this study:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Canton S)</td>
<td>Bloomington Drosophila Stock Center (Indiana University)</td>
</tr>
<tr>
<td>UAS-A53T α-synuclein</td>
<td>Dr. Leo Pallanck (University of Washington)</td>
</tr>
<tr>
<td>1407-GAL4</td>
<td>Bloomington Drosophila Stock Center (Indiana University)</td>
</tr>
<tr>
<td>UAS-(DD2R-RNAi)</td>
<td>Bloomington Drosophila Stock Center (Indiana University)</td>
</tr>
<tr>
<td>TH-GAL4</td>
<td>Dr. Jay Hirsh (University of Virginia)</td>
</tr>
</tbody>
</table>

Fly Crosses

Crosses were made by collecting virgin female flies and male flies and combining them in one bottle. Sex and virginity were verified by allowing both male and virgin female flies to live in separate bottles for four days to ensure that no new larvae were born. Two lines were previously prepared as permanent homozygous lines: 1407-DD2R and TH-A53T α-synuclein. The TH-(DD2R-RNAi) line was prepared by crossing TH-GAL4 and UAS-(DD2R-RNAi) and using the heterozygous F1 larvae.
Larval Locomotion Assay

Larvae were collected at 90 hours after egg-laying for the locomotion assay. A stage was created by boiling 2.5% agar in ddH2O, adding 5 drops of India ink for contrast, and pouring the mixture into a petri dish to solidify. Third instar larvae were loosened from the food using ddH2O and placed individually on the stage to be tested. First, the larvae were allowed to move freely for one minute to allow them to acclimate to the stage. Next, the larva was recorded from above using a Moticam3 digital camera recording at approximately 10 frames/sec with the Motic Images Plus 2.0 software. The recording time was 30 seconds, with recording being stopped if the larva left the field of view. Several drops of ddH2O were periodically added to the stage to keep it from drying.

Statistical Analysis

Changes in measures of locomotion in response to A53T or rotenone, as well as the degree of rescue were determined with a one-way ANOVA followed by a Bonferroni post-hoc test to compare pairs of groups.
Results

Analysis of locomotion parameters

The program ImageJ (http://rsb.info.nih.gov/ij/) was used to analyze the video files. The videos were first imported to produce a stack of images for each frame. The color threshold was then adjusted so that the larva was a distinct object in contrast with the background.

In order to determine the locomotion speed of the larvae, the MTrack2 plug-in (http://valelab.ucsf.edu/~nico/IJplugins/MTrack2.html) was used to determine the path length. The length was then converted to mm/min by using the number of seconds in the video file as well as a conversion factor between pixels and millimeters, which had been determined previously to be 12.2 pixels/mm given the height of the camera (Varga et al., 2014).

To find the angular speed and pause time of the larvae, software was developed (with the assistance of Jason Bury, B.S. Computer Science and Engineering, University of Toledo) to analyze the raw movement data as exported from MTrack2. The raw data consisted of a set of ordered pairs corresponding to all the points to which the larva moved. To analyze the raw data, the software divided the number of frames in the video by the number of seconds in the video. The resulting number corresponded to the number of frames in one second of video, and the software then sampled the position of the larva at each second. This was done to reduce noise in the video to provide more accurate results; the camera used in this study recorded at approximately 20 frames per second, but other studies that study
various parameters of larval locomotion record at 1-2 frames per second (Saraswati et al., 2004). Lines were then made connecting each of the selected points (Figure 3).

To determine the angular speed, the angle was determined at the intersection of each pair of lines. To do this, the distance formula (1) was used to determine the distance of each of the two lines, as well as the distance of a third line that would produce a triangle between the two lines.

\[
d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}
\]

Given this triangle, the angle of interest (gamma) was determined using the law of cosines (2).

\[
c^2 = a^2 + b^2 - 2ab \cos(\gamma)
\]

This angle was then subtracted from 180° to determine how many degrees the larva had deviated from a straight line in that given second. This analysis was done for each second, and the average in °/sec was subsequently determined by dividing the sum of all of the angles by the number of seconds.

The pause time was determined using the same software. For each line indicating a second of movement, the distance was converted to mm/min using the technique described above. A threshold value would determine whether the larva was considered to be “paused” or “in motion” as even a stopped larva registers some movement due to head shaking, rolling, etc. A value of 45 mm/min was found to correlate well with the pause time seen by a human observer for several videos. The number of seconds in which the larva moved at a speed less than the threshold value was found, and this was then converted to the number of seconds paused per minute.
In figures, pathways were visualized by selecting the 30-second pathways that most closely represents the locomotion parameters of each group. Arrows were placed at the end of each pathway to show the direction of motion.

**Figure 3**: Locomotion paths of larvae were exported from the MTrack2 plugin, which also provided the locomotion speed. The paths were then sampled at one-second intervals to determine average turning rate and pause time.

**L-DOPA effects in the rotenone-treated larvae**

In order to support the ability of the larval *Drosophila* model to translate to human PD, it was necessary to test the ability of a known PD drug to rescue symptoms. L-DOPA is the gold-standard treatment for human PD, and it is vital that a model for testing therapeutic approaches to PD shows a response to the drug. The
rotenone model in larval *Drosophila* has been shown to reliably model human PD, both by causing locomotion deficits, specifically decreasing crawling speed, and inducing the degeneration of DA neurons in the brain (Varga et al., 2014). In this study, three measures were used to quantify the extent of locomotion deficits in order to create a more nuanced picture of how the larva’s motion is affected; these include crawling speed, turning rate, and pause time. Based on observations of locomotion, it was expected that 10µM rotenone administered for 90-96 hours would cause significant deficits in all three of these measures. In fact, the rotenone treatment caused crawling speed to decrease, turning rate to increase, and pause time to increase compared to the wild-type larvae (Figure 4). L-DOPA was then administered at a dose of 100µM (which was previously determined to produce the maximum therapeutic effect) concurrently with the rotenone treatment (Table 1). L-DOPA treatment fully restored each of the three measures of locomotion to wild-type levels, and the path of the L-DOPA treated larva is both longer and straighter than the larva that was not given the treatment (Figure 4). This finding provides evidence that the human PD drug L-DOPA provides a similar benefit in the larval *Drosophila* rotenone model.

**Table 1: Locomotion speed of A53T larvae at various doses of L-DOPA**

<table>
<thead>
<tr>
<th>Locomotion speed (mm/min)</th>
<th>10µM L-DOPA</th>
<th>100µM L-DOPA</th>
<th>1mM L-DOPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locomotion speed (mm/min)</td>
<td>43.3</td>
<td>83.4</td>
<td>78.8</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>18</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 4: Representative pathways of each group (a) show that L-DOPA normalized the locomotion of the rotenone-treated larvae to wild-type levels. When 100µM L-DOPA was administered to rotenone-treated larvae, all measures of locomotion were fully restored, including (b) locomotion speed, (c) turning rate, and (d) pause time. (* = p<0.05; ** = p<0.01; rot = 10µM rotenone; L-DOPA = 100µM L-DOPA)

L-DOPA effects in the A53T larvae

Besides the environmental PD model provided by rotenone treatment, the A53T model can provide insight into the genetic causes of PD. In order to determine whether L-DOPA treatment is equally effective in the A53T compared to the rotenone model, the A53T larvae were administered the same dose of L-DOPA (100µM)
chronically from birth. The A53T larvae, similarly to the rotenone-treated larvae, experience deficits in all three measures of locomotion, showing a lower speed, a greater turning rate, and a greater pause time (Figure 5). When treated with L-DOPA, each of the three measures was significantly improved compared to the untreated larvae; however, there was still a significant difference between the treated larvae and the wild-type larvae, which did not exist for the rotenone model larvae (Figure 5). Notably, there was a higher percent improvement in locomotion speed (62%) compared to turning rate (50%) and pause time (56%). Another observation is that A53T larvae had a similar crawling speed to rotenone-treated larvae, they showed a much higher turning rate and pause time. This may help explain the difference between the two models, which is explored further in the Discussion section. In summary, the A53T model responded positively to L-DOPA treatment, although not to the same extent as the rotenone model.

**Nobiletin effects in the rotenone-treated larvae**

With the effectiveness of L-DOPA shown as a proof-of-concept, the next step was to show the true benefit of this PD model: to test novel substances for their ability to rescue PD symptoms. The chemical that was selected for this study was nobiletin, a citrus flavonoid that has demonstrated some potential benefits to health, including combating PD symptoms, as discussed in the Introduction. A dose of 10µM nobiletin was sufficient to fully rescue all locomotion deficits in the rotenone model, restoring
crawling speed, turning rate, and pause time to wild-type levels (Figure 6). This finding supports the notion that nobiletin may have promise as a PD drug in the future.

**Nobiletin effects in the A53T larvae**

In order to provide a more comprehensive picture of the potential benefits of nobiletin in treating PD in humans, the chemical was also tested in the genetic PD model using A53T larvae. The same dose of nobiletin that was provided to rotenone-treated larvae is effective in rescuing the deficits in the A53T model (Figure 7). In a similar case to L-DOPA, though, the drug did not restore the measures of locomotion fully to wild-type levels (Figure 7). However, nobiletin differed from L-DOPA in the extent that it restored specific symptoms. Unlike L-DOPA, the highest percent improvement was in pause time (68%) followed by locomotion speed (63%) and turning rate (58%). This finding shows that nobiletin is effective in treating symptoms in both the genetic and environmental PD models, suggesting that its therapeutic effects are not restricted to one specific type of PD.
Figure 5: Representative pathways of each group (a) show that L-DOPA partially restored all measures of locomotion in the A53T larvae. When 100µM L-DOPA was administered to A53T larvae, (b) locomotion speed, (c) turning rate, and (d) pause time were improved but still differed significantly from wild-type larvae. (* = p<0.05; ** = p<0.01; rot = 10µM rotenone; L-DOPA = 100µM L-DOPA)
Nobiletin effects in 1407-(DD2R-RNAi) larvae

One benefit of using *Drosophila* as a model is the ability to target genetic changes to specific cells using the UAS/GAL4 system (as described in the Introduction). This powerful tool was utilized in this study to try to determine the mechanism by which nobiletin rescues locomotion deficits in the larvae. Because the...
primary cause of PD pathology is the degeneration of dopaminergic neurons in the substantia nigra pars compacta and the subsequent decrease of dopamine available to postsynaptic neurons, a common target of PD drugs is the dopamine receptors, including the D2 receptor, a G protein-coupled receptor that causes inhibition of the postsynaptic cell. *Drosophila* express a homologous receptor known as the *Drosophila* D2-like receptor (DD2R). Using UAS-(DD2R-RNAi) to decrease the expression of DD2R in target cells, the 1407-GAL4 line was used to express the gene pan-neuronally. The rotenone model was used in order to determine whether nobiletin would be effective in the 1407-(DD2R-RNAi) larvae (the A53T model could not be used in this case because it requires a different GAL4 driver, which would cause undesirable interference). The 1407-(DD2R-RNAi) larvae experienced deficits in crawling speed and pause time but not turning rate when rotenone was administered (Figure 8). More importantly, nobiletin was ineffective in rescuing either of the affected locomotion deficits (Figure 8). These data suggest that nobiletin acts via DD2R to rescue locomotion deficits in the PD models.
Figure 7: Representative pathways of each group show that administration of nobiletin partially rescued the locomotion of A53T larvae but that the rescued group still had a shorter locomotion path and more irregular movement than the wild type group. Similarly to L-DOPA, when 10µM nobiletin was administered to A53T larvae, all measures of locomotion were partially restored, including (a) locomotion speed, (b) turning rate, and (c) pause time, although they did not reach wild-type levels. (* = p<0.05; ** = p<0.01; nob = 10µM nobiletin)
Figure 8: Representative pathways (a) shows that all groups had irregular paths, although the control group covered more total distance. Nobiletin was ineffective in treating any symptoms caused by rotenone treatment in 1407-(DD2R-RNAi) larvae (b, d). Notably, rotenone did not cause any changes in turning rate (c). (** = p<0.01; rot = 10µM rotenone; nob = 10µM nobiletin)

Nobiletin effects in TH-(DD2R-RNAi) larvae

Although it was now known that nobiletin works via DD2R, which cells specifically provide the target site for its effects was still unknown. The excitability and firing rate of dopaminergic neurons themselves is modulated via D2 receptors,
known as autoreceptors (Beaulieu & Gainetdinov, 2011). Agonism of these autoreceptors has already been shown to decrease the degeneration of DA neurons in culture (Wiemerslage et al., 2013). In order to construct a model deficient in D2 autoreceptors, larvae containing a TH-GAL4 driver (specific to DA neurons) were crossed with UAS-(DD2R-RNAi) larvae. This model showed deficits in crawling speed, turning rate, and pause time when exposed to rotenone; however, when 10µM nobiletin was administered, there was no rescue of any symptoms (Figure 9). These data suggest that D2 autoreceptors are both necessary and sufficient for mediating the rescue effects of nobiletin in the rotenone model.
Figure 9: Representative pathways (a) show that both rotenone-treated groups had shorter and more irregular movements than the control group. Nobiletin was ineffective in treating any symptoms caused by rotenone treatment in TH-(DD2R-RNAi) larvae (a, b, c). (* = p<0.05; ** = p<0.01; rot = 10µM rotenone; nob = 10µM nobiletin)
Discussion

*Drosophila* larvae provide a useful model of human PD, demonstrating altered locomotion and the progressive degeneration of DA neurons (Varga et al., 2014). In addition, *in vitro* studies of *Drosophila* DA neurons expressing mutated human alpha-synuclein have shown the development of Lewy body-like structures, a common marker of human PD (Park & Lee, 2006). With its wide range of genetic tools, speed of development, and complex locomotion, the *Drosophila* larval model provides an exceptional tool for researchers to better understand PD.

However, the larval model has not been used to study PD treatments, despite the fact that these various advantages would be useful in quickly screening drugs as well as probing their mechanisms. In order to determine whether the larval PD-like condition was related to human PD, it needed to respond to standard treatment. Establishing this relationship would provide more evidence that novel treatments discovered using this model have the potential to be beneficial for human patients.

In this study, as a proof-of-concept, L-DOPA was used to determine whether a known human PD drug is also effective in *Drosophila* larval models of PD. Furthermore, as an example of the potential of this model to screen drugs, the chemical nobiletin was explored as a treatment for PD. Nobiletin is a flavonoid found in the peels of citrus fruits that has been shown to improve symptoms of PD in one study using PD model mice as well as improving symptoms in Alzheimer’s models (Yabuki et al., 2014). Because of its understudied nature, one of the aims of this study
was to determine whether nobiletin is also effective in the Drosophila model and also to use the UAS/GAL4 system to determine its specific mechanism of action.

L-DOPA was successful in rescuing locomotion parameters in both the rotenone and A53T models used to model PD in this study. Both showed a significant improvement in all three measures of locomotion as a response to treatment with 100µM L-DOPA. As described earlier, L-DOPA treats the symptoms of human PD by being converted to dopamine by DDC, which then compensates for the reduced dopamine levels in the nigrostriatal pathway due to the degeneration of DA neurons (Shulman et al., 2011). This results suggests that, similarly to human PD, defects in larval locomotion occur in both the A53T and rotenone models as a direct result of dopamine deficiency, which is likely due to the progressive degeneration of DA neurons that has been demonstrated previously (Varga et al., 2014). Therefore, this finding gives evidence to the claim that the Drosophila larval PD-like condition is analogous to human PD both in how it develops and how it is treated, opening the door to using this model to test novel drugs.

With L-DOPA treatment established as a proof-of-concept, the larvae of both PD models were subsequently treated with nobiletin, which demonstrated a similar ability to rescue all the measures of locomotion. This finding, given the previously demonstrated analogy between human and Drosophila PD treatment, adds to the body of evidence that nobiletin may have potential as a drug used to treat human PD. Given this result, the next goal was to elucidate the mechanisms by which nobiletin rescues locomotion.
With DD2R-RNAi expressed pan-neuronally using the 1407-GAL4 driver, nobiletin was no longer able to rescue any of the parameters of locomotion in the rotenone model (however, the turning rate showed no reduction in response to rotenone, which could be explained by the already existing locomotion deficit of the 1407-(DD2R-RNAi) phenotype). As described in the introduction, many drugs that are successfully used in the treatment of human PD are in the category of dopamine agonists; that is, they mimic dopamine at its receptors as another way to replace the reduced output from the dying DA neurons. Therefore, a natural first step was to use a model with altered DA receptors, specifically DD2R, the *Drosophila* analog of the D2 receptors (Draper et al., 2007). The D2 receptors are the target of PD drugs like pramipexole and ropinirole (Münchau & Bhatia, 2000). Given this finding, it seems that the improvement in condition in response to nobiletin treatment is also mediated via this receptor, which potentially places in the category of DA agonists.

As a further step, DD2R-RNAi was only expressed in a subset of neurons, specifically the DA neurons using the TH-GAL4 driver, and it was once again found that nobiletin had no effect in rescuing any of the dysfunctions in locomotion produced by rotenone. These data suggest that part of the mechanism of nobiletin is via D2 autoreceptors. Given what is known about D2 autoreceptors, there are several possible ways that it prevents the locomotion dysfunction seen in the rotenone model (Figure 10).

Nobiletin may be neuroprotective; that is, it may prevent the degeneration of DA neurons from occurring in the first place. Ropinirole and pramipexole have been
found to prevent the mitochondrial permeability transition that plays a role in cell death (Parvez et al., 2010). In addition, the D2 agonists quinpirole and bromocriptine were able to reduce DA neuron degeneration via the DD2R autoreceptor in an MPP+ model (Wiemerslage et al., 2013). D2 autoreceptors have been shown to provide a negative feedback mechanism for DA neurons, reducing their excitability by inhibiting adenylyl cyclase (Beaulieu & Gainetdinov, 2011). This causes a subsequent decrease in dopamine release, which may be neuroprotective; because dopamine degradation produces reactive oxygen species, the reduced dopamine production may prevent future oxidative damage to the cell (Parvez et al., 2010). On the other hand, in the only other study of the role of nobiletin in PD treatment, the mouse MPTP model, when administered nobiletin, was found to have improved locomotion but showed no rescue of DA neurodegeneration in the SNpc, which was found by measuring tyrosine hydroxylase levels (Yabuki et al., 2014). This finding could be due to the model used (MPTP vs. rotenone), but it is also possible that nobiletin’s primary mechanism is not via neuroprotection.

D2 autoreceptors also cause long-term changes in DA neurons that might compensate for PD-induced neurodegeneration. Although D2 receptor are known as inhibitory receptors, as discussed above, in the longer term, D2 autoreceptors actually enhance the speed and regularity of the firing of DA neurons by altering gene expression (Hahn et al., 2006). In addition, pramipexole was found to cause desensitization of D2 autoreceptors after chronic administration; by this mechanism, it may prevent the inhibitory effect of these receptors, causing a net increase in
excitability and dopamine neurotransmission (Chernoloz et al., 2009). In the previous mentioned study by Yabuki et al., nobiletin was found to rescue the reduced dopamine levels in the striatum and increase striatal dopamine release (2014). This effect may be a downstream effect of nobiletin’s action in modulating dopamine neurotransmission in DA neurons.

Figure 10: Potential mechanisms of nobiletin via the Drosophila D2 presynaptic receptor. Drugs that affect D2 autoreceptors have previously been shown to have a variety of downstream effects, including direct inhibition of DA release, changes in gene expression, and long-term effects on the pattern of DA release. Future studies using whole brain immunostaining and electrophysiology could help ascertain the exact mechanism by which nobiletin exert its rescue effects.
Interestingly, there was a difference in the degree of response between the rotenone and A53T model when the same dose of L-DOPA or nobiletin was used. For the rotenone model, L-DOPA or nobiletin treatment successfully rescued symptoms to the point that the rotenone-treated larvae were indistinguishable from wild-type larvae. On the other hand, treatment of the A53T larvae with the same dose of each drug was able to partially rescue all symptoms, but a significant difference still remained between the A53T larvae and the wild-type larvae. This fact could be attributable to several possible reasons. First, A53T larvae display a greater amount of DA neuron degeneration compared to rotenone-treated larvae, which has been proposed to be due to uneven penetration of the drug into the brain (Varga et al., 2014). Second, although A53T larvae showed a similar locomotion speed to rotenone-treated larvae, they displayed much high defects in turning rate and pause time, which may explain why the same dose was less effective. In future experiments, higher doses could be tested to see if they show a greater improvement in any symptoms. Finally, it is possible that the difference in locomotion is due to genetic background effects that the A53T larvae might have but that the rotenone-treated larvae would not have. Past experiments have shown that TH-GAL4 locomotion speed (without UAS) does not differ from that of wild-type larvae, which suggests that the transgene does not have a strong effect in altering locomotion (Varga et al., 2014). However, other measures of locomotion (turning rate and pause time) were not examined in these studies, which may account for the difference. Nevertheless, although there was a difference in extent, both drugs did succeed in having a therapeutic effect in both PD models.
Additionally, in the A53T model, there appeared to be a difference in relative effectiveness in rescuing the three locomotion parameters between L-DOPA and nobiletin. L-DOPA was most effective in rescuing locomotion speed and less effective for turning rate and pause time. On the other hand, although nobiletin produced a similar percent improvement in locomotion speed to L-DOPA, it showed a much higher improvement in both turning rate and pause time. As explained in the introduction, in human PD, L-DOPA is generally less effective in treating postural instability and freezing of gait compared to bradykinesia (slowness of movement). Given that nobiletin seemed to have more effectiveness in rescuing parameters beyond locomotion speed in this study, this result may suggest that nobiletin could be used as an adjunct or primary therapy to treat symptoms that are unresponsive to L-DOPA in human PD.
**Future Directions**

Future experiments can focus more on the potential of substances to prevent the neurodegeneration seen in PD, rather than just on their ability to treat the symptoms of the disorder. In this study, the agent causing the PD-like condition and the potential therapeutic agent were administered at the same time (at birth). One potential way to determine whether a drug like nobiletin has a neuroprotective effect is to administer it at a higher dose later in life to see whether it produces the same therapeutic effect even when some neurodegeneration has already occurred in the larva due to the A53T gene or rotenone treatment. In addition, treated larvae could be examined using whole brain immunostaining to determine whether the treatment reduces the number of TH-positive neurons that have died. Using an *in vitro* model would allow researchers to quantify the degeneration of TH-positive neurons as well as to measure the degree of Lewy body formation.

Future studies of nobiletin can explore other potential sites of action that may play a role in the substance’s therapeutic effects. One potential site may be the D1 receptor, to which, similarly to the D2 receptor, *Drosophila* has homologs (Draper et al., 2007). Prior studies have shown that the D1 and D2 receptors work in synergy in the brain to coordinate motor control and, in some neurons, are even present as heteromers (Hasbi et al., 2011). This may indicate that the mechanism of action of nobiletin also includes the D1 receptor, which could be studied by using RNAi to reduce expression of the D1 receptor either by itself or at the same time as the D2 receptor.
One helpful addition to this system would be to devise a model for dyskinesia in the *Drosophila* larvae. As explained in the introduction, dyskinesia is one of the most prominent side effects of L-DOPA that discourages its use in early PD and encourages the use of dopamine receptor agonists. Therefore, it would be useful to determine whether *Drosophila* larvae treated with L-DOPA experience similar side effects, and, if so, whether other novel drugs can treat the disorder without inducing dyskinesia. At this point, no behavior in *Drosophila* larvae has been associated with dyskinesia, although models exist for non-human primates and rodents (Morin et al., 2014). One possible way to search for these behaviors is to administer very high doses of L-DOPA (above therapeutic levels) to larvae to search for aberrations in locomotion. Because the present system has the ability to record larval movement at a high frame rate, it is possible that to find subtle differences in locomotion, such as twitching, that may indicate dyskinesia. Using these behaviors, it could be possible to screen for drugs that minimize side effects in human PD treatment.
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