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Article

Development of a Novel Treatment Strategy to treat Parkinsonian Neurodegeneration by targeting both Lewy Body Aggregation and Dopaminergic Neuronal Degradation in a *Drosophila Melanogaster* Model

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SUMMARY

Parkinson's Disease (PD) is characterized by the progressive degradation of dopaminergic neurons in the substantia nigra of the brain and is triggered by both environmental and genetic factors. PD is characterized by symptoms that can range from muscle dysfunction to severe mood disturbances. The pathology of PD is two-fold: (1) degradation of dopaminergic neurons due to mitochondrial calcium overload and (2) deposits of α-synuclein that increase cytosolic calcium levels in the brain. In this study, we tested a therapy that targets both pathologic manifestations of PD in fruit flies. Specifically, we used combinations of the drugs Ruthenium red (RuR), a mitochondrial channel uniporter inhibitor, and Ambroxol, a pharmacological chaperone of the lysosomal enzyme glucocerebrosidase that digests α-synuclein, to treat PD in fruit flies. We then measured the climbing ability, ATP content, and glucocerebrosidase activity in the fruit flies. While the drugs showed positive results individually, we found that the drugs had a synergistic effect when used together that resulted in a statistically significant increase in climbing ability, ATP content, and glucocerebrosidase activity. The results of this study indicate that these drugs could be used in treatments for PD.

INTRODUCTION

Parkinson's Disease (PD) is a chronic neurodegenerative syndrome of the central nervous system that affects approximately 1% of all individuals over 60 years of age. PD is primarily associated with symptoms of motor dysfunction, including shaking, rigidity, slowness of movement, and difficulty walking (1). The etiology of PD remains poorly understood, but several environmental and genetic factors are known to contribute to increased risk of PD. Exposure to certain chemicals and mutations of various genes can also produce parkinsonian symptoms (2). However, due to our lack of knowledge of its specific etiology, parkinsonian disorders are characterized primarily by two neuropathologic findings: (1) the degradation of pigmented dopaminergic neurons in the substantia nigra pars compacta and (2) the presence of Lewy body aggregates and Lewy neurites (3).

The presence of Lewy body aggregates is a feature in all postmortem analyses of human parkinsonian degeneration. While PD in fruit flies does not produce the same deposits of protein, inserting the Synuclein Alpha (SNCA) gene into the fruit fly genome leads to the production of human α-synuclein protein and to parkinsonian neurodegenerative effects in flies. Lewy body aggregates, or clumps of protein that often include the protein α-synuclein, have been shown to contribute to both motor and non-motor symptoms of PD (4). Alterations of the SNCA gene which cause misfolding of the α-synuclein protein and those which cause excess production of α-synuclein have been found to cause parkinsonian symptoms and these misfolded or excess α-synuclein proteins cluster together in Lewy bodies (5). While very little is known about these deposits, most research indicates that the deposits have negative effects on the human nervous system. α-synuclein has been implicated in the release of harmful pro-inflammatory cytokines, which exacerbate dopaminergic neurodegeneration by activating microglia and facilitating neuronal death (6). Furthermore, α-synuclein aggregates bind to and activate the SERCA calcium pump in vitro, leading to increased mitochondrial calcium concentrations by passage of the ions through the Mitochondria-Attached Membranes (MAM) and the Mitochondrial Channel Uniporter (MCU) (7). This research suggests that targeting these protein deposits may be critical for effective PD therapy.

Furthermore, additional research indicates that the dissolution of protein aggregates may also be critical for effective PD therapy. The neurotransmitter dopamine is oxidized into neuromelanin when a burdened neuron cannot sequester the dopamine into vesicles before its antioxidant defenses take over and this process increases with age. Due to the accumulation of neuromelanin, the lysosomes cease to function and are unable to digest α-synuclein (8). α-synuclein is normally present in the cell in small concentrations, but this lysosomal enzymatic dysfunction leads to accumulation of the protein. One key lysosomal enzyme that is implicated in this process is the enzyme glucocerebrosidase (GBA) (9). Mutations of the GBA enzyme increase the risk of PD approximately 20-fold, indicating that GBA dysfunction may contribute to these protein deposits and parkinsonian disorders (10). Increasing α-synuclein digestion by improving lysosomal function could be an effective therapy for the reduction of α-synuclein levels. One drug that has potential to reduce protein aggregation is Ambroxol (C13H18Br2N2O). Ambroxol is a drug used in the treatment of respiratory diseases associated with excessive mucus. Ambroxol has

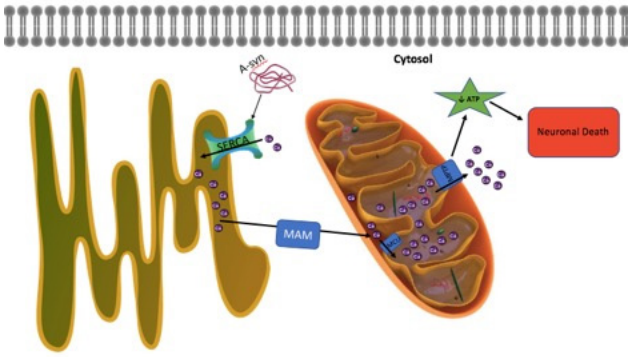

Figure 1. Dysregulation of calcium ions leading to neuronal death. This diagram shows how the α-synuclein protein activates the calcium pump SERCA and causes an influx of calcium ions inside the endoplasmic reticulum (ER). Through the mitochondrial-attached membranes (MAM), the calcium ions enter the mitochondria and through the mitochondrial calcium uniporter (MCU), pass through the inner mitochondrial membrane.

recently been shown to increase activity of GBA and has been used as an effective treatment for neuronopathic Gaucher disease, which is a lysosomal storage disease characterized by a deficiency of GBA. Oral Ambroxol reduced symptoms in Gaucher's patients in a pilot study, indicating that this drug crosses the blood-brain barrier (11). Although much research has been done on substances that reduce α-synuclein production and aggregation, few are able to cross the blood-brain barrier.

Recently, oxidative stress has been implicated as one of the environmental causes of parkinsonian neurodegeneration. Oxidative stress is an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects. Research on common oxidative stressors, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrabydropyridine) and rotenone, demonstrated their ability to cause parkinsonian pathology (12). One study linked PD symptoms in patients to consumption of the illicit drug MPTP, and postmortem analyses revealed destruction of the substantia nigra (13). In addition, many studies have confirmed the presence of oxidative stress in postmortem animal and human models of PD (14). In another study, dosing fruit flies with potent concentrations of the drug rotenone generated parkinsonian phenotypes such as neuromotor dysfunction due to oxidative stress (15). Any process that decreases oxidative stress and lowers the production of reactive oxidative species (ROS) could reduce or prevent Drosophila neurodegeneration.

New research has implicated mitochondrial calcium dysregulation in the pathogenesis of PD (16). The production of oxidative stress through the opening of the mitochondrial permeability transition pore (mPTP) is explored in this study. The mitochondrial calcium uniporter (MCU) is a calcium channel located on the inner mitochondrial membrane that allows the passage of calcium ions from a cell's cytosol to the mitochondria. The MCU is the primary regulator for calcium influx and key to calcium homeostasis, and has been proven to control the rate of energy production and cell death (17). The excessive calcium ion concentration in the mitochondria is what causes the dopaminergic neuronal death in PD (**Figure 1**). Interestingly, the mutation of genes such as PINK1 and Parkin, which control the opening of the MCU, is a common genetic factor for PD (16). Excessive mitochondrial calcium influx triggers the opening of the mPTP on the inner mitochondrial membrane and triggers the release of harmful ROS, leading to neuronal death by excitotoxicity. Blocking the passage of calcium ions through the MCU is one method to prevent excessive calcium influx and the opening of the mPTP. In a cardiac injury model, mice lacking Mcu, a subunit of the MCU, are protected against Calcium overload by preventing activation of the mitochondrial permeability transition pore (18). Ruthenium red (RuR), an MCU inhibitor, could have a similar mechanism and therefore prevent neuronal death.

We used the organism Drosophila melanogaster in this study to model PD by using both transgenic flies and drug-induced wild-type flies. To model familial Parkinson's in humans, we used α-synuclein transgenic flies that produce Lewy body aggregates. To model sporadic PD in humans, we used an induced model in flies through the introduction of rotenone into the fruit fly medium. This method allows for accurate evaluation of both idiopathic and familial PD. Although both have similar pathophysiology, their etiology remains obscure, so this study will improve knowledge of both forms of PD . The model Drosophila melanogaster was selected due to their rapid reproductive rate and relatively low maintenance expenses, allowing for reliable testing of several experimental conditions. Moreover, models of PD in fruit flies demonstrate the same neuromotor dysfunction observed in humans, which allows us to correlate the neurodegeneration between species (19). Although Lewy bodies do not appear in the wild-type Drosophila model of PD, inserting the SNCA gene for α-synuclein into the mutant fly genome generates the same protein deposits and neurodegeneration found in human brains.

The purpose of this study was to develop a drug therapy targeting parkinsonian symptoms in fruit flies exposed to oxidative stressors or genetically induced to model PD. We hypothesized that the fruit flies given both Ambroxol and RuR therapy in the highest concentrations would display better climbing abilities, higher ATP levels, and higher GBA activity compared to the untreated group. To assess the effects of Ambroxol and RuR on parkinsonian degeneration, we utilized three main assays: a negative geotaxis assay, an ATP bioluminescence assay, and a GBA activity assay.

This research will contribute to the development of a sustainable treatment for PD and advance our understanding of oxidative stressors.

RESULTS

All in all, the goal of this project is to record and analyze the effects of the two drugs tested here on two different forms

Figure 2. A picture of the negative geotaxis assay apparatus. The apparatus was constructed using two vials and masking tape. A mark at a point 8.5 cm above the bottom of the vial was made.

of PD in Drosophila melanogaster. We hypothesized that the drugs would improve neurological and cellular function and to assess the effects of RuR and Ambroxol, we measured the climbing abilities, ATP levels, and GBA activity of fruit flies exposed to media hydrated with solutions of 500 μM, 1 mM and 5 mM of Ambroxol and 100 μM, 250 μM and 500 μM of RuR, both separately and with combinations of both the solutions. Results from flies exposed to media with drug treatments were compared to results from flies who were fed medium without any drug treatment. Multiple trials of conditions were conducted to ensure preciseness and accuracy of data. For the negative geotaxis assay, three trials of 10 flies per experimental condition were used. For the ATP bioluminescence assay and GBA enzyme activity assay, we generated 3 sample replicates per condition, which consisted of homogenate made from 20 flies each.

For the negative geotaxis assay, we set up vials with 10 fruit flies and measured the percentage of flies that crossed a mark drawn 8.5 cm from the bottom of the vial (**Figure 2**). We conducted 3 trials per vial before, during, and after the 10- day study (Day 0, 5, and 10). The negative geotaxis assay utilizes the natural tendency of flies to fly up against gravity when agitated to measure their locomotive capabilities. Negative geotaxis is an innate escape response in fruit flies. This assay is sensitive to deficits of motor coordination and can therefore be used to test the effects of RuR and Ambroxol on parkinsonian motor degeneration. We placed 10 flies in a vial and tapped the vial to dislodge the fruit flies to the bottom of the vial. We allowed the flies to climb up the side of the vial for 10 seconds. The number of flies above a certain point on the vial is then measured. Fewer flies climbing up the sides of the vial indicates faster disease progression. Ambroxol and RuR had a significant impact on the flies' neurological function based on the results of the negative geotaxis assay (**Figures 3** and **4**). While most of the treated and untreated PD model flies gradually lost motor function over time, treatment with Ambroxol and RuR significantly increased the number of flies that climbed past the mark (p-value < 0.05). Moreover, treatment with these drugs at higher concentrations had a greater impact on locomotive capabilities in treated flies than treatment at a lower concentration, further suggesting that the observed improvement in locomotive capabilities was due to

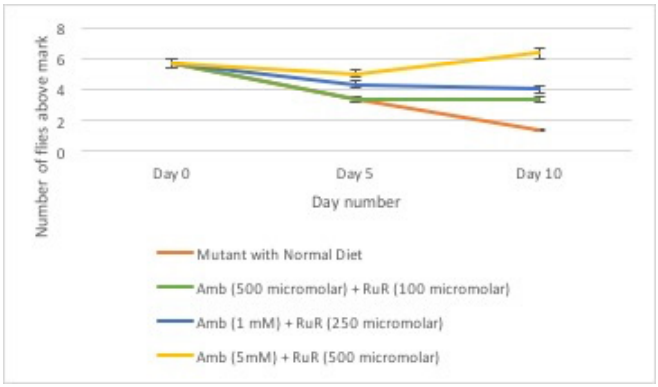

Figure 3. Negative Geotaxis Assay of the Mutant (Mut) Flies cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations. The average number of mutant flies above the 8.5 cm mark during the negative geotaxis assay. Three trials of ten flies for each condition (normal diet, 500uM Ambroxol + 100uM RuR, 1mM Ambroxol + 250uM RuR, and 5mM Ambroxol + 500uM RuR) were conducted at Day 0, 5, and 10. Error bars represent a 95 percent confidence interval.

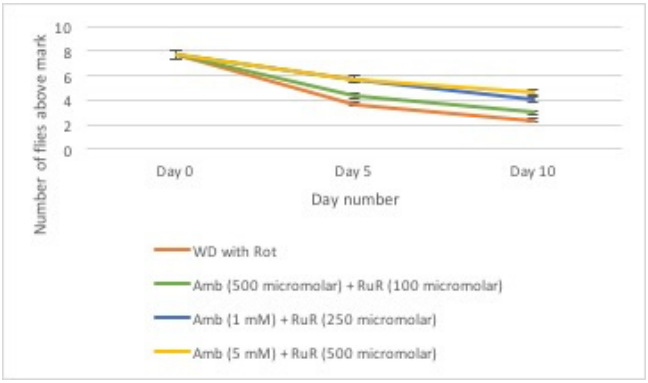

Figure 4. Negative Geotaxis assay of the Wild-Type (WD) Flies with Rotenone (Rot) cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations. Flies were tapped down the bottom of the apparatus shown in Figure 2 and were allowed to climb back up for 10 seconds. The number of flies above a mark 8.5 centimeters below the bottom of the apparatus were counted. 3 trials of 10 flies were conducted for each experimental condition. Error bars represent a 95 percent confidence interval.

treatment and not due to age-related or other random factors. The treated flies exhibited improved locomotive capabilities compared to their normal (mutant flies with normal diet and wild-type (WD) flies with rotenone) counterparts.

The ATP bioluminescence assay uses firefly luciferase, a monomeric protein that catalyzes luciferin oxidation using ATP as a co-substrate. Through the conversion of luciferin to oxyluciferin, light is produced in the presence of ATP. The luminescence can be quantified to yield a value for ATP content in the samples. The ATP assay test was conducted to analyze the mitochondrial potential of the treated flies. Based on the theory of preservation of mitochondrial integrity by inhibiting the MCU, more ATP should be produced for more functional mitochondria. Inhibiting the MCU alters calcium

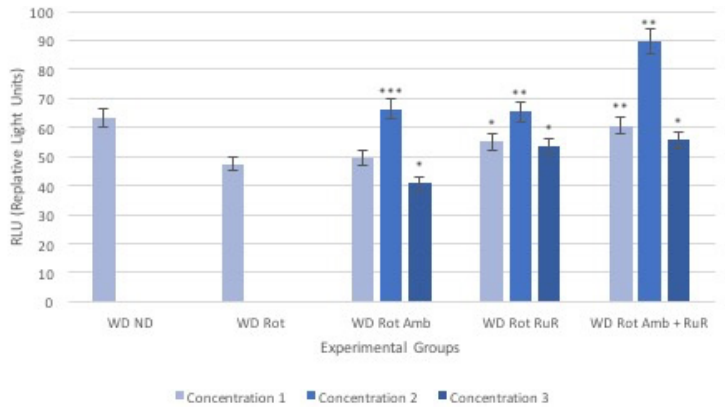

Figure 5. ATP Bioluminescence Assay of Wild-Type (WD) flies with Rotenone (Rot) cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations and Normal Diet (ND). Average ATP activity in mutant flies was measured for three concentrations (1 = 500uM Ambroxol and/or 100uM RuR, 2 = 1mM Ambroxol and/or250 uM RuR, and 3 = 5mM Ambroxol and/or 500uM RuR). Averages were from homogenates of three trials of 20 flies, and error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

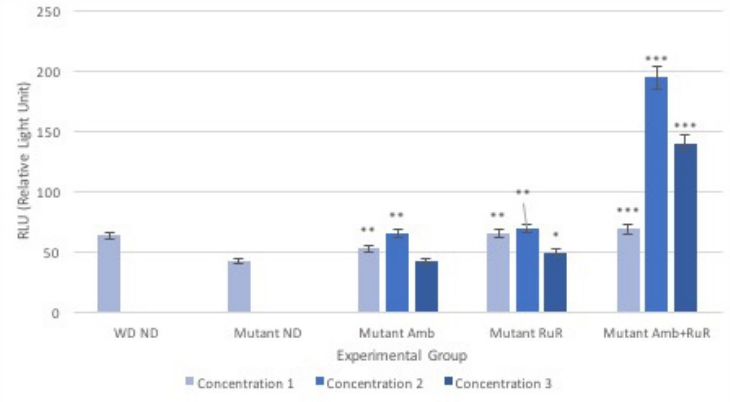

Figure 6. ATP Bioluminescence Assay of Mutant (Mut) flies cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations and Normal Diet (ND). Average ATP activity in mutant flies was measured for three concentrations (1 = 500uM Ambroxol and/or 100uM RuR, 2 = 1mM Ambroxol and/or 250uM RuR, and 3 = 5mM Ambroxol and/or 500uM RuR). Averages were from homogenates of three trials of 20 flies, and error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

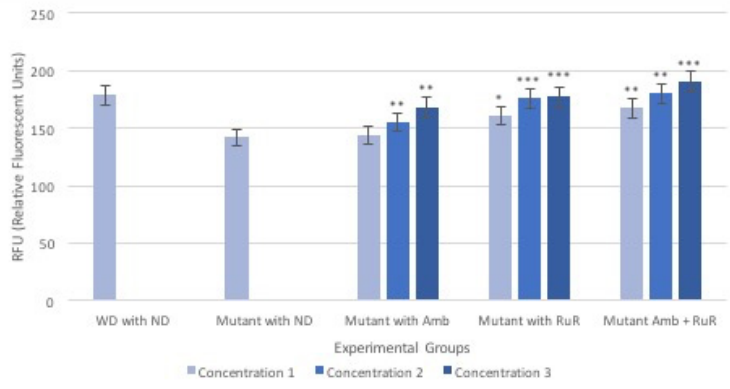

Figure 7. GBA Activity Assay of Mutant (Mut) flies cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations and Normal Diet (ND). Average glucocerebrosidase activity in mutant flies was measured for three concentrations (1 = 500uM Ambroxol and/or 100uM RuR, 2 = 1mM Ambroxol and/or 250uM RuR, and 3 = 5mM Ambroxol and/or 500uM RuR). Averages were from homogenates of three trials of 20 flies, and error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

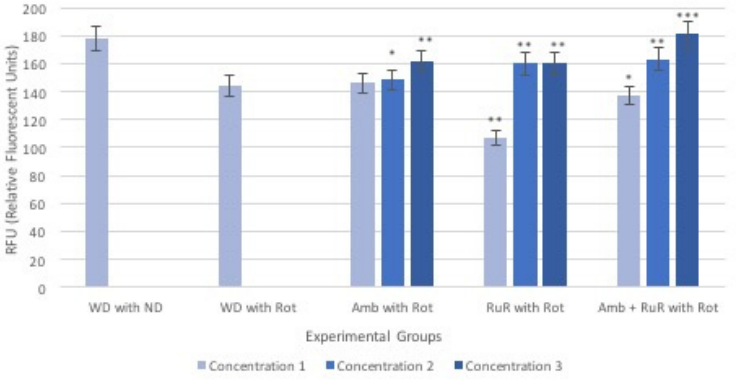

Figure 8. GBA Activity Assay of Wild-Type (WD) flies with Rotenone (Rot) cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations and Normal Diet (ND). Average glucocerebrosidase activity in mutant flies was measured for three concentrations (1 = 500uM Ambroxol and/or 100uM RuR, 2 = 1mM Ambroxol and/or 250uM RuR, and 3 = 5mM Ambroxol and/or 500uM RuR). Averages were from homogenates of three trials of 20 flies, and error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

and prevents mPTP opening, therefore preventing mitochondrial death and allowing the mitochondria to keep producing ATP. The group given RuR and Ambroxol had significantly higher ATP values, and therefore more functional mitochondria than the untreated samples(p-value < 0.05) (**Figures 5** and **6**). Importantly, the middle concentrations of the treatments yielded higher ATP values compared to the low or high doses. This points to a concentration related-toxicity as amounts of the drug increase. More research should be conducted to determine the optimal dosage for this therapy.

In the GBA activity assay, the activity of GBA, a critical lysosomal enzyme that functions in the digestion of the protein α-synuclein, which aggregates in protein deposits in PD is measured. Enhancing lysosomal function provides an important path to dissolving these deposits and lowering the inflammation that speeds neuronal degradation. GBA activity was significantly increased in most groups treated with both Ambroxol and RuR (p-value < 0.05) (**Figures 7** and **8**). While treatment with a single therapy did improve GBA function, combined treatment surpassed the effect of either drug alone. However, low concentrations of RuR and Ambroxol + RuR produced a negative effect on GBA activity in the samples with sporadic PD, but do not show this effect in samples with familial PD. While this could be due to human error, an alternative hypothesis suggests a fundamental difference between the progression of idiopathic and familial PD.

DISCUSSION

The purpose of this experiment was to develop a novel treatment for PD that targets both aggregation of α-synuclein and neuronal degradation. In this study, we tested the synergistic effects of RuR and Ambroxol in a PD model of fruit flies. While the addition of either drug alone demonstrated neuroprotective effects, the combination of these drugs demonstrated greater neuroprotective effects than either of the drugs alone. However, it does have to be said that when given in its highest concentrations, the drugs both alone and together did not have the most optimal ATP production for that experimental group. This indicates that there is a safe medium concentration in which the drugs can be used to their maximum potential. Since the drugs I am testing here have not really been used before for much medical use, my concentrations were estimates. More research should be done into which specific concentrations are the most effective. While as a whole, the data did show the neuroprotective effects of the drugs when given together as proven by significantly higher climbing, ATP production, and GBA activity, concentration related toxicity must be studied further due to the relatively unknown nature of the drugs used in this study.

In this study, we demonstrated that RuR increased ATP production, increased GBA activity, and improved neurological function. While we expected that RuR would influence ATP production by increasing mitochondrial viability, we did not expect RuR to influence GBA activity. It is possible that RuR leads to higher lysosomal activity by activating autophagy pathways that digest damaged mitochondria. The role of PINK1, a gene that functions to ensure lysosomal autophagy of damaged organelles is completed, in PD corroborates this idea. When the PINK1 gene is not present or its protein is mutated, there is a visible parkinsonian phenotype present (20). Lysosomal activity depression has a close correlation with PD progression, as these data show.

There is an urgent need to produce a long-lasting therapy for PD. With an incidence of PD that is still increasing due to continuing harmful pesticide use, more people will come to need a permanent treatment (21). Instead of being forced to rely on expensive cocktails of dopamine precursors that will eventually lose effectiveness, a therapy utilizing these drugs has a much higher chance of preserving neuronal function. The current most common, and only temporary treatment for PD is called the levodopa-carbidopa therapy. This involves the introduction of a dopamine precursor, levodopa, into the body and the brain is able to produce dopamine artificially from this precursor. However, this treatment is not a permanent one, as (1) introducing dopamine into the body does nothing to combat the loss of dopamine production by the brain, (2) temporarily reduces symptoms while actual neuronal degradation is still ongoing, and (3) eventually loses effectiveness as time goes on. Combating the underlying neuronal degradation is important to stop disease progression and this therapy has potential to do this. This project proves that the use of *Drosophila melanogaster* as a model for sporadic PD can help us gain an understanding of its pathophysiology. While the genetic pathway of familial PD is mostly understood, the pathway for sporadic PD remains a mystery. Utilizing a pesticide model provides a viable way of understanding the etiology of sporadic PD. Understanding how it is caused can provide new avenues and methods to treat and potentially cure the disorder.

A major limitation in this project was the time it took for the flies induced to have PD to reproduce. To create the original PD model flies, a driver fly line GAL4 and another fly line UAS were crossed to develop an α-synuclein transgenic fly line. While this fly line is traditionally crossed with itself to grow its population, we found that this PD model flies exhibited a very slow growth rate. To solve this, the GAL4 and UAS crosses were maximized to collect mutants rather than culture the PD model flies in their own vials. Another limitation of this study is the drug delivery method via media. Some of the variations between trials may be explained by the fact that some flies tend to eat more medium than others and the amount of the drug-hydrated medium that an individual fly consumed is impossible to account for and measure. Differences in body weight could have contributed to the differences in the assay values generated from the homogenate. To compensate for this problem, a Bradford assay was conducted and the samples were diluted in TE buffer to normalize signal to protein content to improve assay precision.

In future experiments, the potential of drug therapies using these two drugs to drastically increase ATP production, even past wild-type values should be explored to create new therapies for disorders like fibromyalgia and chronic fatigue syndrome, or malfunctions of ATP synthase. Fibromyalgia and chronic fatigue syndrome are both characterized by low ATP levels due to mitochondrial dysfunction and increasing ATP levels provides a potential solution to these problems (22). Researchers could also explore this therapy on a long-term basis and observe the effect on total long-term mitochondrial integrity, ATP production, and calcium homeostasis. Brain scans could be analyzed to determine the impact of this therapy on long-term dopaminergic neuron survival. While such scans of fruit flies cannot be taken, specific neuronal areas of interest in the substantia nigra could be observed in murine models, which have a form of PD very similar to the human disease, complete with Lewy body deposit. Currently, Ambroxol is the active ingredient of the drug Mucosolvan and is used as a treatment for respiratory disorders associated with excessive mucus by stimulating the release of surfactant. Since Ambroxol is already used in the treatment of human disease and it can cross the blood-brain barrier, Ambroxol is a strong contender PD treatment (11). While Ruthenium has never been used in a clinical setting or as a therapy in humans, this research shows that it has potential for use in the treatment of PD and further research is warranted.

MATERIALS AND METHODS

Generation of α -synuclein transgenic Drosophila melanogaster

Adult female virgin neural Gal4 promoter transgenic flies and adult male UAS-human α -Syn flies were placed into a vial. A 6:10 female to male ratio was found to maximize egg production. After 5 days, all the adult flies in the vials were removed. All the flies that appear in the vials after will be mutants for the SNCA gene and have a form of familial PD. Flies were transferred out of the vial after anesthetizing them with carbon dioxide gas.

Generation of induced PD models for Drosophila melanogaster

Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to 2/5ths of the volume of the vial. The dry medium was hydrated with 15 mL of 150 μM rotenone (Cayman Chemicals). Approximately five grains of yeast were added and the vial was covered with a vial plug (Carolina Biological).

Control group vials

Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to two-fifths of the volume of the vial. The dry medium was hydrated with 15 mL of distilled water. Approximately 5 grains of yeast were added and the vial was covered with a vial plug (Carolina Biological).

Ambroxol vials

For this experiment, drug concentrations of 500 μM, 1 mM, and 5 mM Ambroxol hydrochloride (Sigma Aldrich) were used. Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to two-fifths of the volume of the vial. For each concentration, the dry medium was hydrated with 15 mL of the drug solution. Around 5 grains of yeast were added and the vial was covered with a vial plug (Carolina Biological). Wildtype flies, fruit flies cultured with rotenone, and α-synuclein transgenic fruit flies were transferred into the drug vials and fed the medium for 10 days.

RuR vials

For this experiment, drug concentrations of 100 μM, 250 μM, and 500 μM RuR (Cayman Chemicals) were used. Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to two-fifths of the volume of the vial. For each concentration, the dry medium was hydrated with 15 mL of the drug solution. Around 5 grains of yeast were added and the vial was covered with a vial plug (Carolina Biological). Wild-type flies, fruit flies cultured with rotenone, and α-synuclein transgenic fruit flies were transferred into the drug vials and fed the medium for 10 days.

Ambroxol and RuR vials

For this experiment, the individual drug solutions were combined into one beaker and gently shaken to mix the solutions. Three groups were used: one using 500 μM Ambroxol HCl and 100 μM RuR, one with 1 mM Ambroxol HCl and 250 μM RuR, and one with 5 mM Ambroxol HCl and 500 μM RuR. Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to two-fifths of the volume of the vial. For each group, the dry medium was hydrated with 15 mL of the combined drug solution. Around 5 grains of yeast were added and the vial was covered with a vial plug (Carolina Biological). Wild-type flies, fruit flies cultured with rotenone, and α-synuclein transgenic fruit flies were transferred into the drug vials and fed the medium for 10 days.

Negative Geotaxis Assay

Using carbon dioxide anesthesia, we sorted the flies into groups of 10. The flies were allowed an hour to recover from anesthesia. To create the apparatus shown in Figure 8 that was used for this assay, we arranged 2 empty polystyrene vials (Carolina Biological) facing each other and joined them with tape. A mark 8.5 cm above the bottom of the vial was placed. For each condition, 10 flies were transferred into the vial and taped in. After an hour, the flies were tapped down and the number of flies that climbed above the 8.5 cm mark were measured. The assay was repeated for all trials and

sample conditions. Three trials of 10 flies were conducted for each experimental condition.

GBA Enzyme Activity Assay

First, the flies were anesthetized with FlyNap (Carolina Biological). We suspended 20 fruit flies in RIPA buffer with a protease inhibitor cocktail and homogenized the mixture with a glass dounce homogenizer. The samples were centrifuged at 15000xg for 20 minutes and the supernatant was aspirated. We conducted a Bradford protein assay and samples were diluted to a protein concentration of 5.5 µg/mL. 40 µL of homogenate was placed in wells of a 96-well microplate and 20 µL of citrate-phosphate buffer was added to each well. The mixture was incubated for 10 minutes and the reaction was terminated with 60 µL of a glycine-NaOH stop solution. The plates were read with a 520/20 filter on a microplate reader (Synergy HTX). Three trials were conducted in this manner for each condition.

ATP Bioluminescence Assay

The flies were first anesthetized with FlyNap (Carolina Biological). We suspended 20 fruit flies in RIPA buffer with a protease inhibitor cocktail and homogenized the mixture with a glass dounce homogenizer. The samples were centrifuged at 15000xg for 20 minutes and the supernatant was aspirated. We conducted a Bradford protein assay and samples were diluted to a protein concentration of 5.5 µg/mL. For this assay, the Promega Cell-titer Glo system was used. 50 µL of homogenate were loaded into wells of a 96-well plate and contents were equilibrated to room temperature for 30 minutes. 50 µL of Promega Cell-Titer Glo reagent (Promega) were then loaded into each well and samples were allowed to incubate for 10 minutes. Luminescence was recorded using a microplate reader (Synergy HTX). Three trials were conducted in this manner for each condition.

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