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Literature Review on Drosophila Research in Parkinson's Disease

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Introduction

Parkinson's disease (PD) is one of the most prevalent neurodegenerative diseases. It is characterized by its main symptom of a tremor that usually starts in one limb. The four cardinal symptoms of PD are tremor, rigidity, bradykinesia, and postural instability. While the four cardinal symptoms are all motor symptoms there are non-motor symptoms for PD which include cognitive changes, sleeping disorders, lightheadedness, early satiety, and mood disorders. PD is characterized by dopaminergic (DA) neuron loss and the accumulation of Lewy bodies (LB) which are predominantly composed of α-synuclein (α-Syn) protein.

α-Syn is a protein found abundantly in the brain. It is characterized by an amphipathic lysine-rich amino terminus which plays a role in modulating its interactions with membranes. This protein is also characterized by its acidic carboxy-terminal tail, which plays a role in regulating its nuclear localization and interactions with metals, small molecules and proteins (Lashuel et al.). The misfolding of α-Syn can cause aggregates allowing damage to the cells that it is found in, which has been shown to be a cause of PD. Mutation in α-Syn was first noted in 1997, where an amino acid substitution change was noted producing an autosomal-dominant pattern of inheritance for PD (Stefanis). There are two types of PD, familial PD where a mutation can be passed on through generations. Sporadic PD, the second type of PD, is when the mutations occur in patients without the familial connection to PD. Mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) have also been linked to the cause of autosomal dominant PD. Additionally, LRRK2 has been linked to tau and α-Syn pathologies, suggesting that possibly LRRK2 mutations are players in cell degeneration or cell death. Mitochondrial dysfunction can also lead to PD, therefore the pathway that leads to this dysfunction needs to be explored more.

Drosophila as an animal model

Drosophila melanogaster is known to be one of the most common model organisms used for different biomedical science research purposes. Advantages to using the Drosophila model is its low cost, fast generation time, easy to care for, and is an excellent tool for genetic manipulation (Tolwinski). Since 75% of human disease genes have homologues in *Drosophila,* it is a powerful model organism to study human neurodegenerative diseases. In the *Drosophila* model, transgenic lines are created with mutated human α-Syn genes which display similar symptoms/characteristics of PD found in humans. Symptoms include the decreased locomotor function, and characteristics include the loss of DA neurons (Aryal and Lee).

α-Synuclein

α-Syn toxicity may be one of the causes for PD as accumulation of α-Syn protein can lead to the formation of LB and cause cell damage or cell death. The cause of α-Syn protein accumulation is not well known. MicroRNAs (miRNAs) mediate the expression of many genes and are involved in cell homeostasis. Dysregulated microRNAs have been a suggested link to PD, and many of these miRNAs are found in the blood and brain of PD patients (Kong et al.).

In one study, using the *Drosophila* model, PD induced flies were produced that had phenotypes of deteriorating climbing ability consistent with symptoms of PD flies. To determine the effect that these types of miRNAs have on PD, analysis of all the miRNAs that were expressed in the PD flies and the control flies were quantified. 154 miRNAs were identified, and 5 miRNAs (dme-miR-133-3p, dme-miR-137-3p, dme-miR-13b-3p, dme-miR-932-5p, dme-miR-1008-5p) were found to be more expressed in the PD flies (Kong et al.). The targets for these miRNAs were found to be enriched in neuron- related biological processes. Examining targets in the neuroactive ligand-receptor interaction pathway, they found that the five targets associated with the upregulated 5 miRNAs were downregulated in the PD flies, which showed that these 5 miRNAs are dysregulated. (Kong et al.). The suggested explanation for miRNA dysregulation is the toxicity that occurs with α-Syn forming aggregates.

In the literature there are studies that look to understand the pathway or mechanism of a disease. One such study involving α-Syn's mechanism studied Glucocerebrosidase deficiency as a possible pathway. Mutations in the GBA_1 gene have been shown to have a tight connection with α-Syn toxicity (Suzuki et al.). Homozygous mutations in the GBA_1 gene have been shown to lead to decreased enzyme activity (Glucocerebrosidase (GCase)) and tend to lead to Gaucher's disease which have a higher tendency to develop parkinsonism; these patients tend to have α-Syn positive Lewy bodies in their brains (Suzuki et al.). PD patients with heterozygous mutations in the GBA_1 gene tend to develop diffuse Lewy body pathology which is linked to earlier onset of the disease. The molecular mechanism of the connection between a deficiency in Gcase and α-Syn induced PD *in vivo* was not well known. The objective of the study was to discover the relationship between this decreased activity of GCase and α-Syn toxicity as well as the molecular mechanisms involved using the *Drosophila* model (Suzuki et al.). To investigate GCase deficiency effects on α-Syn toxicity transgenic RNAi flies that have inverted repeat RNA that targets the *Drosophila* homolog of the GBA_1 gene were generated. The *dGBA1a-IR* (dGBA1a-RNAi flies) or *dGBA1b-IR* (dGBA1b-RNAi flies) flies showed 81-92% decrease in GCase activity (Suzuki et al.). Wild type α-Syn expressing flies were crossed with the dGBA1a-RNAi and dGBA1b-RNAi flies to obtain progeny that have co-expressing α-Syn and *dGBA1a-IR* (Suzuki et al.). A climbing assay was performed to determine the locomotor dysfunction. The progeny's climbing ability was significantly declined from two to three weeks after

eclosion and rapidly decreased from then on. Immunohistochemical staining for tyrosine hydroxylase which is essential in the synthesis of dopamine (DA) showed that the RNAi flies have fewer DA neurons than the control flies, demonstrating the tendency to exacerbate the α-Syn toxicity (Suzuki et al.). To see if an accelerated accumulation of α-Syn is what causes the toxicity, an immunoblotting analysis was performed. With the GBA_1 knockdown, there was no significant increase in α-Syn, showing that the GCase deficiency does not lead to the accumulation of α-Syn (Suzuki et al.). Instead it was suggested that the number of α-Syn is not what ties into its toxicity. A proteinase K (PK) treatment was applied to the experimental strain lines which showed GCase deficiency may promote conformational changes in α-Syn to PK-resistant α-Syn, which could lead into the α-Syn toxicity. This accumulation of PK-resistant α-Syn suggested that GlcCer, a substrate of GCase, could possibly affect the conversion of α-Syn to its PK-resistant form. Staining and chromatography showed that the GlcCer accumulates when there is a deficiency of GCase (Suzuki et al.). PK digestion with GlcCer showed that GlcCer directly promotes the conversion of α-Syn to its PK- resistant form (Suzuki et al.). Fly lines that contained inverse repeats of the *Drosophila* homolog of *β-galactosidase* were also observed. This knockdown of *β-galactosidase* was analyzed with its conversion of α-Syn to its PK-resistant form. The results were very similar to those of the GlcCer conversion effects. The main difference was that dimer formation was not affected. Results showed that the *β*-gal deficiency mediates the conversion of α-Syn to its PK-resistant form, then increasing the toxicity of α-Syn (Suzuki et al.). The study showed that GCase deficiency played a role in the mechanism of α-Syn toxicity.

Another study that looked at the mechanism of α-Syn toxicity studied the role of vacuolar protein sorting 35 (Vps35). This protein is a part of a cargo recognition subunit of a retromer, aspartyl protease cathepsin D (CTSD). This retromer is a major player in the degradation of long-lived proteins such as α-Syn which suggested that VPS_{35} is directly involved with this pathway. The study's idea was to interfere with the VPS_{35} which then would stop the maturation of the CTSD protein, possibly allowing for the accumulation of the α-Syn (Miura et al.). To create VPS_{35} deficient cells in the fly model, two siRNAs were used to target VPS_{35}. This created an increase in pre-mature pro-CTSD and a decrease in the overall mature CTSD in the cells (Miura et al.). To see if CTSD does promote the degradation of α-Syn, CTSD was then overexpressed in the cells, which showed an increase in mature CTSD in the cells and a significant decrease in the long version α-Syn (Miura et al.). To see if the deficiency in this VPS_{35} retromer links to the formation of these PD phenotypes (loss of motor skills), VPS_{35} was silenced in cells which then caused an increase in α-Syn. The eyes of the flies were then analyzed and found to have a different phenotype than that of the control flies, resulting in experimental group to have eye degeneration (Miura et al.). VPS_{35} deficient flies had higher age- dependent loss of climbing ability (Miura et al.). Thus the link between

VPS_{35} and α-Syn which leads to PD- like phenotypes can be shown in this study. However, their mechanisms are not well known and needs further studies.

Other types of studies focus on the suppression of PD symptoms in *Drosophila*. One study focused on synphilin-1, another component of Lewy bodies. synphilin-1 is a major part of LB which is a pathological aspect of PD. synphilin-1 also interacts with α-Syn which when mutated causes PD. synphilin-1 and α-Syn each by themselves causes their own host of issues, and toxicity. However, when these two proteins are co-expressed, they seem to cancel each other out in the *Drosophila* model (Vargas et al.). The first round of testing in this study was to direct its expression pan-neurally by means of Elav-GAL4 driver. The results showed that the overexpression of α-Syn and synphilin-1 are very similar in that both showed slight vacuolization and marble-like inclusions in the cytoplasm (Vargas et al.). The co-expression of these two proteins showed a suppression of the vacuolization and marble-like inclusions phenotype and one that is closer to the wild-type phenotype. When this transgene expression is driven in DA neurons, the survivability of these neurons showed that the overexpression of just one of these proteins causes a toxic effect on the neurons whereas the co-expression of these proteins promotes an environment where more of the neurons can survive (Vargas et al.). The motility of the flies that overexpressed just one protein was compared to those that co-expressed and control flies. The results showed a significant difference in motility between those that expressed one protein and those that co-expressed both proteins. The co-expression flies seem to reverse the adverse effects of the one protein flies which have a less likely outcome of survival and motility (Vargas et al.). *Drosophila* electroretinogram is an electric field measurement that has transients appear at light stimulation. α-Syn or synphilin-1 overexpressing flies when treated with L-Dopa/carbidopa reverts the effect of the transgenes (Vargas et al.). Overexpression of either of these two proteins damaged DA neurons and not other photoreceptors, whereas the co-expression of both suppressed PD- like symptoms.

Another study focused on the use of a kinase to suppress the PD symptoms in the *Drosophila* model. PINK1 (PTEN-induced putative kinase 1) has been localized to the mitochondria and is speculated to be involved in the Mitochondria's protection. Mutations in this gene have been known to lead to oxidative stress, therefore it has been thought that PINK1 has a role in preventing oxidative stress. In the same pathway, parkin has been shown to be able to rescue α-Syn induced PD- like phenotype. The possibility that parkin and PINK1 act in a similar manner suggests that PINK1 can protect or rescue an αSyn induced PD phenotype. A motility assay was preformed and flies with overexpressed PINK1 showed suppression of PD phenotype by losing their climbing ability. In flies with overexpressed α-Syn, rough eye phenotype could be observed. This phenotype was partially rescued in the overexpressed PINK1 flies (Todd and Staveley). However, PINK1 overexpression in control flies also led to a partial suppression of the rough eye phenotype, which means PINK1 also counteracted

the effects of Gal4 (Todd and Staveley). In the α-Syn fly model retinal degeneration could occurred, with intact retina at 3 days and then premature deteriorated at 30 days. When PINK1 is overexpressed, the retina was intact at 3 days and past 30 days (Todd and Staveley), showing the suppression of the damaging effects of the α-Syn.

One last study that focused on α-Syn suppression in the *Drosophila* model using ubiquitin linkages. PD is pathologically marked by mitochondria dysfunction, as well as oxidative stress, deposition of aggregates (LB), and degeneration of DA neurons. LB are known to be made of α-Syn proteins. It is known that two pathways target oxidized damaged or misfolded proteins for degradation, those being ubiquitin proteasomal system (UPS) and autophagy lysosomal pathway (ALP). These pathways also have been known to be associated with neurodegeneration. The main objective of the study was to determine if UPS or different types of ubiquitin linkages affect α-Syn toxicity in the PD *Drosophila* model (Lee et al.). To test the role of ubiquitin linkages with the suppression of α-Syn toxicity, the retinal degeneration phenotype in the Drosophila model was observed. Wild type α-Syn was expressed alone in one set of flies and as previously known, the results showed the degeneration of rhabdomeres in the eyes of the flies (Lee et al.). However, in the flies that were expressed with wild type α-Syn and ubiquitin linkage proteins there was a bit of contrast, those that co-expressed either K48 or K63R with the wild type α-Syn showed suppression of α-Syn induced toxicity phenotype, degeneration of rhabdomeres in the eyes (Lee et al.). The other two ubiquitin linkages that were co-expressed with the wild type α-Syn (K48R and K63) differed in results as the degeneration of rhabdomeres in the eyes still did (Lee et al.).

One phenotype that is commonly observed in the Drosophila PD model is the loss of climbing ability which is age dependent. The flies were co-expressed with wild type α-Syn and the mutant ubiquitin linkages to explore the link between mutant ubiquitin linkages and suppression of α-Syn toxicity. Those that had K48 or K63R had a suppression effect on the α-Syn induced phenotype and a higher level of climbing ability at an older age was observed. Those with the K48R or K63 co-expressed had a much lower level of climbing ability at an older age, comparably like that of the flies that just had the wild type α-Syn expressed (Lee et al.). The research team believed that this suppression effect of α-Syn induced phenotype was dependent on K48. The K48 ubiquitin dependence suggested that the way in which this works is through the facilitation of degradation of cellular proteins. To test this hypothesis, a Ub-R-GFP degron was constructed, and when the transgenic flies co-expressed wild type α-Syn, Ub-R-GFP and K48 promoted the degradation of Ub-R-GFP more than that of the other co-expressions of K33 and K63 (Lee et al.). However, the amount of α-Syn was unaffected by these constructs added to the transgenic flies. A similar experiment was conducted with GFPu and similar results were observed (Lee et al.). Since the degradation of these proteins was enhanced with those flies that expressed wild type or K48, the proteasome activity in these types of flies was tested. However, the

proteasomal activity is inconsistent with what was thought to have happened. Overall, the study found that the expression of ubiquitin linkages, specifically K48 was able to offer neuroprotection against α-Syn toxicity that occurs in the cell (Lee et al.).

LRRK2

Another cause of PD is a mutation that occurs within LRRK2. This mutation has been shown to cause autosomal dominant PD (Liu et al.). Transgenic expression of LRRK2 and its mutants in the *Drosophila* model bring about loss of dopaminergic neurons and behavioral deficits which make them great models for PD. One study aimed to explore how inhibitors for LRRK2 could possibly rescue the loss of DA neurons and PD- like phenotypes. Inhibitors like GW5074 and sorafenib were shown to have effects on the LRRK2 and were able to rescue PD like phenotypes in the *Drosophila* model (Liu et al.). The model of *C. elegans* showed that the population that expressed LRRK2 had significant DA degeneration whereas those with LRRK2 inhibitor treatment showed significant DA survival and rescue (Liu et al.). To test if LRRK2 induced PD is dependent on kinase activity, a mutation was introduced that abolished enhanced kinase activity. As the flies increase in age the amount of degenerated DA neurons increases as well. Climbing assays and survival of flies with these two inhibitors expressed were measured. The two inhibitors impacted the transgenic flies, in that survival increased and climbing impairment decreased. The GW5074 inhibitor was slightly stronger than that of the sorafenib inhibitor (Liu et al.). Both inhibitors were also found to reduce the amount of lost DA neurons and decrease the amount of kinase activity in the population. The GW5074 being slightly stronger in all these areas then that of sorafenib (Liu et al.).

Another study that looked at treatment on LRRK2 induced PD aimed to determine how LRRK2 interacts with Vps35 along with Vps29 and Vps 26 (Linhart et al.). The PD symptom in LRRK2 mutants is black lesions in the eye, this is a marker for neuron death occurring in later eye development. Transgenic flies that expressed a higher level of Vps35 or Vps26 rescued this black lesion phenotype that occurs with the LRRK2 mutant (Linhart et al.). Loss of DA neurons is a pathological marker for PD which creates the motor symptoms that are seen in PD patients, *Drosophila* motor skills can be measured using a negative geotaxis climbing assay. LRRK2 mutant flies had a lower climbing ability compared to the control flies while LRRK2 mutant flies with overexpressed Vps35 or Vps26 were able to recover their climbing ability (Linhart et al.). The basic survival of these flies was also observed and those with VPs35 or VPs26 overexpression had a significantly longer survival than those without (Linhart et al.). Other LRRK2 mutants, were also observed and the results showed similar results, further solidifying these results. The rescue of climbing ability then is not dependent on the kinase dominant mutant, providing evidence that LRRK2 may play a role in the retromer dependent pathway in PD (Linhart et al.). Knockdown of Vps26 was observed

and had a very significant phenotype in the eye (black lesions) which was found to be non-additive (Linhart et al.). Non-additive effects were also found when Vps35 or Vps29 were knocked down, which then impaired the locomotor function of these flies (Linhart et al.). LRRK2, based on these results, is in the same pathway as these core components of the cargo recognition subunit. Rotenone is a pesticide and can be used to model PD. Applying a rotenone treatment to control flies brought down the survival rate of those flies. Overexpressing Vps35 in normal flies with rotenone treatment mildly protected the flies (Linhart et al.). Co-expression of LRRK2 and Vps35 protected the flies from the rotenone toxicity, much more than any of the other treated groups (Linhart et al.). Climbing assay on these flies had similar results. Knocking down Vps35 did not have a significant effect on the flies climbing ability or survival in comparison to the other groups of flies (Linhart et al.). Vps35 and LRRK2, based on these results, interact in DA neurons, possibly importantly under cellular stress.

Another study investigated LRRK2 mutation and its link to PD treatment investigating melatonin as a solution. Melatonin is a naturally produced compound in many organisms which regulates the wake/sleep cycle (Ran et al.). Although the suggestion to use melatonin as a possible therapy for PD or other neurodegenerative diseases has been made, the mechanisms of action remain elusive. The aim of the study was to identify the molecular mechanisms of melatonin as a therapy for PD in the *Drosophila* model (Ran et al.). Using both behavioral tests and other assays for analysis, data in the study suggested that the long-term memory of PD flies would be affected. When long-term memory was studied in the transgenic flies, flies that expressed LRRK2 had a significantly reduced capacity in long term memory compared to the control flies. However, flies with melatonin treatment had a significantly long-term memory, close to the level of the control flies (Ran et al.). The mechanism of how melatonin works in affecting the LRRK2 has been suggested to be by regulating the extracellular calcium channel activity (Ran et al.). Current density of the calcium channels was observed in different groups and the groups with expression of LRRK2 had a significantly increased current density compared to that of the control group (Ran et al.). The group that had melatonin treatment has decreased current density when compared to the non-melatonin treatment group. The mean open time was also lower in the melatonin treatment group (Ran et al.). Overall, the study showed that melatonin treatment rescued the effects of LRRK2 induced dysregulation, including rescuing long-term memory, suggesting that melatonin could possibly be used as a novel therapy for PD.

hUCP2 (human uncoupling protein 2)

Another study aimed to study the role that mitochondrial uncoupling proteins (UCPs), specifically ucp2, has in neuroprotection in the *Drosophila* model of PD (Islam

et al.). UCPs promote proton leakage, which disrupts the proton gradient in the ETC, resulting in uncoupled substrate oxidation and ATP phosphorylation. UCP2 expression was found to be induced after mimics of epilepsy, stroke, and seizures in mouse models. A study was preformed to determine the neuroprotective role that human UCP2 (hUCP2) in a *Drosophila* model for PD. As chronic exposure to rotenone leads to DA neuron death, transgenic flies that expressed hUCP2 were treated with rotenone along with a control group without treatment (Islam et al.). Those with hUCP2 were found to be neuroprotection against DA degeneration when compared with the control group. As DA degeneration by rotenone exposure can lead to motor deficiency, negative geotaxis assays were preformed and those with hUCP2 expression showed a higher retention of climbing ability (Islam et al.). ATP levels were also examined and hUCP2 flies have higher ATP than control flies. This higher amount of ATP in the hUCP2 flies could be a result of higher mitochondrial function or mitochondrial biogenesis. Using real time PCR and transmission electron microscopy, the team was able to determine that other processes besides mitochondrial biogenesis is what is producing this elevated level of ATP (Islam et al.). To determine if mitochondrial function is at play, quantitative real time analysis was done and found that there was an increase in complex 1 activity but not complex3 (Islam et al.) Spargel a known protein that regulates mitochondrial activity but not biogenesis, found to be significantly higher in the hUCP2 flies (Islam et al.). Spargel linked protein Tfam was also found to be significantly higher. These results suggested hUCP2 reduced rotenone oxidative damage and plays a neuroprotective role in the Drosophila model of PD.

Conclusion

Overall, the literature in *Drosophila* research on Parkinson's disease is diverse. The main genetic cause of PD would seem to be the α-Syn component. The aggregation of these misfolded proteins causes cellular death and the loss of the DA neurons. The studies show that there are ways to rescue the PD effect of this pathology. Some of those ways include co-expression of both α-Syn and other proteins or overexpression of other proteins. Other studies that investigated α-Syn pathology also showed insight into the interactions or mechanisms that α-Syn takes in PD models. Including the how deficiency of GCase and Vps35 can contribute to the pathology of PD.

The second most prevalent genetic cause of PD being that of LRRK2 mutations. This disrupted kinase ends up being a player in loss of DA neurons as well. The studies have shown through the *Drosophila* model of PD that there are ways to rescue this pathway and ways to possibly create therapies. Those possible options being shown in the results of Vps35, inhibitors and melatonin as treatment options.

Lastly looking at the Drosophila model of PD with respects to hUCP2 a neuroprotective approach. Overall many approaches to looking into treatment for PD are conducted, which seem to be all centered around rescuing the phenotypes or symptoms in this model. The way that PD is being investigated in this model, as seen seems to be bringing about some great results.

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