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The Effects of SKF-835666 on Levodopa-Induced Dyskinesia in *Drosophila melanogaster*

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ABSTRACT

Although levodopa is considered the "gold standard" medication for Parkinson's disease, it is flawed as almost 90% of patients contract a side effect known as Levodopa Induced Dyskinesia (LID) after 10 years of treatment. LID manifests as involuntary movements and muscle spasms and presently lacks a cure. Previous studies on *Drosophila melanogaster* models have found that epigenetically inhibiting the ADCY2 gene reduces LID symptoms as represented by abnormal involuntary movements. Consequently, this study sought to assess the therapeutic potential of SKF-83566, an ADCY2 inhibitor, in ameliorating LID symptoms within a *D. melanogaster* model. The primary objective was to quantify the drug's impact by implementing Abnormal Involuntary Movements (AIMs) assay. This study found that wild-type flies on a diet of 10 mM of levodopa had a statistically significant increase in AIMs scores when compared to normal flies, implying that they successfully modeled LID. Furthermore, the data from this study supports that SKF-83566 alleviates LID symptoms, as flies with LID had lower AIMs scores when given SKF-83566. Moreover, findings from this study indicate that the locomotion of healthy *D. melanogaster* remained unaffected when exposed to a 5 mM dosage of SKF-83566 over a 7-day period. This study presents a novel administration procedure of SKF-83566 which could be applied in future research with *D. melanogaster*. The demonstrated effectiveness of the compound in reducing LID symptoms within a *D. melanogaster* model highlights its potential as a treatment option for LID, which could be researched further in the future.

Introduction

Pathogenesis and Significance of Levodopa-Induced Dyskinesia (LID)

Figure 1. Development of Levodopa-Induced Dyskinesia after 10 years of treatment.

Parkinson's disease (PD) is the fastest-growing and second-most common neurodegenerative disorder, accounting for 15% of worldwide dementia cases. PD, which originates in the basal ganglia, the section of the brain responsible for voluntary motor control, is caused by the degeneration of dopaminergic neurons, resulting in the deficiency of dopamine, an important neurotransmitter. While PD is not a quick-acting or fatal disease, it can lead to fatal incidents due to its symptoms. PD starts as a slight tremor but progresses to near paralysis, leading the patient to lose control of coordination and muscles. PD has no current cure, and the most widely available palliative treatment option is levodopa which can effectively alleviate PD symptoms (Meade et al., 2019). Levodopa is a synthetic form of dopamine that can bypass the blood-brain barrier and is then decarboxylated into the organic form of dopamine. Levodopa treatment, often consisting of an orally ingested tablet, alleviates PD symptoms and allows patients with PD to live normally. Although levodopa is an effective treatment, almost 90% of patients experience a side effect of the treatment known as Levodopa Induced Dyskinesia (LID) after 10 years of treatment (Bordone et al., 2021). LID severely reduces the quality of life of patients due to symptoms such as muscle spasms and uncontrollable movement. As a result, some patients are forced to stop using levodopa and experience a return in PD symptoms.

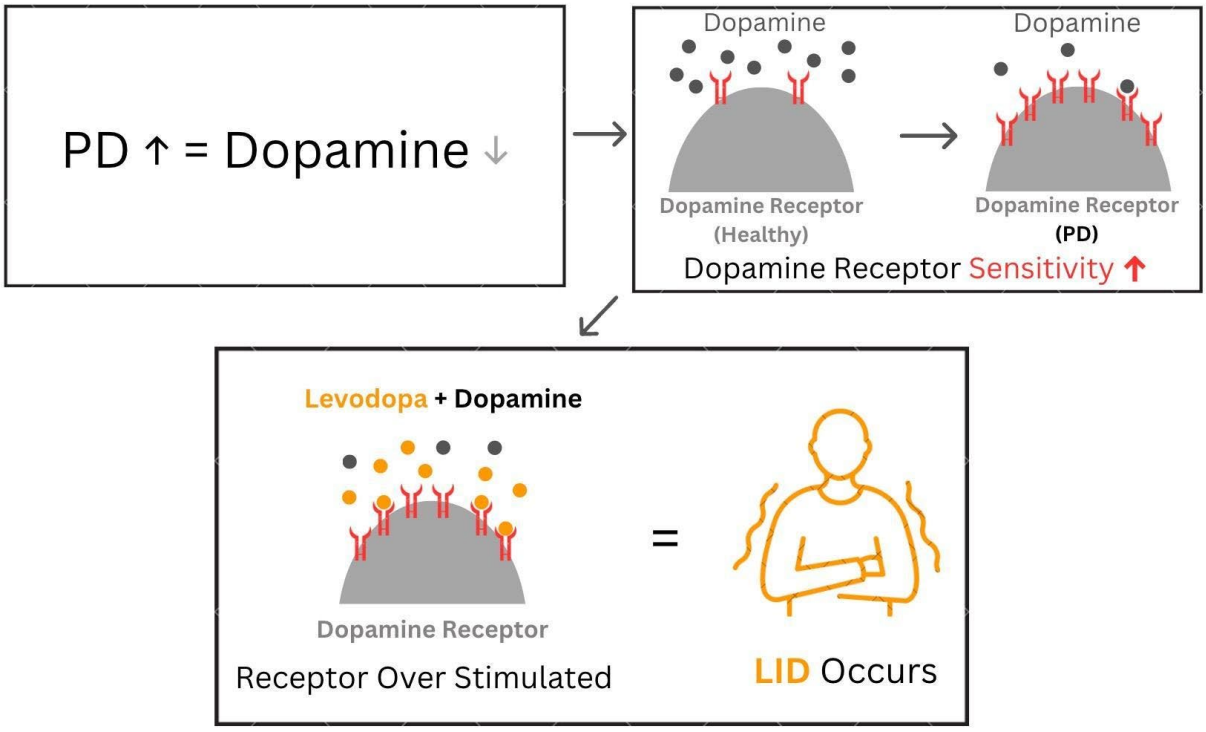

Figure 2. Pathogenesis of Levodopa-Induced Dyskinesia.

Drosophila Melanogaster as Model Organism for LID

D. melanogaster, also known as the fruit fly, is a commonly used model for several human neural diseases. About two-thirds of human disease genes are found in *D. melanogaster*, allowing readily available access to genetic manipulation of a model species in order to treat disorders. Additionally, *D. melanogaster* has low maintenance costs and a short life span, making it an accessible model organism. In addition, a previous study constructed a *D. melanogaster* model of LID. This model was determined to have LID based on its locomotor defects after being treated with levodopa (Blosser et al., 2020). While the study incorporated a Parkinson's model of *D. melanogaster*, the study conducted

by Yoon et al. determined that a Parkinson's model was unnecessary. Instead, a LID *D. melanogaster* model can be induced by placing wild-type flies on a levodopa diet for a long period of time.

Purpose, Variables, Novelty, and Justification

The purpose of this research was to determine if LID symptoms could be reduced pharmaceutically using SKF-83566. The study's hypothesis posited that administering SKF-83566 in the diet of *D. melanogaster* flies modeling LID would decrease LID symptoms as assessed by the Abnormal Involuntary Movement (AIM) assay. This hypothesis was based on SKF-83566's recognized role as an ADCY2 inhibitor, which in turn regulates the function of D1-like receptors. Since LID was characterized by oversensitized D1-like receptors, downregulating the receptor function would result in decreased LID symptoms (Spigolon & Fisone, 2018). Additionally, previous studies have demonstrated a direct correlation between the knockout of ADCY2 and reduced AIM scores, further supporting the anticipated decrease in AIM scores upon administering SKF-83566 to wild-type flies with LID (Yoon et al., 2022).

Unlike previous studies, this research aimed to lower symptoms of LID through pharmaceutical means. Previous studies have been limited to epigenetic modification to reduce symptoms (Yoon et al., 2022). Furthermore, drug treatment is significantly more viable for future research in mammalian models.

The independent variables are the presence of SKF-83566 in the fly's diet and the presence of LID in flies. LID can be induced in wild-type flies through supplementation of a 25 mM concentration of levodopa for 7 days.

The dependent variable is the AIMs score of the *D. melanogaster,* which is found through conducting an AIMs assay. This assay is done by observing a fly's movement over a 5-minute period and quantitatively analyzing its abnormal locomotive behaviors through a Matlab Behavioral Microarray code.

Methods

Materials

D. melanogaster utilized in the research were wild-type flies (w[1118]) obtained from Bloomington Drosophila stock center (#27898).

All equipment and fly food chemicals (except levodopa and SKF-83566) utilized in this study were borrowed from the Academy of Science laboratory. Chemicals such as levodopa and SKF-83566 were purchased from ThermoFisher Scientific US (catalog number #167530050) and GlpBio (catalog number #GC15016), respectively.

The AIMs assay stages were 3-d printed (a white 7 cm x 7 cm x 0.7 cm rectangular prism with a 0.2 mm deep, 6 cm diameter circular well in the center). Data was recorded using an iPhone camera capable of recording at 30 fps, HD, with 1x zoom.

Groups

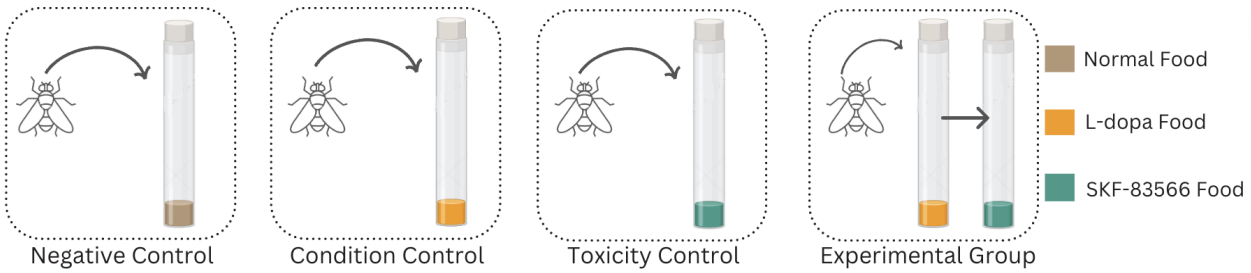

Figure 3. Summary of the creation of four different groups utilized in the study

This experiment consisted of 4 groups, 3 of which were controls. The first group was the negative control, consisting of unmutated wild-type flies that did not receive any treatment plan. This control was established to provide a healthy D. melanogaster locomotion standard in the Abnormal Involuntary Movement (AIM) test. This control group was projected to have low AIMs scores since the flies were not fed either the levodopa or the SKF-83566 and therefore were projected to have normal locomotion. The second control group was the condition control which consisted of unmutated wild-type flies on a treatment diet of levodopa. This control group was designed to induce LID in the flies which provided a standard for high AIM scores and could then be compared with the experimental group to observe a potential change. This group was expected to have high AIM scores as the flies will experience dyskinetic symptoms without the treatment of SKF-83566. The last control group was the toxicity control which encompasses unmutated wild-type flies on a diet of SKF-83566. This control group was designed to test the effects of SKF-83566 on the locomotion of wild-type flies to ensure it has no adverse effects. This group is expected to have similar locomotion compared to that of the negative control. The last group is the experimental group which contains flies supplemented with levodopa, which induces LID, and SKF-83566. This group, once compared with the other controls, will test this study's hypothesis.

The experiment will consist of ten trials each for all 4 groups. From each trial, ten flies will be tested per group at one time, which amounts to a total of 400 flies over all the trials and groups. The average of the AIMs scores will be utilized as the data for that trial.

Culturing and Maintaining Flies

D. melanogasters were placed in a plastic vial with fly food and covered by a flug. The vials were placed in a 22ºC environment in a 12:12 light-dark cycle, resulting in an approximate lifespan of 14 days. *D. melanogasters* were tapped every 3 weeks when the stock was being maintained, and parents were tapped every 4 days when the stock needed to be expanded. The vials were regularly checked for mites, dryness, and mold. Vials with dry food were rehydrated by lightly soaking the flug in distilled water. Moldy vials were immediately disposed of. To dispose of vials due to mold or because the vial had become too old, the vials were placed in an ultra-low temperature freezer for 15 minutes to humanely dispose of the flies.

Fly Food Preparation

The process began by using an electronic balance scale, weight boats, and a scoopula to measure the required dry ingredients: 6.75 g of yeast, 3.9 g of soy flour, 28.5 g of yellow cornmeal, and 2.25 g of agar. All the dry ingredients were combined and mixed in a 1 L beaker using a glass stirring rod. Next, the wet ingredients were measured using a 100 mL graduated cylinder and poured into a separate 1 L beaker in their respective volumes: 390 mL of distilled water and 30 mL of light corn syrup. The dry ingredients were then added to the beaker with the wet ingredients and mixed until the mixture became relatively homogeneous without clumps. The mixture was heated in intervals of 30 seconds in a microwave, with 5-10 seconds of mixing between each heating period. Once the mixture started boiling, the beaker was removed from the microwave and stirred regularly as the solution cooled to prevent clumping. To further prevent contamination, a cheesecloth was placed over the top of the beaker and secured with weights. After the solution stopped producing vapor, 1.88 mL of propionic acid was poured and mixed into the solution as a preventive measure against mold and fungus. The food was then cooled until it hardened. Finally, the food was poured into approximately 40 plastic vials, covered with flugs, and stored in a refrigerator to increase longevity.

Levodopa was given by adding 1.97 grams of the drug to the dry ingredients. 0.525 grams of ascorbic acid was also added to levodopa food to slow down oxidation. SKF-83566 was supplemented to *D. melanogaster* at a concentration of 5 mM. SKF was dissolved in DMSO and combined with sucrose to produce a 5 mM SKF and 5 mM sucrose solution. This solution was added to a napkin on the side of experimental and toxicity control vials. To further ensure consumption of SKF-83556, flies were starved for 1 hour every day with only the SKF-83566 solution. For

consistency purposes, the negative and positive control flies were also starved for 1 hour, however, without the SKF-83566.

Cold Sorting

The study exclusively involved male *D. melanogaster*, approximately 2-4 days old, selected through conventional age-matching procedures. To sort the flies, a cold sorter was employed. Initially, the *D. melanogaster* were gently tapped into empty vials, which were subsequently placed in containers with ice to induce temporary immobilization. The cold sorter was adjusted to a temperature of 2°C, and a weigh paper was positioned atop the cooling plate. Once the device reached the desired temperature, the flies within the vials were poured onto the weigh paper and separated using a sorting feather based on distinctive characteristics: female flies were identified by their ovipositor, male flies were distinguished by their dark, rounded abdomen with genital claspers. The male flies were then transferred back into food vials and allowed to regain consciousness. As for the female flies, they were humanely disposed of in accordance with the disposal procedure (refer to Fly Maintenance Section).

AIMs assay

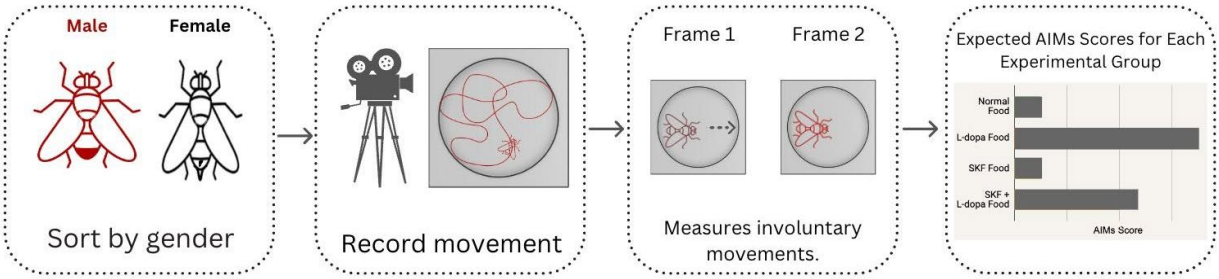

Figure 4. Summary of methodology utilized for data collection

Male flies were transferred to an empty vial and temporarily incapacitated by placing the vial in ice. Then a sorting feather was utilized to transfer the male *D. melanogaster* into the AIMs assay which was then covered with a transparent glass plate. The assays had a diameter of 6 centimeters. The flies in the assays were then placed in a dark environment for 1 hour to acclimate to their environment and to limit motion until re-exposed to light where they would then begin moving around the stage. Once the flies were placed back into the light after acclimation, a 5 minute video was taken of the AIMs assay. The procedure was conducted with 10 flies at the same time to compose one trial per group.

Once videos were taken, Ctrax, Matlab, and Ctrax's behavioral microarray were downloaded. Then the videos were imported into Ctrax for locomotion analysis. Each video was cropped so it only contained one fly and the video file format was converted to AVI. In Ctrax, the high threshold was set at 20 and the low threshold was set at 10. Additionally, the tracking settings were adjusted so only one blob was allowed to be detected. Then the background and target shape were computed and all 9000 frames were analyzed and downloaded as an .mat file. After that, the command "compute_perframe_stats" was entered and a .mat file from Ctrax was opened. Then the following code was entered which outputted an AIMs score using the flies instantaneous velocity and mean velocity over 250 frames to count the number of abrupt, abnormal movements. The threshold value used was 0.4 as found in previous studies (Yoon et al. 2022).

```
window_size = 250;
threshold = 0.4;
aim = 0;

for i = (floor(window_size/2) + 1):(length(trx.velmag_ctr) - floor(window_size/2))
    if (log10(trx.velmag_ctr(i) * 0.2 / mean(trx.velmag_ctr((i - floor(window_size/2)):(i + floor(window_size/2))))) > threshold
        aim = aim + 1;
    end
end

fprintf('Fly Aim score: %f\n', aim);
```

Figure 5. Code inputted into MATLAB to produce AIMs score from video after fly was tracked in Ctrax program

Safety Precautions

This research study involved the use of chemicals, heat, and glassware, which made it imperative to adhere to proper safety precautions. Chemicals such as propionic acid and SKF-83566 were kept clear of the skin and eyes as they can cause severe irritation. It was essential to handle these chemicals while wearing appropriate personal protective equipment (PPE) such as lab coats, gloves, and goggles. Furthermore, since propionic acid is flammable it was protected from heat sources and flames. Chemicals such as levodopa were stored in a cool location and disposed of properly to prevent environmental contamination. When preparing fly food, researchers used rubber heat-resistant mitts to prevent burns. PPE must be worn during procedures, such as those involving the production of fly food.

Results

Table 1. Average AIMs Scores per Trial for Each Group

	Average AIMs Scores										
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	**Mean**
Negative Control	2.9	3.7	2.8	1.9	2.5	3.5	5.3	3.2	3.1	3.6	3.25
Condition Control	9.4	13.7	5.9	14	16	12.9	9.6	9.5	13.2	6.8	11.91
Toxicity Control	5	5	3.2	1.9	5.4	3.8	4.1	3.4	5.1	4.4	4.13
Experimental	5.7	6.7	5.1	6.5	6.2	8	5.6	8.2	7.2	6.4	6.56

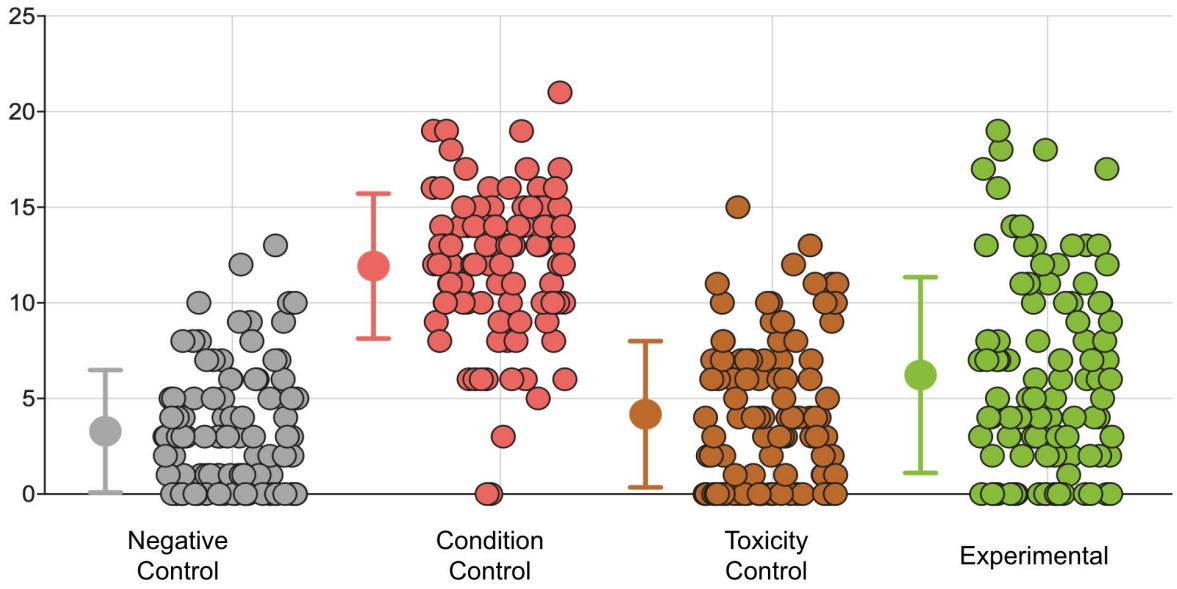

Figure 6. Dot Plot and Standard Deviation of AIMs Scores for Each Group

Table 2. P-values for Each Group Compared from a One Way ANOVA test

	p-value
Negative Control vs Condition Control	0.0002*
Negative Control vs Toxicity Control	0.9612
Toxicity Control vs Condition Control	0.0007*
Condition Control vs Experimental	0.0148*
Negative Control vs Experimental	).3830

**Starred values are statistically significant with p<0.05*

Review of Hypothesis, Results, and p-values

It was predicted that the condition control would model LID when on supplemented 25 mM of levodopa for a 7 day period. This prediction was supported by Table 1 where the mean AIMs score for the negative control (Wild type flies) of 3.25 was lower than the mean AIMs score for the condition control (LID) of 11.1. In previous studies, flies on a chronic levodopa diet at a concentration of 10 mM had an AIMs of 7 which aligned with the findings in this study since the concentration was increased to 25 mM meaning the AIMs should be higher (Yoon et al, 2022). Furthermore, in Table 2, the p-value between the negative control and the condition control was 0.0002, which is statistically significant with an α of 0.05, which shows that LID was likely induced in wild type flies on a levodopa diet.

It was also found that SKF-83566 had no impact on locomotion in *D. melanogaster*. In Table 2, the p-value between the negative control (wild type flies) and the toxicity control was 0.9612, which is not statistically significant. In addition, in Table 1, the mean AIMs value for the negative control was 3.25 which is relatively similar to the mean AIMs value of the condition control of 4.13. In previous research, wild type flies had an AIMs score of 0 with a

threshold value of 0.4. In this study a concentration of 25 mM was used so the AIMs is expected to be higher. Furthermore, the p-value comparing the toxicity control (SKF-83566) and the condition control (LID) was 0.0007 which is statistically significant. That further supports that SKF-83566 is not toxic as it did not produce an AIMs score similar to the control condition.

It was hypothesized that SKF-83566 would have reduced symptoms of LID because SKF-83566 would pharmaceutically inhibit ADCY2. This hypothesis was supported from Table 1 where the mean AIMs score for the experimental group (LID + SKF-83566) of 6.56 was lower than the mean AIMs score for the condition control of 11.1. Moreover, from Table 2, the p-value comparing the condition control and the experimental group was 0.0148 which is statistically significant. Furthermore, the p-value for the negative control (wild type flies) and the experimental group was 0.3830, which is not statistically significant. This supports that SKF-83566 alleviates symptoms of LID. However, according to Table 1, the mean AIMs value of the experimental group of 6.56 is almost double that of the negative control group, which means AIMs value of 3.25. As a result, it is likely that SKF-83566 reduced locomotive LID symptoms significantly, but did not completely treat them.

Discussion

Potential Assumptions and Errors

This research contained certain assumptions to allow for data collection. One assumption was that flies actively consumed SKF-83566 since it was mixed with sucrose. However, the sucrose concentration might not have been high enough, leading to less consumption of SKF-83566. However, this assumption was minimized by starving flies for 1 hour so they would be forced to eat the SKF-83566 solution.

Another assumption was that flies in the positive control were induced with LID due to a chronic levodopa diet. However, this assumption was addressed as adding levodopa to the positive control's food was the only difference between the negative control and positive control and the most likely explanation for the increase in AIMs scores.

A third assumption was that the AIMs assay effectively measured LID symptoms. This assumption has been made since previous studies used AIMs as a quantitative measurement of LID.

A potential error in this research is that the data collection was conducted in different locations. However, to minimize the effect of this, data collection was only done between 11 am to 3 pm.

Limitations and Future Work

This study has yielded promising results that offer potential avenues for further investigation. While the observed reduction in LID symptoms is notable, it is important to acknowledge that complete treatment was not achieved. To enhance future research, it is advisable to optimize the concentration of SKF-83566, considering that only a 5 mM concentration was tested in this study. Additionally, exploring the effects of SKF-83566 in other model organisms, such as mice, could provide valuable insights and broaden our understanding of its therapeutic potential.

Significance and Impact

This research has significantly contributed to our understanding of the pathogenesis of LID by elucidating its underlying mechanisms. The findings highlight the efficacy of SKF-83566, an ADCY2 inhibitor, in effectively reducing LID symptoms. Consequently, these results provide compelling support for the association between LID and D1-like dopamine receptors. The implications of this study present a promising avenue for future research and eventual treatment of LID.

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