Video Article

Methods to Characterize Spontaneous and Startle-induced Locomotion in a Rotenone-induced Parkinson's Disease Model of *Drosophila*

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Abstract

Parkinson's disease is a neurodegenerative disorder that results from the degeneration of dopaminergic neurons in the central nervous system, primarily in the substantia nigra. The disease causes motor deficiencies, which present as rigidity, tremors and dementia in humans. Rotenone is an insecticide that causes oxidative damage by inhibiting the function of the electron transport chain in mitochondria. It is also used to model Parkinson's disease in the *Drosophila*. Flies have an inherent negative geotactic response, which compels them to climb upwards upon being startled. It has been established that rotenone causes early mortality and locomotion defects that disrupt the flies' ability to climb after they have been tapped downwards. However, the effect of rotenone on spontaneous movement is not well documented. This study outlines two sensitive, reproducible, and high throughput assays to characterize rotenone-induced deficiencies in short-term startle-induced locomotion and long-term spontaneous locomotion in *Drosophila*. These assays can be conveniently adapted to characterize other *Drosophila* models of locomotion defects and efficacy of therapeutic agents.

Video Link

The video component of this article can be found at http://www.jove.com/video/51625/

Introduction

Locomotion deficiencies are a major symptom of Parkinson's disease and are largely caused by deterioration of dopaminergic neurons of the substantia nigra[1]. Rotenone is a ketonic insecticide that has been studied extensively to model Parkinson's motor deficits in *Drosophila*[2-6]. Rotenone causes oxidative damage by blocking the oxidative phosphorylation pathway, which ultimately causes cell death[7]. Dopaminergic neurons are more prone to rotenone toxicity, making the effects of the chemical primarily motor based[2,7]. By inducing Parkinson's disease symptoms in flies, we can better understand the disease and remedy its symptoms[6,8-11]. *Drosophila* provides a good model for studying this effect because they are genetically tractable, easy to maintain, and have a rapid life cycle.

Several studies have shown that rotenone causes short-term startle-induced locomotion defects in *Drosophila*—when flies are maintained on rotenone-supplemented food, they show a slower negative geotactic response after startle[2-6]. Their failure to climb upwards in a vial apparatus as quickly as control trials is indicative of startle-induced locomotion defects.

The effect of rotenone on long-term, spontaneous movement is not well described. *Drosophila* activity monitors (DAMs) have been successfully used to monitor movement in *Drosophila* circadian rhythm studies[12,13]. Flies are placed in individual tubes, which are loaded into the DAM. This apparatus is equipped with an infrared sensor, which counts the number of times a fly breaks the infrared beam. These counts can be used as a measure of undisturbed locomotion and activity[12,13]. By placing flies in a DAM, the effect of rotenone on their long-term locomotion can be characterized. This study describes methods to measure short-term startle-induced locomotion and long-term spontaneous locomotion in order to better understand the effects of rotenone mediated motor deficiencies. Characterization of locomotion deficiencies mimicking Parkinson's disease are important because they allow for the study of other compounds which may reverse these locomotion defects.

Protocol

1. *Drosophila* Startle-induced Locomotion Assay

1. Drug Treatment
    1. Sedate to immobilize desired number (approximately 8-12) of 1-3 day-old male flies using CO_2 and transport them to vials containing the drug-supplemented food. Note: Another anesthetic *e.g.,* ether or ice can be used to sedate flies to enable counting and handling.
    2. Allow flies to recover from sedation for 20 min (or until recovery) with the vial in a horizontal position (to prevent flies getting stuck on food) and then place the vial upright in a 12 hr dark, 12 hr light incubator at 25 °C for the remainder of the experiment.

2. Experimental Set Up
    1. Divide this double vial setup into three equal sections of 6.33 cm by marking circles around the vials with a permanent marker.
    2. After 3 days of drug exposure, transfer flies without anesthetizing into the bottom vial and quickly place the top vial over the opening. Tape the two vials together with clear tape.
    3. Allow flies to acclimate to the new environment for 15 min.
    4. Place vials on a white background and set up a digital camera at an appropriate distance from the double vial apparatus with a timer in view. Ensure the entire apparatus is visible in a single picture frame and that all flies are in focus. To maintain consistent frames between trials, mark the location of camera and vial.

3. Mobility Assay
    1. Clearly display the trial number, drug treatment, and timer in camera view.
    2. Firmly tap the double vial apparatus against the countertop 3 times and ensure that all flies fall to the bottom of the vial. Simultaneously start the timer.
    3. Every 5 sec for 1 min, take a picture of the apparatus. Note: Alternatively, a video could be captured and paused at appropriate intervals for measurements.
    4. Allow flies to recover undisturbed for 1 min.
    5. Repeat 2 more times with 1 min recovery time between each trial. Note: Each apparatus should take 5 min to complete data collection. Maintain similar force of tapping between trials. Multiple (at least 3) apparatus can be easily handled simultaneously.

4. Data Analysis
    1. Review pictures and record the number of flies in each section over time. Calculate the percentage of flies in each section over time. Notes: Repeat this entire procedure with the same flies at 2 or 3 time points of interest, for example, day 3, 5, and 7. If too many flies die throughout the experiment it is possible to scale up the original trial number to compensate for the mortality. Use appropriate statistical analysis to compare the data.

2. *Drosophila* Spontaneous Locomotion Assay

1. Food Preparation
    1. Reconstitute 3 g of instant *Drosophila* medium with 15 ml of de-ionized water and desired rotenone (or another drug of interest) dosage.
    2. Once the food mixture has become firm (about 5 min), carefully load the food to be approximately 1 cm high into manufacturer supplied transparent tubes (5 mm X 65 mm). Add the drug infused food to the tubes by carefully placing the tubes vertically in the food and twisting them until they can be removed with the food inside the tube. Note: It is helpful to place a finger on the opening of the tube to create a vacuum. Food should not contain any air bubbles or have an uneven surface as the flies can become stuck.

2. Experimental Setup
    1. Place a plastic cap on the end of tube nearest the food. Push the plastic cap on the tube as little as possible, as it can create an air bubble in the vial if pushed to forcefully.
    2. Sedate 1 day-old male flies using CO_2 and carefully insert 1 male fly into each tube with a paintbrush. Repeat depending on the number of desired trials.
    3. Plug the end of the tube farthest from the food with a small cotton ball, which can be hand rolled from larger store bought cotton balls.
    4. Allow flies to recover with the tubes in a horizontal position for 15 min and ensure that all flies are alive and active. Insert the tubes into DAM and make sure that all the tubes are in the same position relative to DAM. Note: It is possible to place them with the area of monitoring at the middle of the vial, or to push all the vials to the side, so that the end of the tube is being monitored. Note: See discussion for variations on this method.

3. Data Collection
    1. Place the DAM in a 12 hr dark, 12 hr light incubator set to 25 °C. Connect the DAM to the data collection system. Open the DAM software and under preferences select bin length to 10 min. Start data collection and allow the program to collect data for 7 days. Note: Bin length can be adjusted if required.
    2. Data Analysis
    Note: Process the data to obtain counts per min as a measure for long-term spontaneous locomotion.
        1. Open DAM file scan program and access monitor data by clicking select input data.
        2. Select appropriate monitor range and select bin length to 10 min intervals.
        3. In output file type choose channel files. Leave all other options as default.
        4. Click scan data and save to a designated folder.
        5. Import data in a circadian data analysis software to obtain counts per min. Note: For data analysis Clocklab software is commonly used. Other options are also available.

Representative Results

Drosophila Startle-induced Locomotion Assay

Wildtype, *canton-S*, flies showed a robust negative geotactic response with only approximately 88% and 5% of flies in the top and bottom sections respectively, of the double-vial apparatus after 30 sec (**Figure 1**). Flies exposed to 125 µM and 250 µM rotenone for 3 days showed a slight decrease in the number of flies in the top section and slight increase in the number of flies in the bottom section. Flies exposed to 500 µM rotenone showed significant defect in the negative geotactic response ($p < 0.05$ ANOVA, Bonferroni pair wise comparison) as evidenced by

fewer flies in the top section and more flies in bottom section as compared to control flies (**Figure 1**). This defect in negative geotactic response due to inability to swiftly climb in the apparatus is indicative of a defect in startle-induced locomotion.

***Drosophila* Spontaneous Locomotion Assay**

Wildtype, *canton-S*, flies showed 0.57 counts per minute as a measure of spontaneous mobility on the fourth day in the DAM (**Figure 2**). Flies exposed to 125 µM rotenone showed a similar level of spontaneous locomotion. By contrast, flies exposed to 250 µM and 500 µM rotenone showed approximately 50% lower measures ($p < 0.05$ ANOVA, Bonferroni pair wise comparison) of spontaneous locomotion (**Figure 2**). These flies moved at about 0.20 counts per minute, which is indicative of a rotenone-induced defect in spontaneous locomotion.

To account for initial discrepancies (if any) in locomotion not caused by rotenone exposure, we subtracted locomotion data between day 4 and day 3. Control flies showed an increase of about 0.1 counts per minute in spontaneous locomotion between days, while flies exposed to 125 µM rotenone exhibited a slight decrease of about 0.15 counts per minute (**Figure 3**). Flies exposed to 250 µM and 500 µM rotenone displayed more severe decreases in locomotion between days, with differences being approximately 0.3 and 0.5 ($p < 0.05$ ANOVA, Bonferroni pair wise comparison) counts per minute respectively. This data suggests a deficiency in spontaneous locomotion over time with exposure to rotenone and confirms single day analysis mentioned above--flies exposed to higher dosages of rotenone showed a decrease in spontaneous locomotion. Taken together, these methods reliably measure rotenone-induced deficiencies in spontaneous and startle-induced locomotion.

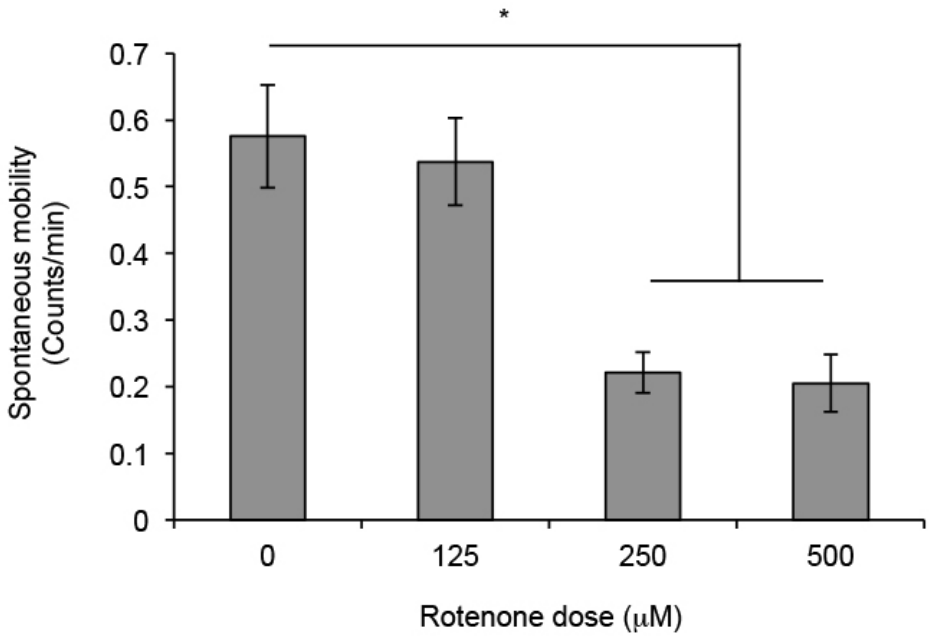

Figure 1. Startle-induced locomotion plot of flies exposed to increasing doses of rotenone. Wild type, *canton-S*, male flies were exposed to different dosages of rotenone for 3 days and surviving flies (8-12) were then tapped into the bottom of the double vial apparatus. Flies exposed to 500 µM rotenone show a significant decrease in the percent of flies in the top (**A**) and increase in the percent of flies in the bottom section (**B**) of the apparatus after 30 sec. This is indicative of a deficiency in startle response in flies exposed to rotenone. Columns represent the average percentage of 6 independent experiments. Error bars represent the standard error of mean; * $p < 0.05$ ANOVA, Bonferroni pair wise comparison.

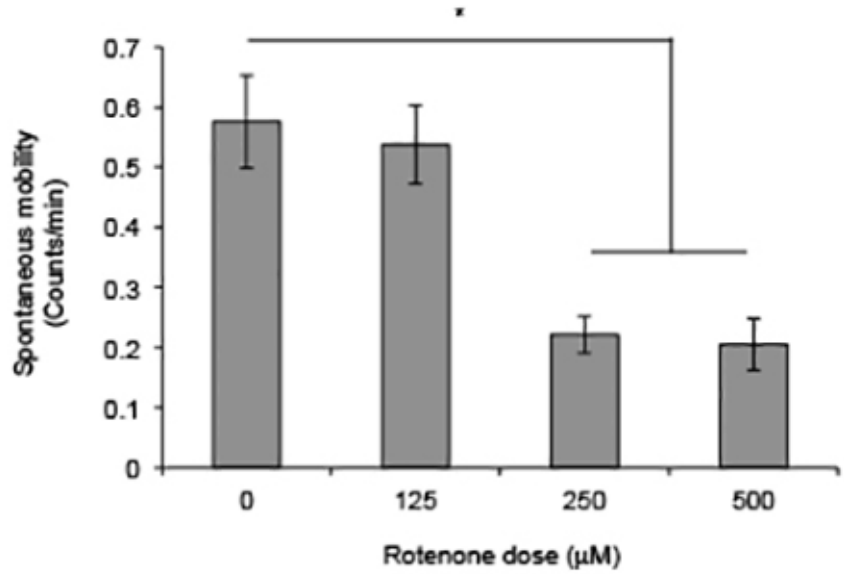

Figure 2. Spontaneous locomotion plot of flies exposed to increasing doses of rotenone. Wild type, *canton-S*, male flies were exposed to different dosages of rotenone and counts per min on the fourth day after exposure were plotted. Counts were measured in a DAM. Flies exposed to 250 μM and 500 μM rotenone show a reduction in counts per min. This is indicative of a deficiency in spontaneous locomotion in flies exposed to rotenone. Columns represent the average counts per min on fourth day of 5 independent trials. Error bars represent the standard error of mean; * $p < 0.05$ ANOVA, Bonferroni pair wise comparison.

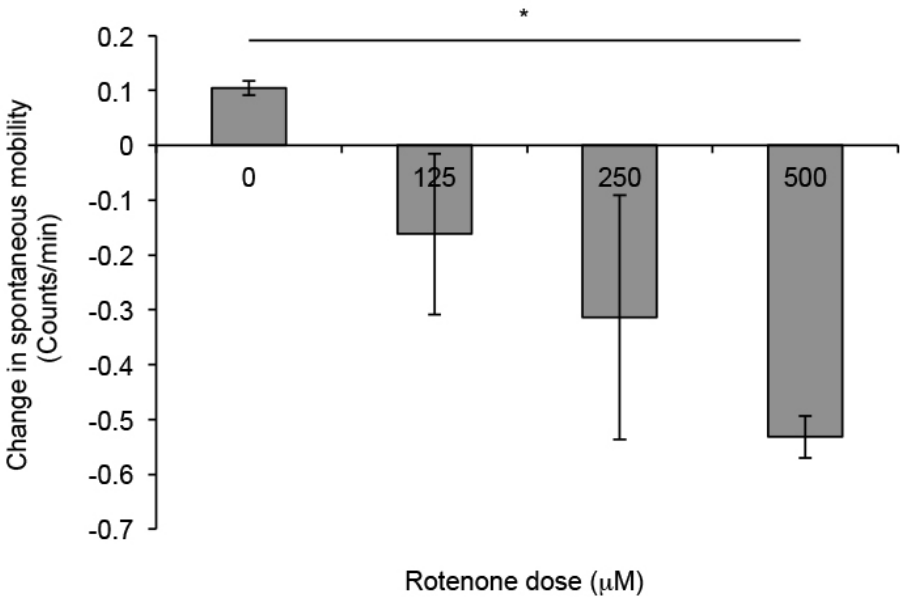

Figure 3. Change in spontaneous locomotion plot of flies exposed to increasing dosages of rotenone. Wild type, *canton-S*, male flies were exposed to different dosages of rotenone and the difference in counts per min on the third and fourth day after exposure were plotted. Counts were measured in a DAM. There is a dose dependent trend for decline in spontaneous locomotion with flies exposed to higher doses having a more negative change in locomotion. This is indicative of a decrease in movement. Columns represent the average change in locomotion per minute of 5 independent trials. Error bars represent the standard error of mean; * $p < 0.05$ ANOVA, Bonferroni pair wise comparison.

Discussion

In this study, we describe two procedures for measuring both long-term spontaneous locomotion and short-term startle-induced locomotion in a rotenone-induced *Drosophila* model of Parkinson's disease. One can also measure these locomotion characteristics in flies exposed to other pharmacological agents known to model Parkinson's disease *e.g.,* paraquat[14], genetic models of Parkinson's disease *e.g.,* alpha-synuclein mutants[15], and other fly models of diseases affecting locomotion. For both methods, alternative methods and modifications can be considered.

Flies can be anesthetized using ice, which could alleviate limitations of CO_2 anesthetization for example lag in data collection to let CO_2 effect wear off.

In the startle-induced locomotion assay, since flies show circadian variation in mobility, it is important to collect data at the same time of the day between experiments. It is also important to introduce flies into the testing apparatus without anesthesia. Contrary to most startle response locomotion assays, which rely on a snapshot of passing a threshold in a predetermined amount of time[10,11,15,16], our approach, similar to rapid iterative negative geotaxis (RING) assay[17,18], monitors the movement at successive multiple instances over a period of time. This approach of continuous monitoring of the distribution of flies in different zones may resolve subtle differences between treatment groups. Additionally, our approach of calculating the percentage of flies in multiple zones of the arena may help minimize the contribution of outliers in the data.

We also systematically decided at which time point to take data to compare the treatment groups. After taking data every 5 sec for 1 min, we plotted the data and found that the most notable differences between treatments could be seen at 30 sec. After this time point, flies exposed to rotenone are able to compensate for their locomotor defects. Therefore we recommend users to optimize the timing of data acquisition to best resolve the differences between their control and experimental sets. This approach also has the advantage of determining relative locomotor deficiencies between pharmacological agents and/or genetic models. For example, a more toxic chemical than rotenone may show most notable differences earlier than 30 sec time point.

For the long-term spontaneous locomotion assay, since flies are anesthetized to introduce them in the tubes, do not consider data from first 24-48 hr to allow for the anesthesia to wear-off and acclimation of flies in the monitor tubes. Another consideration for this assay is the relative position of the tube and the motion sensor in DAM, which we think can impact the spontaneous locomotion data. We placed the tubes containing flies in the monitor so that the sensor was monitoring the one-third span of the tube farthest from the food and not the middle of the tube as is usually done in traditional use of DAM in circadian studies. This allowed us to examine the ability of the flies to traverse at least two-thirds of the tube's length and led to more consistent data. It is likely that the activity counts can be impacted in rotenone-fed flies due to movement bursts and/or twitching phenotype. Other possible confounding factors for the activity counts could be a gustatory and/or an olfactory aversion/attraction towards rotenone and other chemicals of interest to the user. Therefore additional video tracking[17,19] can be employed to complement the DAM data for a more thorough analysis of the locomotion phenotype.

In a scenario where experimental flies have similar activity counts as compared to control flies, it is possible that they differ in the circadian distribution of movement since flies are more active around light on/off and off/on transition in a 12 hr light-12 hr dark cycle. Therefore, it would be helpful to determine the counts per min at multiple time points and bin lengths in a 24-hour period to determine the exact distribution of locomotion. In conclusion, this assay, due to its ability to assess movement characteristics not limited to movements in response to startle, will provide new insights into locomotion defects and characterization of remedial strategies.

Disclosures

The authors have nothing to disclose.

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