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Measuring the effects of larval life stress on adult anxiety behavior in *Drosophila melanogaster*

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Abstract

Anxiety is the body's homeostatic response to stressful situations. While anxiety is caused by factors such as pain, it has been categorized as a response that helps distinguish dangerous situations. Disruption during developmental stages directly affects an individual's mental health and social behavior. This is seen in the offspring of pregnant women when exposed to famine during pregnancy, resulting in increased risks of coronary heart disease, diabetes, obesity, and other conditions. In this report we tested the hypothesis that *Drosophila melanogaster* that are frequently exposed to stress during early developmental stages will have amplified levels of anxiety as adults. This was conducted by using bright light stress stimuli and through the usage of optogenetics pain to cause anxiety like behavior in the flies. It was found that wild type Ore-R flies placed under stress stimuli as larvae showed increased anxiety behavior when compared to the flies that were not exposed to stress stimuli. These results confirm that stress during early developmental stages can have severe behavioral effects later in life.

Introduction

Anxiety is the body's response to stressful situations. While anxiety may be caused by several factors, including pain, it has been categorized as an emotional response that helps assess dangerous situations. Anxiety-inducing stimuli in early life can have negative effects on an individual's development (Mohammad et al., 2016). Disruption during the developmental stages directly affects an individual's mental health and social behavior. Children of pregnant women exposed to stresses such as famine during pregnancy are more susceptible to coronary heart disease, diabetes, obesity, microalbuminuria and accelerated cognitive aging as adults (Babenko et al., 2015). In *Drosophila melanogaster*, the effect of early life stress on late-stage anxiety behavior has yet to be studied. This behavioral study will explore if exposure to anxiety-inducing stimuli in early life will influence the levels of anxiety expressed in adult fruit flies.

In this study, we assessed anxiety using two different behavioral assays, one being wall following assay (WAFO) and another being locomotive assay, which are both well-established anxiety behavior measurements in *D. melanogaster*. Fruit flies are known to avoid the center of an area due to distress and anxiety which is termed centrophobism (Iliadi,2009). By taking advantage of centrophobism, the wall following assay was developed where the flies under stressful or anxiety-inducing conditions show an increase in WAFO activity. They tend to cling to the walls of the arena more often than the non-anxious flies. The other measure of anxiety is the locomotive assay, where the decrease of locomotive activity is an indicator of anxiety behavior for fruit flies (Ostrowski et al., 2018). It has been proven that motivated behavior, such as walking, can be altered by induced stress. It was found that electric shock and high-temperature exposure over several minutes decreased flies' walking activity that lasted around eight hours. Locomotive activities in *D. melanogaster* are established as the most direct way to assess anxiety behavior (Ostrowski et al., 2018).

There are several ways to induce anxiety in *D. melanogaster* which includes exposure to bright light stimuli and by optogenetic induction of pain. Bright light induces stress in *Drosophila.* Decreases locomotor activity in larvae, suggesting an increase in anxiety levels (Min and Condron, 2005). Another way to generate anxiety is to induce pain through the usage of optogenetics. Optogenetics involves the genetic encoding of light-sensitive proteins called opsins, into cell membranes of neurons to allow for temporal control of cellular activity by photo-stimulation. There are many light-sensitive opsins, we used Chrimson, a red light sensitive opsin, to activate pain neurons as a way to induce anxiety in the animals in this study (Allsop et al., 2014).

In the current study, we examined whether anxiety-inducing stimuli during the developmental stages of *D. melanogaster* will affect late-stage anxiety behavioral patterns. We hypothesize that *D. melanogaster* that are frequently exposed to stress during early developmental stages will have amplified levels of anxiety as adults. It is known that animals exposed to stress will learn to avoid or adapt to the phenomena that caused anxiety. There is evidence regarding changes in gene expression when exposed to an anxiety stimulus (Sørensen et al., 2005). Another study explored the possible relationship between prenatal stress exposure in

mammals to links to ADHD, schizophrenia, autism, anxiety, or depression later in life, and how epigenetic alterations have a large influence on mental health in the later stages of life (Babenko et al., 2015). Here, we studied the effect of early life stress on *Drosophila* larvae on late stage anxiety behavior in the adult fruit flies. This was done through the usage of optogenetic pain and bright light stress. Furthermore, their anxiety behavior was assessed through locomotive and WAFO assays.

Materials & Methods

D. melanogaster has been the model organism for extensively studying animal behavior and neurobiology. *D. melanogaster* works well as a model organism due to its capability to produce large amounts of progeny during its short eight-week life cycle. Having a short life cycle is ideal for this behavioral experiment because it allows for adequate exposure of anxiety-inducing stimuli in the early larval stage and observation of anxiety-like behavior in the adult-stage.

Wild Type Flies

The Oregon-R wild type fruit flies used in the ongoing study were supplied by Dr. Kwangwon Lee's laboratory at Rutgers-Camden University. The stock was maintained under 25-degree Celsius, in cornmeal-based fly media, in which they were allowed to breed and mature. The larvae that have hatched in the media will be used for experimental treatments. After the female fly lays eggs, it takes 24 hours for the larvae to hatch, after which they start to roam around the fly media. It was reported that *D. melanogaster* larvae mature into adult form in four to five days after female fruit flies lay the eggs (Stefan et al., 2012).

Larval Selection

There are different developmental stages of larvae forms, called instars. Larvae in the third instar stage were identified as the largest of larval form, and were utilized for experimental treatments. Differentiation was made between 2nd and 3rd instars based off their spiracles. In the 2nd instar, the anterior spiracle is club-like, while in the 3rd instar it is branched. The posterior spiracles of the third instar also have a dark orange ring at their tip, which is lacking or weakly present in the 2nd instar. This band was used to identify 3rd instars when they are feeding and only their posterior spiracles were visible. Larvae were then collected from the vials using a Drosophila sorting brush from Carolina Biological, plastic handle, 6-½mm. They were then put into labeled 60mm x 15mm fisher brand stackable lid petri dishes for experimental treatments.

Optogenetics Flies

Optogenetic organisms 79598 opto ChR are highly sensitive to the light, due to expression of the light-sensitive opsins proteins (Guru et al., 2015). Normally, ChR (Channelrhodopsin-2) can induce an action potential in ChR expressing neurons through the usage of blue light. However, for this experiment Chrimson protein, a type of channelrhodopsin was used, which gave control over the activation of the light sensitive-ion channel. Chrimson is a fluorescent protein which is activated by red light instead of blue light. The main advantage of Chrimson is that we were able to use red light for stimulation without the worry of it being seen by the flies since they can not see red light. We expressed Chrimson only in pain neurons via the UAS-Gal4 system in ppk-GAL4/UAS-chrimson drosophila. Ppk is a gene only expressed in class 4 sensory neurons which are considered nociceptors. Class 4 sensory neurons are highly branched multidendritic sensory neurons in *Drosophila* larvae that function as nociceptors that are necessary for behavior responses to noxious mechanical stimuli (Zhong et al., 2010). Thus, our system will allow for specific activation of pain neurons as a way to induce anxiety in these animals.

The optogenetic strain ppk-GAL4/UAS-chrimson (79598) opto ChR received from the Bloomington Stock Center is a Gal4/ UAS system. Gal4 is a transcription factor which activates transcription of its target genes by binding to UAS regulatory sites. In *D. melanogaster*, these two elements are carried in separate lines 32078 (encodes the ppk-gal4) and 55136 (encodes the UAS-Chrimson). When these two lines are crossed the progeny, 79598 opto ChR is expected to have Gal4 expressed in pain neurons. Due to this gene expression, red light exposure activates pain neurons. As a result, these flies are a successful model organism to examine anxiety behavior, since pain is known to cause anxiety (Allsop et al., 2014). Optogenetic stock cultivation and larvae identification was accomplished under the same protocol that was used to maintain Ore-R flies.

Stressors

Two types of stress-inducing stimuli were be given to the organisms at the larval stage during the duration of the experiment. These stress stimuli included bright light for the wild type flies and then optogenetic pain for the optogenetic stock. In a previous study, it was found that bright light causes anxiety in larvae due to their photophobic nature (Min and Condron, 2005). Because of the stress from light, larvae showed decreased locomotor activity. Hence, the reason why bright light was used to cause stress during the larval stage. The bright light source was provided by the Olympus SZX7 Stereo Microscope, the microscope uses LED lights which are of 1500 lumens.

Third instar larvae were placed in small 60mm x 15mm fisher brand stackable lid petri dish with 0.5ml of water to prevent

them from desiccating during the exposure time. Then they were placed under a bright light for intervals of 10 minutes, 3 times for every 24 hours. Ten minutes of exposure time was chosen because it has shown to give the maximum behavior response in larvae (Min and Condron, 2005). The bright light stimuli were repeated until larvae initiate pupation. Near the enclosion stage of flies, the stimulus was stopped and they were allowed to mature into adults, which is the stage where their anxiety levels will be measured.

For the 79598 opto ChR strain, the larvae were exposed to a red light instead of bright light. The red light was provided by a LED light of 627 nm wavelength,1100 lumens. Red light served as a pain activating stressor during the early developmental stage, due to Chrimson being expressed in larval pain neurons. The same procedure that was used for wild type flies to induce stress will be followed. First, larvae were placed in 60mm x 15mm petri dishes with 0.5 ml of water to prevent desiccation. Then they were placed under red light for intervals of 10 minutes, 3 times for every 24 hours until they initiate pupation. Then the stimuli were stopped, allowing the organisms to grow into adults, at which their anxiety levels will be measured.

Measurement of anxiety phenotype

The stress levels were measured by video recording the adult flies using an iPhone camera. The .mov format obtained from the iPhone recording was converted to .avi since it is the correct format the fly tracking software utilizes. There are multiple analysis programs that can be used to track *D. melanogaster* behavior. Such programs are: Ctrax, Biotrack, IDTracker, and Noldus Ethovision (Chao et al., 2015). In this experiment, Ctrax was the best option for tracking the organisms wall following behavior due to anxiety- inducing stimuli. The Ctrax software tracked the path traveled by each individual fly. First, the video was analyzed using the Ctrax software which then will produce a matLab file with the position (x and y coordinates) of the fly in the arena. Then the positions of the flies were plotted, and the percentage of time spent along the wall was calculated. To quantify this, within 11 points from the left or right wall in the x coordinates was considered being along the wall, and 11 points within the top or bottom wall in the y coordinates was considered being along the wall. This is because 11 points in one direction equated to approximately the length of a fruit fly.

In order to prepare the flies for video recording they were placed in a 40 × 40 mm 3-D printed square arena which is further divided into 4 chambers resulting in 20x20 mm chambers with a height of 1.5 mm. *D. melanogaster* is considered centrophobic, like rodents, so they naturally tend to cling to the walls of the container they are kept in, especially when stressed (Iliadi, 2009). The intervals of time that the experimental flies stay near the walls of the square arena were be compared to the control. As a result, their time spent near the walls was utilized as an indicator of anxiety levels.

The change in locomotion behavior and the time the flies spent moving in the square arena was also an indicator of anxiety. As a result, the time spend moving will be recorded the during a duration of 10-minute videos. Any change in the fly's displacement was considered as a response. It was reported that the flies not exposed to previous stress tend to have more locomotive activity in an enclosed arena compared to flies exposed to stress, which show slow or no locomotive activities at all (Ostrowski et al., 2018). A t-test was performed to examine the significant of values obtained from the stress and non-stressed groups. Where p-value <0.05 is considered statistically significant.

Experimental Plan

The experiment was conducted with four groups of flies: a control group of wild type Ore-R flies, a stress group of wild type Ore-R flies, as shown in Figure 1. A control group of optogenetic 79598 opto ChR flies, then finally a stress group of optogenetic 79598 opto ChR flies. Every group was bred and their larvae were collected and separated into 60mm x 15mm fisher brand stackable lid petri dishes. The control group was allowed to grow into the adult phase without any stressors, and their behavior was observed via video tracking software and locomotive assay. The larvae from the stress group of wild type flies were put under stress from bright lights. They were exposed to bright light for 10 minutes, 3 times with 10-minute intervals in between, every 24 hours until pupation. When they reached the adult phase, their movements were also observed via video tracking software along with locomotive assay. The larvae from the optogenetic flies' group were put under stress from a red light that caused them to feel pain, which will induce anxiety. They were exposed to the red light for 10 minutes at a time, 3 times with 10-minute intervals in between every 24 hours until pupation. When this group reached the adult phase, their movements were observed as well via tracking software and locomotive assay.

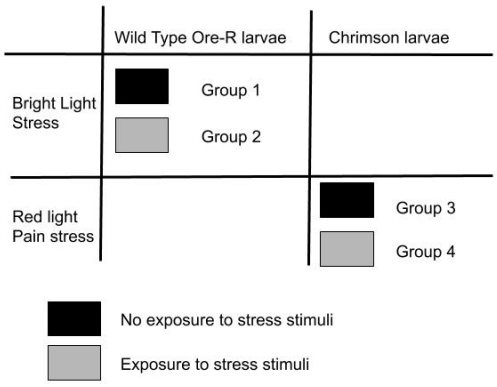

Figure 1. Experimental design

Then, the amount of time each group of adult flies spent close to the walls were compared. This gave us an indication of the levels of anxiety in each group. From this data, it can be determined whether administering stress stimuli during the larvae stage of *D. melanogaster* will amplify anxiety levels later in life. The locomotive assay also served as an indicator of stress, when the control no stress flies were compared to experimental stress flies.

Results

Locomotive Assay of the Control and Stress Groups

To test if bright light during larval stage caused a locomotive change in adult life, we measured the flies time spent walking during the duration of 10-minute video recordings. It was found that wild type Ore-R stress group had decreased locomotor activity compared to the wild type Ore-R control group (Fig. 2A). Ore-R stress groups locomotive behavior was statistically significant from that of the Ore-R stressed group (t-test, p-value: 0.0000823, $p < 0.05$, n=6).

To test if optogenetic pain during larval stage caused locomotive change in adult life for 79598 opto ChR strain, the flies locomotive activities were also observed. In 79598 opto ChR, the opposite trend was discovered where the control group displayed decreased locomotor behavior activities compared to the stressed group which showed increase in locomotor activities (Fig. 2B). However, optogenetic 79598 opto ChR stress group locomotive behavior was not statistically significant from that of the 79598 opto ChR control group (t-test, p-value: 0.9390060, $p > 0.05$, n=6).

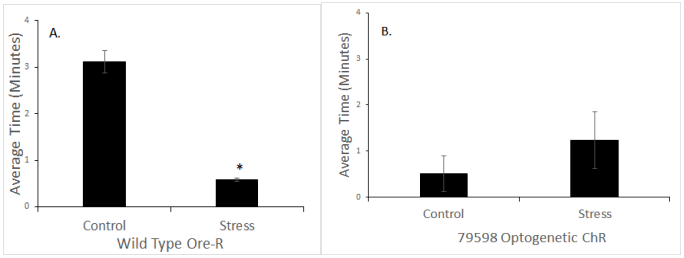

Figure 2. A) Locomotive assay of control and stress group for wild type Ore-R. B) Locomotive assay of control and stress group for 79598 optogenetic ChR flies.

Wall following (WAFO) of the Control and Stress Groups

In order to test if bright light stress stimuli will cause a change in wall following behavior (WAFO) compared to the control groups in Ore-R, the fly movements were recorded for 30 minutes. Then these were tracked by C-trax giving x and y coordinates of the flies positions. The x and y positions were plotted displaying their movements in the chambers (Fig. 3). In Figure 3A and 3B, the WAFO of the wild type control fly and stress fly can be visualized. Comparing the control fly versus the stress fly it can be observed that the control fly (Fig. 3A) moved the whole area of the arena. While the stress fly (Fig. 3B) only tracked along the walls of the arena. Then to quantitatively assess this observation, the percentage of time spent moving along the walls of the arena were found. In comparing Ore-R control and stress group, it was found that Ore-R control group was not statistically significant from that of stress group (t-test, p-value: 0.142854, n=4) (Fig. 3C).

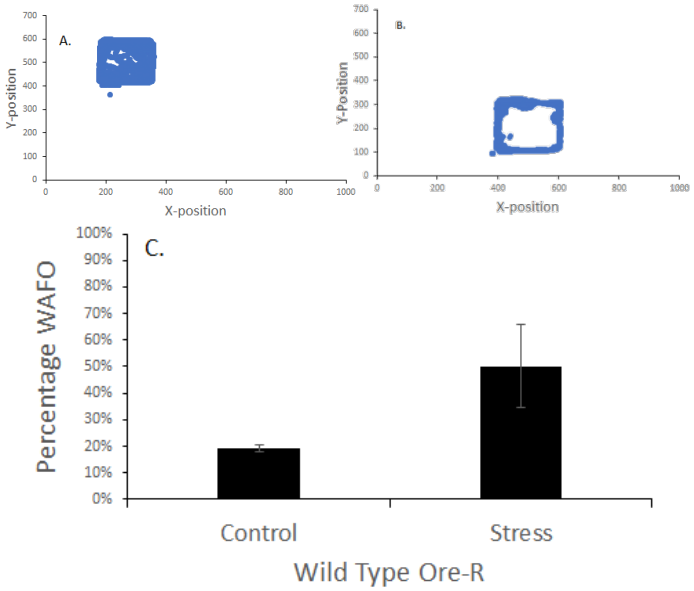

Figure 3. A) Shows the x and y coordinates of WAFO activities obtained by C-trax of the no stress control Wild type Ore-R fly. B) Shows the x and y coordinates of WAFO activities of the stress Wild type Ore-R fly. C) Quantitatively assesses the WAFO behavior of n=4 flies by calculating the time spent near the wall in a 20x20 mm chamber, for control and stress flies.

Then to test if the optogenetic pain for ChR flies caused a change in WAFO behavior in control and stress group, their movements were also tracked by C-trax for a duration of 30 minutes. Figure 4A and 4B displays the WAFO movement of a ChR fly. In this case,it can be observed that the control fly (Fig. 4A) moved the whole area of the arena, however it do not have a clear wall following pattern while on the other hand the stress fly (Fig. 4B) shows a very defined tracking along the walls and the whole area of the arena. Qunantivally, similar observation was seen in the optogenetic flies as in wild type, where the control group was not statistically significant from that of the stress group (t-test, p-value: 0.137332, $p > 0.05$, n=4) (Fig. 2D). However, there was a trend present where the WAFO activity of the stress group was higher than that of the control group for both Ore-R and the 79598 opto ChR strains.

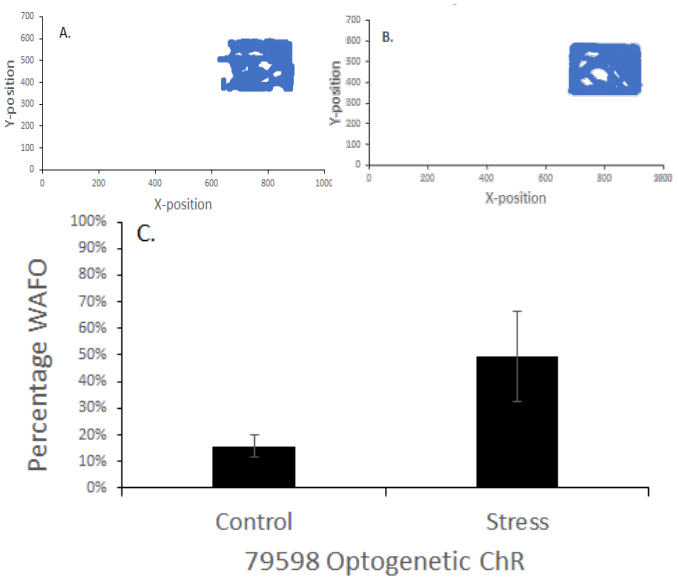

Figure 4. A) Shows the x and y coordinates of WAFO activities obtained by C-trax of the no stress control 79598 opto ChR fly. B) Shows the x and y coordinates of WAFO activities of the stress 79598 opto ChR. C) Quantitatively assesses the WAFO behavior of n=4 flies by calculating the time spent near the wall in a 20x20 mm chamber, for control and stress flies.

Discussion

Our study demonstrates that early life stress can induce anxiety-like behavior later in life. This confirms that exposure to bright light as larvae can inherently induce higher levels of anxiety as adults in Wild type Ore-R strain, as seen in the locomotive assay. This means the wild type flies serve as a successful model to measure the effect of anxiety behaviors. The decrease of locomotive behavior has been studied as an anxiety response caused by excessive heat shock in *Drosophila* (Ostrowski et al., 2018). However, in our study, the same patterns in locomotive activities were indicative as an anxiety response caused by bright light stimuli in early stages of life instead. This indicates that stress stimuli like bright light and heat shock will cause *D. melanogaster* to experience a decrease of locomotive activity due to anxiety. In previous studies it also can be seen that stresses such as electric shock (Ries et al., 2017) and induction of anxiety through continuous exposure to vibrating stress stimuli has caused a change in their locomotor behavior (Batsching et al., 2016).

For the 79598 optogenetic Chrimson flies, there was no significant difference between the control and the stress group in the locomotive assay. This insignificance can arise from the fact that ambient white light could also serve as a stress stimulus for them because the ambient light contains red wavelengths of light, which could activate the Chrimson opsin expressed in pain neurons of the optogenetic flies. This pre-exposure to indirect red light may have caused the pain neurons to be activated even before they were experimentally exposed to red light. As a result, the optogenetics flies did not serve as a model strain for testing the induction of anxiety by activating pain neurons through the usage of red light.

Even though WAFO behavior did not yield any statistically significant results, a positive trend was observed in the WAFO assay. Where the control groups for both of the strains had lower percentage of WAFO compared to the stress groups which showed greater WAFO activities. The insignificance in the data could arise from the low sample size, n = 4. However, due to great variance in the tracking of the video and the low sample size no conclusions can be made. It is because both the graphs in Figure 3C & Figure 4C show non-overlapping error bars but have insignificant p-values ($p > 0.05$). Although, the p-values were not significant, a power analysis showed that n=9 of the optogenetic organisms would be sufficient enough to obtain significance in our WAFO data at power level of 0.80.

D. melanogaster has been a model organism in a variety of experiments due to its genetic similarities with humans. For this reason, alterations to the genes of the organism may result in a change in behavioral response (Sørensen et al., 2005). Thus, this experiment assumed that the larval stage in *D. melanogaster* was analogous to infancy in humans. After confirming that administering stress to fruit flies at a larval stage influences the organism as an adult. It can be noted that exposing humans to stress during infancy, will play a role in their behavior as adults. As the experiment demonstrated, anxiety-like behavior was indicated by decreased locomotion. In humans these anxiety behaviors may translate into social, verbal, or even mental disorders.

This study can be utilized to assess future experiments in the same field of interest. For example, to further understand the locomotive behavior of anxious flies, it is worth looking into how increasing and decreasing the exposure time to stress stimuli can further affect their locomotive response. Another potential option may be to attempt reversing the effects of the amplified anxiety in the fruit flies. Additionally, an assessment can be made to test if any permanent damage is caused to the organism due to increased levels of stress and the effects of stress on progeny. A study also can also be made on the effects on the longevity of the organism due to the increased level of anxiety. Finally, epigenetic effects on the progeny of the stressed group can also be explored. It has already been suggested that social experiences like isolation can alter the epigenetic landscape involved in transcription and neural function (Agrawal et al., 2018). However, here we

can explore how anxiety experiences can alter the epigenetic landscape.

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