The relationship between GABA powder and the learning and short-term memory of *Drosophila melanogaster* with a Fragile-X Mutation

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Abstract

Fragile-X Syndrome (FXS) is a rare genetic X-linked disorder that occurs when there is a mutation in the Fmr1 gene. Normally the Cytosine-Guanine-Guanine (CGG) sequence in the Fmr1 gene is only repeated 5 - 40 times, but in people affected by FXS, the CGG sequence repeats more than 200 times. These excessive repeats prevent the Fmr1 gene from creating more FMRP protein, this affects the synaptic function and plasticity in the brain, therefore resulting in trouble in effectively communicating between neurons. Gamma-aminobutyric acid (GABA) is a chemical inhibitory neurotransmitter that decreases the stimulation of nerve cells in the brain, which can help slow down brain processes in those diagnosed with FXS. The purpose of this research is to determine a relationship between the increased levels of GABA and learning and short-term memory consolidation within flies that are affected by FXS. It was hypothesized that if GABA is consumed, then *Drosophila melanogaster* with FXS will have greater learning and short-term memory consolidation. The Aversive Phototaxic Suppression Assay (APS) was used to test the learning and short-term memory of the *Drosophila melanogaster*. After analyzing the results collected from Mann-Whitney tests, there was a statistically significant difference between the Wild-type flies and the FXS flies that fed on 0 μM GABA, which shows that the FXS flies did have trouble with learning and short-term memory. Additionally, there was no statistically significant difference between the Wild-type flies that fed on 0 μM GABA and the Wild-type flies that fed on 30 μM GABA, which shows that the GABA treatment targets those with FXS. In conclusion, the hypothesis was accepted because there was a statistically significant difference between FXS flies that fed on 0 μM GABA and FXS flies that fed on 30 μM GABA. In the future, the effect of GABA on learning and short-term memory could also be conducted on other organisms, such as mice, to allow for a better understanding of the

connection between short-term memory consolidation and the role of GABA as they have similar processes to humans in terms of aging, similar endocrine systems, and similar responses to infections and diseases.

Introduction

1.1 Significance of Fragile-X Syndrome

Fragile-X syndrome (FXS) is an intellectual disorder that is on the autism spectrum. About 1 in 7000 males and 1 in 11,000 females in the general population have this disorder. Fragile-X is more common in males because males have only one X chromosome (XY), which is mutated. On the other hand, females may have fewer effects of Fragile-X because they have two X chromosomes (XX), and the unaffected chromosome may reduce the effects of the mutated one (Bartholomay et al., 2019). FXS occurs when there are more than 200 repeats of the CGG (cytosine-guanine-guanine sequence) at the bottom of a chromosome (normally, this sequence is only repeated 5 to 40 times). As seen in Figure 1 below, these repeats prevent the Fmr1 gene from creating more FMRP protein; the lack of this protein interrupts the functions of the nervous system (Razak et al., 2020). People with FXS have certain physical features such as large ears, large faces, broad foreheads, and scoliosis. Other symptoms of FXS include balance problems, shaky hands, numbness, unstable mood, cognitive problems, and memory loss. When people learn, they form connections between their neurons, and if people have low levels of FMRP, these connections may be interrupted or hard to form (Kennedy, 2013). The FMRP protein causes changes in synaptic function and brain plasticity, making it difficult for neurons to be able to form connections between each other.

Figure 1: The Cause of Fragile-X Syndrome

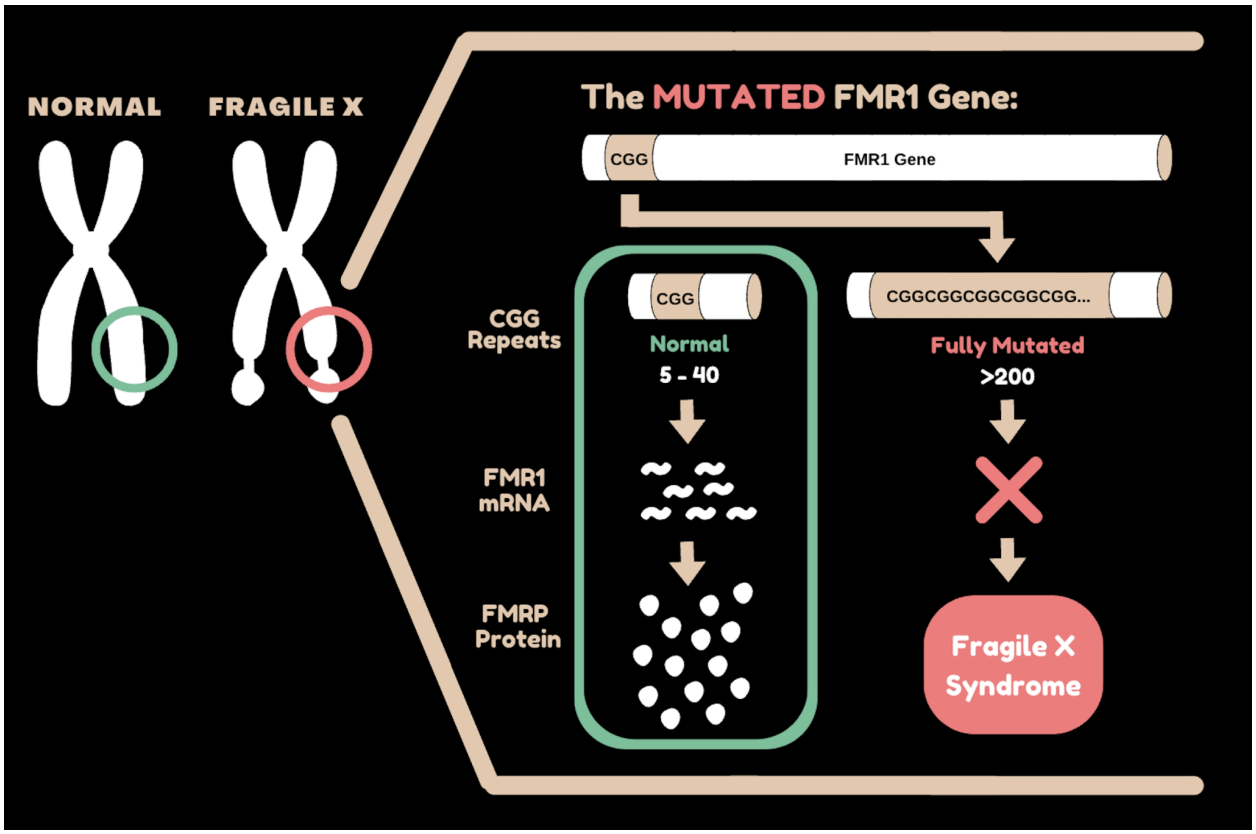


Figure 1 above represents the difference between a normal X chromosome and a mutated Fragile-X chromosome. A regular X chromosome has a Fmr1 gene that has only 5-40 Cytosine-Guanine-Guanine (CGG) repeats, while a mutated X-chromosome has a mutated Fmr1 gene with more than 200 CGG repeats, which does not allow the creation of the FMRP Protein, leading to Fragile-X Syndrome.

1.2 Role of Gamma-Aminobutyric Acid (GABA)

GABA is a chemical inhibitory neurotransmitter that controls the stimulation of the nerve cells, and it also has anti-anxiety and anti-seizure effects. Some functions of GABA include slowing the brain down by blocking signals in the central nervous system, producing a calming effect, and helping control nerve cell activity regarding issues such as anxiety, stress, and fear. GABA inherently controls the stimulation of the nerve cells, which helps regulate the

connections between neurons (Bryson, 2020). GABA is being tested as a potential treatment as it regulates neural connections.

1.3 Drosophila Model Used

Drosophila melanogaster share 75% of their DNA with humans. The dfmr1 gene in *Drosophila melanogaster* exhibits a high sequence homology with human genes like FMRP (Drozd et al., 2018). The strain of *Drosophila melanogaster* that will be used is Fmr1[Delta50M] (#6930) due to its repeated use in prior research projects (Zhao, 2021). This specific strain has an fmr1 allele deletion, thus modeling FXS as it is similar to the FMR1 deletion in humans. The deletion of the FMR1 results in the low production of the FMRP protein, and the low levels of this protein results in FXS. Additionally, for the wild-type control group, w[1118] flies (#3605) were used. These flies have the partial deletion of the *white (w)* gene. The w[1118] strain for wild-type flies was used due to the similar genetic background between them and the Fmr1[Delta50M] (Fragile-X) flies.

1.4 Purpose and Significance of Study

This project will be conducted to test the effect of Gamma-Aminobutyric Acid (GABA) on the memory of *Drosophila melanogaster* with a Fragile-X mutation. The significance of this study is to try to alleviate the symptoms of Fragile-X Syndrome, especially memory loss. If GABA were to improve memory, it could provide an avenue of future research to help with memory loss for people who are diagnosed with FXS. Though, if no correlation between the intake of GABA and learning and short-term memory is found, it would be known that this supplement does not alleviate the symptoms of FXS. The results from this study provide insight into the impact of GABA powder on the neural activity of *Drosophila melanogaster* with Fragile-X syndrome by testing learning and short-term memory.

1.5 Variables and Hypothesis

The independent variable for this experiment is GABA powder, as *Drosophila melanogaster* will be tested on their learning and short-term memory after being given 30 μM of GABA powder. Another independent variable is the presence of Fragile-X in *Drosophila melanogaster* because the learning ability of Fragile-X flies will be compared to wild-type flies. The dependent variable of this research is the learning and short-term memory of *Drosophila melanogaster* because this will be tested to determine if the GABA powder had an effect. To measure the learning and short-term memory in the *Drosophila melanogaster,* the Aversive Phototaxic Suppression (APS) Assay will be used.

The hypothesis for this experiment is if the amount of GABA powder being used increases, then short-term memory consolidation in *Drosophila melanogaster* with a Fragile-X mutation will also increase. Those diagnosed with FXS often deal with weak memory and cognition skills. The weak memory and cognition skills occur due to the lack of the FMRP protein, which occurs when there is a mutation to the Fmr1 gene. The lack of the FMRP protein affects the synaptic function and plasticity in the brain, causing it to have trouble in effectively communicating between neurons. GABA, a chemical inhibitory neurotransmitter, could be used to stimulate the rate at which neurons fire in the brain, causing the person to retain better learning and short-term memory consolidation as the number of synapses can bind connected neurons into a circuit that stores new memory. Since FXS can cause a person to have weak memory and cognition skills, GABA could be used to stimulate the neurons in the brain and form connections, causing the person to retain better short-term memory consolidation.

1.6 Procedural Summary

Using the Aversive Phototaxic Suppression Assay, the memory of the *Drosophila melanogaster* will be tested by training the fruit fly to choose the dark vial when given the option to choose between dark and light. Flies tend to choose the light alley of the assay, though every time they choose that alley, there would be an aversive aroma, in the form of Quinine Hydrochloride. Eventually, the flies would be trained to use the dark alley, which is against their natural positive phototaxis. While testing, if the flies choose the light alley, they fail this test because they do not remember the aroma. If they remain on the light side, they pass (Ong et al., 2020). The Aversive Phototaxic Suppression Assay is an affordable assay that is used to test how genetic manipulations or environmental perturbations alter the short-term memory and response to the tests that will be conducted (Seugnet et al., 2009).

The results of each trial are calculated by taking an average score of the number of flies that choose the dark of the APS of all the participating flies. The results of the experimental group will be compared to the control group of FXS flies with no GABA concentration and wild-type flies with a 30 μM GABA concentration, and the results from the wild-type flies with no GABA concentration will also be compared to the FXS flies with no GABA concentration and wild-type flies with a 30 μM GABA concentration. Since only two groups are compared at a time, Mann-Whitney tests would be used for statistical analysis. These results will show whether or not the *Drosophila melanogaster* in the experimental groups were able to improve their short-term memory consolidation. If there is an improvement, it will support the hypothesis, and it will be known if the GABA powder was able to help neurons form connections within the brain for FXS patients. Normally, those with FXS or an ASD have a slow processing rate,

resulting in a negative impact on their ability to retrieve information from their long-term memory.

1.7 Current Research

Current research shows that GABA has an important role in regulating and synchronizing the neuron signals in the hippocampus. The hippocampus is an area in the brain that is crucial for episodic memory. It has been found specifically in animal models, that losses in the neurons result in hyperactivity in the hippocampus. In the brain, the transmission of GABA is mostly regulated by receptors, which are found in the pre and postsynaptic terminals of neurons. Researchers have also found that synaptic plasticity in the hippocampus is mediated by tonic inhibition, which is produced by the sustained activation of GABA receptors after the release of GABA to the synaptic cleft in a neuron. The GABA system in the hippocampus is accountable for maintaining balance, as well as synchronizing the activity of neurons within the hippocampus and other remote areas of the brain. Limitations in this study include how it is unknown whether the alterations in the levels of GABA can result in the impact of memory loss when observed in healthy adults (Jiménez-Balado, 2021).

Information currently known about FXS states that the lack of FMRP protein hinders the formation of the dendritic spine in neurons, which results in the disruption of neural protein synthesis. The researchers also found that the absence of the FMRP protein resulted in decreased synthesis of both GABA and its receptor. The decrease in GABA synthesis contributes to the disruption of neural plasticity, which refers to the nervous system's ability to respond to an event. It was also found that mild to moderately low levels of the FMRP protein is most often associated with less severe symptoms, while extremely low levels are more associated with severe symptoms, which are more common in males with FXS. The limitation of this experiment

is the lack of improvement in the design of the clinical trials to test the symptoms of FXS (Protic, 2022).

1.8 Novelty

By conducting our experiment similar results to the existing current research was expected. In previous research, researchers were able to build a behavioral assay for their experiment to better understand the molecular basis of sensory processing dysfunction in *Drosophila melanogaster*. Though, in this study, the concentration of GABA was compared to a control group, which will help determine if alterations in the concentration of GABA impact memory loss. Additionally, in this study, multiple trials will be conducted to test the symptoms of FXS, especially memory loss. The novelty of this project is that the effect of GABA powder on the learning and short-term memory of *Drosophila melanogaster* with Fragile-X mutation has never been directly tested before. Through this research project, results and data regarding the effect of GABA on the learning and short-term memory of *Drosophila melanogaster* will be collected. In the future, the effect of GABA could also be tested on other cognitive impairments such as decision-making.

Materials and Methods

2.1 Variables and Constants

The independent variable of this experiment is the 30 μM of GABA powder, as there will be GABA in the batches of fly food given to the wild-type and Fragile-X flies. The presence of GABA powder will determine if the learning and short-term memory of the *Drosophila melanogaster* has changed. Additionally, the independent variable is the presence of Fragile-X in

Drosophila melanogaster, as the learning ability of Fragile-X flies will be compared to that of wild-type flies.

The dependent variable of this experiment is the learning and short-term memory in the *Drosophila melanogaster* since the learning and short-term memory of the fruit flies will be tested in order to determine if the GABA powder had affected it. The dependent variable will be measured using the Aversive Phototaxic Suppression Assay, which tests if *Drosophila melanogaster* remembers their environment (refer to Section 2.7).

While conducting this study, there were a few constants throughout each of the trials. Throughout the study, only male *Drosophila melanogaster* were tested. The *Drosophila melanogaster* were tested seven days after they emerged from their larvae, where they were either feeding on regular fly food or GABA-concentrated fly food for the entire duration. Each vial of flies had 10 flies, which were maintained at a temperature of 22°C and a constant humidity. Other rearing constants include a 12 hour day and 12 hour night cycle for the flies throughout the study. To test the learning and short-term memory, an Aversive Phototaxic Suppression (APS) Assay was used with a constant amount of 0.2 M Quinine Hydrochloride during the testing period. Additionally, during the testing period, the flies spent one minute in the light vial and 30 seconds in the dark vial throughout the study (refer to Section 2.7). Lastly, the time of day where sorting, tapping, and testing took place was between 1:45 P.M. - 3:30 P.M for each of the trials.

2.2 Control and Experimental Groups

There are three control groups for this experiment. The negative control group is the w[1118] strain (wild-type) *Drosophila melanogaster* with regular fly food (no concentration of GABA powder). This control group would be used to compare the effect of GABA powder on

the learning ability of wild-type flies, and to check if the learning and short-term memory of Fragile-X flies are significantly worse than that of wild-type flies. The genetic control group is Fmr1[Delta50M] strain (Fragile-X) flies with no GABA concentration in order to compare the effects of GABA powder on flies with Fragile-X syndrome. It is expected for Fragile-X flies without GABA powder to have significantly worse learning and short-term memory compared to Fragile-X flies that are fed GABA powder. Lastly, the toxicity control group is the wild-type flies with the GABA concentration in order to check if the GABA powder affects the learning and short-term memory of wild-type flies as well. No change in learning and short-term memory is expected in wild-type flies during this study. The experimental group for this study is the Fragile-X flies that have fed on GABA food. The experimental group would be expected to perform much better in the Aversive Phototaxic Suppression Assay compared to the genetic control, as they have fed on the treatment food.

2.3 Materials

The materials used for this study were abstained through the Academies of Science unless otherwise specified. However, the w[1118] (#3605) and Fmr1[Delta50M] (#6930) strains of *Drosophila melanogaster* were abstained from Bloomington *Drosophila* Stock Center. The LED fairy lights used for the Aversive Phototaxic Suppression (APS) Assay and the GABA powder used for the treatment food were obtained from Amazon.com. For the APS Assay, Quinine Hydrochloride, test tubes, tape, filter paper, and vials were used. Additionally, a stopwatch was used from an iPhone to time the testing and training periods (refer to Section 2.7).

To prepare *Drosophila melanogaster* food, water, soy flour, yellow corn meal, light corn syrup, agar, yeast, 10% Propionic acid, GABA powder, a scoopula, a 500 mL beaker, a stirring rod, a weigh boat, an electronic balance, a cheesecloth, vials, and flugs were used. Additionally,

a microwave (*Panasonic NN-SN936W*) was used to heat up the food during the process of combining all the ingredients (refer to Section 2.4). For cold sorting, a cold sorting pad (*serial no. 213200912*) was used to sort the anesthetized flies. Additionally, an ice bucket, weigh paper, feather, and magnifying glass was used to separate the male flies from female flies before transferring to vials before testing (refer to Section 2.6).

Lastly, safety precautions were taken during this study. To prevent damaging clothes from hot fly food, lab coats, goggles, gloves and close-toed shoes were worn. Additionally, when operating the microwave, hot hands were used to prevent skin from burning from the hot fly food.

2.4 Fly Food Preparation (Eliason, 2022)

To make the fly food, first the dry ingredients must be collected and measured. The mass of the dry ingredients are measured using an electronic balance, weight boat, and scoopula. The dry ingredients consist of approximately 6.75 grams of yeast, 3.90 grams of soy flour, 28.80 grams of yellow cornmeal, and 2.25 grams of agar. To find the mass of all the wet ingredients needed to make the fly food a 100 mL graduated cylinder is used. The wet ingredients needed to make the fly food are measured using the graduated cylinder as well. The wet ingredients consist of 30 mL light corn syrup and 390 mL distilled water. After acquiring all the ingredients necessary they must be combined, the dry ingredients must be mixed in the 1000 mL beaker. The wet ingredients are then added to the beaker, preferably corn syrup is added to the mixture first, this way when adding water to the mixture the excess corn syrup in the beaker will be washed out. The wet and dry ingredients are combined using a glass stirring rod. After making sure that the mixture is completely combined, the beaker is then put into the microwave for 30 seconds until it starts boiling (bubbling), once it boils it is then stirred with the glass stirring rod. It is

important to wear safety equipment like goggles and hot hands while boiling the mixture in the microwave. Once it is evident that the mixture has started bubbling, it should be taken out of the microwave and placed on the lab counter, a cheesecloth is placed over the beaker and the food is left to cool to room temperature, although it should not be solidified. Once the mixture has cooled, 1.88 mL of propionic acid is added to the food using a micropipette, it is then mixed into the food using a glass stirring rod. It is important to note that while handling propionic acid, safety equipment such as lab coats, goggles, and gloves should be worn. After the propionic acid is mixed into the food, 10 mL of fly food should be poured into each vial. A cheesecloth must be placed over the vials while the food cools down. The vials should always be covered to prevent flies from contaminating the food. After the food has solidified, the vials should be flugged, if vials with food are not being used they must be flugged and placed in the fridge. Food must be disposed of if it is unusable, for example when it becomes moldy or contaminated. To make treatment food (GABA food) the same procedure for preparation of basic fly food should be followed, however, 1.4332 mg of GABA for 30 µM concentration should be measured and added to the dry ingredients at the beginning of the procedure (refer to Calculation 1).

Calculation 1: 30 µM GABA Concentration Calculations (Jeong, 2021)

1. Conversion of 30 micromolar to molar

$$30\ \mu M \cdot \frac{1\ M}{1000000\ \mu M} = 3.0\times10^{-5}\ M$$

2. Conversion of 3.0×10^{-5} M to grams/liter

GABA molecular weight = 103.12 g/mole

$$3.0\times10^{-5}\ M \cdot \frac{103.12\ g}{mol} = 3.0\times10^{-5}\ \frac{mol}{L} \cdot \frac{103.12\ g}{mol} = 0.003094\ \frac{g}{L}\ \text{concentration}$$

3. Total amount of food needed for research

30 mL corn syrup + 390 mL distilled water + 1.88 mL propionic acid = 421.88mL wet ingredients

6.75 g yeast + 3.90 g soy flour + 28.5 g yellow cornmeal + 2.25 g agar = 41.4 g

1 g = 1 mL dry ingredients

41.4 mL + 421.88 mL = 463.28 mL = 0.46328 L total ingredients

4. Grams of GABA Required

$0.003094 \frac{g}{L} \cdot 0.46328$ L = 0.0014332 g = 1.4332 mg

2.5 Culturing and Maintaining Flies (Eliason, 2022)

To maintain the flystock the vials of flies must be incubated and hydrated, to do this the food must be kept damp but the sides of the vials should be dry. It is also important to make sure that the temperature must be maintained at 22°C so that the fly life cycle can last up to two weeks. The fly vials must be checked regularly to ensure that they are not overpopulated. If there is minimal food and overcrowding of the *Drosophila melanogaster*, it is best to assume that the vial is overpopulated. If removing flies from a vial, they should be anesthetized so that they can be separated into two different vials. While removing flies from a vial to prevent overpopulation, only adult flies should be removed from the vial, but the fly eggs should remain inside. Flies should transfer vials every 3 weeks if they are not being experimented on. To expand a fly stock, they must be tapped to encourage reproduction. Flies must be tapped into different vials once larvae are seen to make sure that the new flies can mate and reproduce.

There are many threats to the fly stocks, some of these threats include dryness, mites, fungus/mold, and contamination. To prevent dryness the food must be kept refrigerated, and food older than 3 weeks should not be used. If there is dryness in the food of the vial it can be treated

using distilled water, only a little should be used to fill in cracks and spaces. To prevent mites from getting into the fly stock utensils, tools, and stations must be wiped with 70% ethanol before sorting the flies. To prevent fungus and mold propionic acid is added to the food, in case there is moldy food the flies in the vial must be tapped into a new vial immediately. To prevent contamination in the lab it is important to check fly appearances when sorting, the food must be covered at all times. In case a fly stock is contaminated, all stocks must be thrown away to prevent it from spreading.

To tap flies into new vials it is important to make sure to have a new vial with fresh food along with the old vial with flies. The flug from the fresh vial must be removed, and it should be positioned upside down over the top of the old vial with the flies. The old vial with flies should be tapped on a surface about 3 times to ensure that flies are at the bottom of the vial. The two vials should be flipped upside down so that the new vial is now faced upright on the bottom. The vials are tapped so that flies in the vial are brought to the bottom of the new vial. Once the flies are at the bottom of the new vial the old vial is removed from the top of the new vial and the top of the new vial is quickly flugged to make sure flies do not escape. It is important to make sure that the old vial is held upside down while flugging the top, this prevents the flies that might have been left in the old vial to escape as well as preventing contamination.

To dispose of flies, they can be freezed at -80°C, and disposed of in the trash bin. If a vial needs to be kept, after being frozen the flies can be tapped out of the vial, the vial can then be washed so that it could be reused.

2.6 Cold Sorting (Eliason, 2022)

To cold sort a fly stock it is essential to make sure that the flies are anesthetized prior to being placed on the cold sorting machine. An ice bucket should be grabbed and filled up with

ice from the fridge. Then the flies that are about to be sorted should be transferred into new empty vials that do not have food. Once the flies have been transferred into an empty vial a timer should be set for 2 min before putting the flies into the ice bucket. While the flies are in the ice bucket, the cold sorter should be plugged in and the temperature should be set between 0°C and 4°C using the up and down arrow keys on the cold sorting machine. Once the cold sorter is running, a weight paper from the drawer should be placed on the cold sorting plate. It is recommended to wet the edges of the paper so that it sticks to the surface of the machine. After 2 min have passed the flies should be asleep, the vials should be opened and the flies should be poured onto the weight paper on the cold sorting machine. Using a feather and magnifying glass the male and female flies are sorted. The male flies can be sorted by looking for a rounded bottom, while female flies have a more pointed bottom. Once sorting is complete, the flies should be returned back to the vial. The feather and magnifying glass should be placed back in the drawer, the ice bucket should be emptied out, and the weight paper on the machine should be thrown away. After disposing and organizing the materials used for sorting, the surface of the cold sorter should be cleaned using 70% ethanol solution and dried off using a paper towel.

2.7 Aversive Phototaxic Suppression Assay and Administration of Quinine Hydrochloride

To build the APS assay some of the materials must be ordered, these materials include LED fairy lights and tape. To make the base of the assay, two vials must be taped together, one of the vials should be covered with the LED fairy lights and the other vials should be covered with dark colored tape. To ensure that flies do not fly out of the apparatus while testing, two flugs should be used to close each end of the vial. To test using the APS assay, it is essential to understand how to administer the Quinine Hydrochloride, as it influences the flies to follow the instructions learned during the testing phase of the APS assay. To make 10 mL of a 0.2 M

Quinine Hydrochloride solution, 0.7218 g of Quinine Hydrochloride was mixed into 10 mL of water (refer to Calculation 2). To train the flies, 10 flies from a vial must be tapped into the light vial, and flugged using a flug treated with Quinine Hydrochloride. The flies are trained for 1 minute, the flies are then tapped into the dark vial and flugged using a regular flug. The flies are trained in the dark vial for 30 seconds. This process of training must be repeated five times. The flies are then tapped down, and the flug is removed so that the light and dark vials can be combined. The two vials should be kept parallel to the ground. It is important to alternate tapping into the light and dark vials per trial to prevent bias. Before recording the number of flies that choose the dark side of the APS compared to the dark side, it is important to wait for a minute to ensure that the flies have time to decide which side they choose.

Calculation 2: 0.2 M Quinine Hydrochloride Concentration Calculation (Ali, 2011)

1. Conversion of 0.2 M to grams/liter

   Quinine molecular weight = 360.9 g/mole

   $0.2\ \text{M} \cdot \frac{360.9\ g}{mol} = 0.2\ \frac{mol}{L} \cdot \frac{360.9\ g}{mol} = 72.18\ \frac{g}{L}$ concentration

2. Total amount of solution needed for research

   4 groups = 10 mL

3. Grams of Quinine Hydrochloride Required

   $72.18\ \frac{g}{L} \cdot 0.01\ \text{L} = 0.7218\ \text{g}$

2.8 Procedural Summary

One vial of flies of the Fmr1[Delta50M] strain (Fragile-X mutation) and one vial of flies of the w[1118] strain (wild-type flies) are ordered from the Bloomington *Drosophila* Stock center. The one vial of Fmr1[Delta50M] and w[1118] flies are expanded in order to obtain enough flies to conduct ten trials for all four groups, having 10 flies in each vial. Then, the

Aversive Phototaxic Suppression (APS) Assay is built using tape, empty vials, and LED fairy lights. This assay was built in the form of a Y-maze, with the left side being covered in dark tape and the right side surrounded by LED lights.

For every new batch of wild-type flies or FXS flies emerging, the males and females are sorted using the cold sorter. Once male flies are sorted from female flies, male flies are grouped into vials with 10 male flies each vial. For this experiment, 40 total trials are tested: 20 wild-type trials and 20 FXS trials. Then the flies are separated into wild-type or FXS groups, they are left to feed on the control or experimental food for 7 days. The APS Assay is conducted for each wild-type trial or FXS trial feeding on control and experimental food (refer to Figure 2), and the results are recorded in a data table (refer to Table 1). Once the 40 trails are completed and the results are collected, Mann-Whitney tests will be used to analyze the data collected from the experiment.

Figure 2: APS Assay Procedural Summary

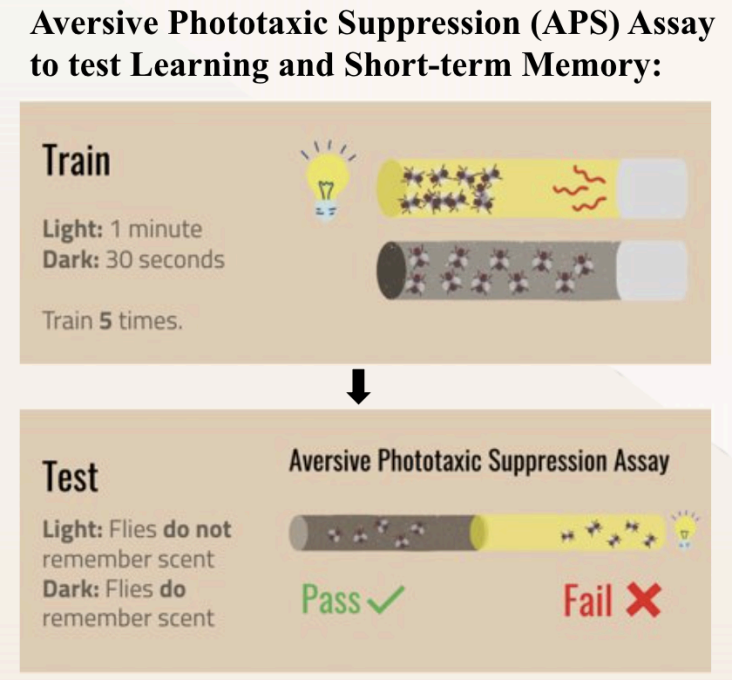


Figure 2 represents the two main steps to conduct an APS Assay. The first step is training, where the flies are trained in the light vial with Quinine Hydrochloride for one minute, and then tapped into the dark vial for 30 seconds. After the flies have been trained five times, the Quinine Hydrochloride is removed and the two vials are joined together during the testing phase. The flies that remain in the light vial fail to remember the scent of the Quinine Hydrochloride, while the flies that remain in the dark vial pass this APS Assay.

Three different Mann-Whitney tests were conducted using INSTAT between all four groups in order to determine if there was a statistically significant difference between the pass rates of the APS Assay in order to determine if the learning and short-term memory of *Drosophila melanogaster* with a Fragile-X mutation significantly increased. The first Mann-Whitney test was conducted between Fragile-X flies with no concentration of GABA and Fragile-X flies with 30 μM concentration of GABA. This would test if there was a statistically significant difference between the pass rates of Fragile-X flies that fed on regular food and Fragile-X flies that fed on GABA food in order to determine if the treatment food did help with the improvement of learning and short-term memory. The second Mann-Whitney test was

conducted between wild-type flies with no concentration of GABA and wild-type flies with 30 μM concentration of GABA. This was conducted in order to determine if the GABA treated food only targets Fragile-X flies, or if it impacts regular wild-type flies as well. The last Mann-Whitney test was conducted between Fragile-X flies with no concentration of GABA and wild-type flies with no concentration of GABA in order to ensure that the learning and short-term memory of Fragile-X flies were significantly worse than regular wild-type flies without feeding on the GABA-treated food.

2.9 Safety, Possible Risk Involved, and Disposal of Materials

The risks involved in this project involve glassware, heat, and the use of chemicals such as GABA and propionic acid. Risks involved with GABA and propionic acid include accidentally inhaling, accidentally swallowing, accidental skin contact, and accidental eye contact. If GABA or propionic acid. is inhaled, the person must be moved to fresh air or supplied with oxygen based on the severity. If GABA is swallowed, the person must drink 2 glasses of water, while if propionic acid is swallowed, the person needs to rinse their mouth thoroughly. Additionally, if GABA or propionic acid comes in contact with the skin, the skin should be rinsed for 15-20 minutes. Lastly, if GABA or propionic acid comes in contact with eyes, eyes should be flushed with water for 15-20 minutes and contact lenses should be removed. Additionally, flies must be anesthetized prior to sorting/experimenting to prevent escape or contamination. If Propionic acid is spilled, it must be neutralized with Sodium Hydroxide. Furthermore, if GABA is spilled, it must be collected and disposed of in a proper disposing bin (not to be flushed down the drain). Affected area must be cleaned up properly to avoid the generation of dust.

The materials used will be disposed of by either being placed in the glass disposal bin, or in the sink to be washed. The chemicals being used will also be disposed of safely by making sure that they go in the waste disposal containers. To dispose of propionic acid, unused product should be left in the container and can not be disposed in household garbage. For the disposal of GABA powder, the product should be left in the original container and not mixed with other waste. Additionally, GABA should not be disposed in household garbage.

Data

3.1 Raw Data Collected

Table 1: Aversive Phototaxic Suppression Assay Data and Pass Rates

Effect of GABA Powder on the Pass Rate of *D. melanogaster*																
Trial	Wild-type + Regular Food				Wild-type + GABA				Fragile-X + Regular Food				Fragile-X + GABA			
	# Flies in Light Vial	# Flies in Dark Vial	Total # Flies	**Pass Rate %**	# Flies in Light Vial	# Flies in Dark Vial	Total # Flies	**Pass Rate %**	# Flies in Light Vial	# Flies in Dark Vial	Total # Flies	**Pass Rate %**	# Flies in Light Vial	# Flies in Dark Vial	Total # Flies	**Pass Rate %**
1	2	8	10	**80**	3	7	10	**70**	5	5	10	**50**	3	7	10	**70**
2	3	7	10	**70**	2	8	10	**80**	4	6	10	**60**	2	8	10	**80**
3	3	7	10	**70**	2	8	10	**80**	6	4	10	**40**	3	7	10	**70**
4	2	8	10	**80**	2	8	10	**80**	5	5	10	**50**	3	7	10	**70**
5	3	7	10	**70**	3	7	10	**70**	6	4	10	**40**	4	6	10	**60**
6	2	8	10	**80**	2	8	10	**80**	5	5	10	**50**	2	8	10	**80**
7	3	7	10	**70**	3	7	10	**70**	5	5	10	**50**	4	6	10	**60**
8	3	7	10	**70**	1	9	10	**90**	4	6	10	**60**	3	7	10	**70**
9	3	7	10	**70**	2	8	10	**80**	6	4	10	**40**	2	8	10	**80**
10	2	8	10	**80**	3	7	10	**70**	5	5	10	**50**	3	7	10	**70**

Table 1 represents the pass rates for each of the trails for each group, which were calculated using Equation 1 below.

Results

4.1 Mean and Median Pass Rates

The pass rates for each of the ten trials for the four groups in Table 1 were calculated using Equation 1. For instance, using Equation 1, the pass rate for Trial 1 for the wild-type and regular food group was 80% (refer to Calculation 3). After the pass rates were compiled in Table 2, the mean pass rate was calculated using Equation 2 below. For example, the mean pass rate for

the wild-type and regular food group was 74% (refer to Calculation 4). Additionally, the median pass rates were recorded using the "=MEDIAN()" command, which would be used during the Mann-Whitney U-tests.

Table 2: Mean and Median Pass Rates Per Trial Group

Effect of GABA Powder on the Pass Rate of *D. melanogaster*				
Trial	**Pass Rate (%)**			
	Wild-type + Regular Food	Wild-type + GABA	Fragile-X + Regular Food	Fragile-X + GABA
1	80	70	50	70
2	70	80	60	80
3	70	80	40	70
4	80	80	50	70
5	70	70	40	60
6	80	80	50	80
7	70	70	50	60
8	70	90	60	70
9	70	80	40	80
10	80	70	50	70
Mean	**74**	**77**	**49**	**71**
Median	**70**	**80**	**50**	**70**

Table 2 represents the compiled pass rates for all four groups. The mean and median pass rates are shown, where the mean pass rates are calculated by Equation 2 below.

4.2 Equations Used for Pass Rate and Mean Calculations

Equation 1: Aversive Phototaxic Suppression Assay Pass Rate Calculation

$$Pass\ rate\ =\ \frac{(number\ of\ flies\ in\ dark\ vial)}{(total\ number\ of\ flies)}\cdot 100\%$$

Equation 2: Mean Pass Rate Per Trial Calculation

$$Mean\ =\ \frac{(sum\ of\ pass\ rate\ for\ all\ ten\ trials\ per\ group)}{(number\ of\ trials\ per\ group)}$$

4.3 Sample Calculations

Calculation 3: Pass Rate Calculation for Trial 1 of Wild-type + Regular Food Group (Refer to Equation 1 and Table 1)

$$Pass\ rate = \frac{8}{10} \cdot 100\% = 80\%$$

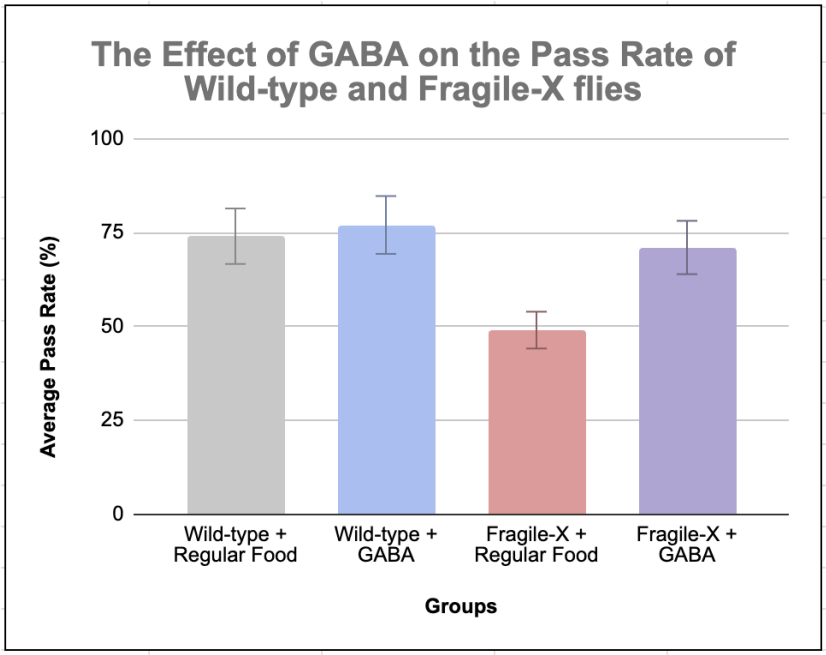
Calculation 4: Mean Pass Rate Calculation for Wild-type + Regular Good Group (Refer to Equation 2 and Table 2)

$$Mean = \frac{80\% + 70\% + 70\% + 80\% + 70\% + 80\% + 70\% + 70\% + 70\% + 80\%}{10} = 74\%$$

4.4 Mean Pass Rate Graph

After the mean and median pass rates were recorded for each of the four groups, the mean pass rates were graphed in Graph 1 below. Based on Graph 1 alone, it can be seen that the mean pass rates for Fragile-X flies that fed on regular food was significantly lower at 49% compared to the rest of the three groups. Additionally, it can be seen that there is a drastic difference between the mean pass rates of Fragile-X flies that fed on regular food compared to the Fragile-X flies that fed on GABA-treated food.

Graph 1: Mean Aversive Phototaxic Suppression Assay Pass Rate



Graph 1 represents the mean pass rates of the four groups, where the x-axis are the four different groups and the y-axis is the average pass rates from Table 2. Additionally, error bars were shown as represented by the black lines at the top of each bar in the bar graph.

4.5 Mann-Whitney U-test Analysis

From the data recorded, three Mann-Whitney tests were conducted using INSTAT, and the p-values and significance for each were recorded in Table 3. The first Mann-Whitney test was conducted to determine if there is a statistically significant difference between the median pass rates of wild-type flies that fed on regular food and wild-type flies that fed on 30 µM GABA-treated food. This Mann-Whitney test was conducted to check if the GABA powder treatment also targets regular wild-type flies in addition to the Fragile-X flies. From the results of the Mann-Whitney test, a p-value of 0.1634 was obtained, which is greater than the significance level of 0.05 (refer to Table 3).

Furthermore, the second Mann-Whitney test was conducted to determine if there is a statistically significant difference between the median pass rates of wild-type flies that fed on

regular food and Fragile-X flies that fed on regular food. This Mann-Whitney test was conducted to check if the symptoms of poor short-term memory consolidation were present in the Fragile-X flies. From the results of the second Mann-Whitney test, a p-value of less than 0.0001 was obtained, which is less than the significance less of 0.05 (refer to Table 3).

Lastly, the third Mann-Whitney test was conducted between Fragile-X flies that fed on regular food and Fragile-X flies that fed on 30 μM of GABA-treated food. This Mann-Whitney test was conducted to determine if the treatment food helped with the learning and short-term memory in Fragile-X flies. The p-value of 0.0001 was obtained from the results of this Mann-Whitney test, which is less than the significance level of 0.05 (refer to Table 3).

Table 3: Mann-Whitney p-value Results

Mann-Whitney U-test Stastical Results		
Groups	**p-value**	**Significance**
Wild-type + Regular Food vs. Wild-type + GABA	0.1634	Not Significant
Wild-type + Regular Food vs. Fragile-X + Regular Food	<0.0001	Significant
Fragile-X + Regular Food vs. Fragile-X + GABA	0.0001	Significant

Table 3 represents the p-values and significance for each of the three Mann-Whitney tests that were conducted. For each of the Mann-Whitney tests, the significance level of 0.05 was used. A p-value for a Mann-Whitney test below 0.05 was considered significant, while a p-value for a Mann-Whitney test above 0.05 was considered not significant.

Discussion

5.1 Analysis of Results and Conclusions

The purpose of this study was to determine if GABA powder would increase the learning and short-term memory of *Drosophila melanogaster* with a Fragile-X mutation. It was

hypothesized that if the amount of GABA powder being used increases, then short-term memory consolidation in *Drosophila melanogaster* with a Fragile-X mutation will also increase.

Based on Table 2, the mean pass rates for the wild-type flies that fed on regular food was 74%, while the mean pass rates for wild-type flies that fed on GABA-treated food was 77%. Even though there was a slight increase in the mean pass rates, the results recorded in Table 3 state that there is no significant difference between the median pass rates of wild-type flies that fed on regular food and wild-type flies that fed on GABA food. Since the p-value was 0.1634, which is greater than the significance level of 0.05, there is sufficient evidence from the sample data to support that there is no statistically significant difference between the median pass rates of wild-type flies that fed on regular food and wild-type flies that fed on GABA-treated food. Therefore, it was concluded that the GABA powder did not affect the learning and short-term memory of the wild-type that fed on GABA.

Furthermore, based on Table 2 and Graph 1, the mean pass rates from wild-type flies that fed on regular food and Fragile-X flies that fed on regular food were significantly different. The mean pass rates for the wild-type flies that fed on regular food were 74%, while the mean pass rates for the Fragile-X flies that fed on regular food were 49%. The difference between the two pass rates portray that the Fragile-X flies had drastically lower pass rates compared to the regular wild-type flies. The results recorded in Table 3 support this as well. Since the p-value of less than 0.0001 is less than the significance level of 0.05, there is sufficient evidence from the sample data to support that there is a statistically significant difference between the median pass rates of wild-type flies that fed on regular food and Fragile-X flies that fed on regular food. Therefore, the learning and short-term memory of Fragile-X flies was significantly worse than regular wild-type flies.

Most importantly, the hypothesis that the learning and short-term memory of Fragile-X flies will increase with the consumption of GABA powder was accepted. Based on Table 2 and Graph 1, where the pass rates of Fragile-X flies that fed on regular food was 49%, and the pass rates of Fragile-X flies that fed on GABA-treated food was 71%. There was a significant increase in the learning and short-term memory based on the pass rates alone obtained from Table 2 and Graph 1 for Fragile-X flies that did feed on GABA-treated food. A similar conclusion could also be drawn from Table 3. Since the p-values of 0.0001 was less than the significance level of 0.05, there is sufficient evidence from the sample data to support that there is a statistically significant difference between the median pass rates of Fragile-X flies that fed on regular food and Fragile-X flies that fed on GABA food. Therefore, the learning and short-term memory of Fragile-X flies that fed on 30 μM GABA food was significantly better than the learning and short-term memory of Fragile-X flies that fed on regular food, and therefore, the hypothesis was accepted.

5.2 Previous Research

Previous research on this topic discusses the use of *Drosophila melanogaster* as a model to study Fragile-X associated disorders. The research article talked about how *Drosophila melanogaster* is the best mammal model to study various different neurodevelopmental disorders, specifically Fragile-X syndrome (Trajković, 2022). The information from this research article correlated with our study as it helps validate the fact that the use of the *Drosophila melanogaster* model best simulates the effect of the treatment and how it affects the symptoms of FXS. As humans and *Drosophila melanogaster* share 75% of their common DNA, it can be inferred that similar effects will be seen in humans that go through the same treatment as the flies.

Another study that was conducted examined the use of GABA and glutamate concentration to predict learning and achievement in human development (Zacharopoulos et al., 2021). This article relates to our study as it discusses the relationship between GABA and various cognitive abilities. In our study, GABA was observed to see how it affected mathematical achievement. In this study it was intended to use GABA to help improve the learning and short-term memory of *Drosophila melanogaster* with a Fragile-X mutation. The data collected from the experiment in the article shows that GABA and glutamate concentrations enhanced the plasticity of a given cognitive function, this data shows that there is a higher likelihood of the GABA treatment working on the FXS flies. This was further supported by our research, as there was a significant difference between Fragile-X flies that fed on regular food and Fragile-X flies that fed on GABA-treated food, as the p-value for the respective Mann-Whitney test was 0.0001. Therefore, the learning and short-term memory of Fragile-X flies that fed on 30 µM GABA food was significantly better than the learning and short-term memory of Fragile-X flies that fed on regular food.

5.3 Errors and Limitations

There are limitations to this experiment, the most evident limitation would be the amount of time we had to complete the experiment. As we only have 100 minutes each block to complete the trials, most of the experimentation was done outside of class to collect accurate data. Another limitation in this study would be the amount of Quinine Hydrochloride used on the *Drosophila melanogaster* during the APS assay. Initially, only 0.1 M Quinine was supposed to be used, however the 0.1 M did not have much of an effect on the *Drosophila melanogaster* during the trials as they all went to the light side of the APS instead of staying in the dark side. Therefore, we changed the amount of Quinine Hydrochloride used to 0.2 M, which seemed to have a

significant effect on the *Drosophila melanogaster*, as they were able to stay in the dark side of the APS after being trained to do so.

This study also had many areas where errors might have occurred throughout the experiment. Some of these instances include the amount of fly food in each vial. Usually only an inch of the vial should be filled with food, but sometimes there was an excessive amount of food in the vial. Another error would be the chunks of food in some of the vials, in the GABA food these chunks would result in some *Drosophila melanogaster* to have more concentration of the GABA than other flies in the vial. The chunks of food were most likely caused by improper mixing while making the fly food. During the process of fly food making, some of the food vials got moldy due to an inadequate amount of propionic acid being mixed in. Another error would be the flies flying away during the process of cold sorting. To cold sort the flies, they had to be anesthetized and then placed on the sorting pad to sort between male and female flies, however, during the sorting some of the flies awoke and flew away. This resulted in more fly vials to be sorted to collect the adequate amount of *Drosophila melanogaster* required for testing.

5.4 Future Work

Experimentation for this study can be continued by looking into the dosage of GABA administered to see if it can be increased to achieve better learning and short-term memory consolidation in *Drosophila melanogaster* affected by FXS. Eventually, experimentation should also be done in a controlled experiment with other organisms, like mice as they have similar processes to humans in terms of aging, similar endocrine systems, and similar responses to infections and diseases, to see if results remain consistent. This research will allow further understanding of the connection between GABA and short-term memory consolidation in those affected with FXS.

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