JOURNAL OF
EMERGING INVESTIGATORS

Article

Drosophila melanogaster: A model to observe behavioral effects of mutated Foxp

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SUMMARY

FOXP syndrome is a neurodevelopmental disorder that can cause movement deficiencies and abnormal behavioral traits. Defects in FOXP production can cause defects in motor skills and daily behaviors in *Drosophila*. In common movement disorders, such as Huntington's disease and Chorea, tetrabenazine has been used as a possible drug, as it is a common drug used to help alleviate such symptoms. Cyrene was used to dissolve the drug, tetrabenazine, rather than DMSO because it is a much safer and greener option. Additionally, Cyrene was used as a possible control group for the *Drosophila* to determine if Cyrene itself had effects on the Drosophila's behavior. We hypothesized that the tetrabenazine treatment would lead to an improvement in the motor skill assays compared to the untreated group. We performed three different assays to assess motor skills: larval crawling, negative geotaxis, and courting behaviors. In the larval crawling assay, the tetrabenazine treated mutant larvae crossed fewer gridlines than the untreated larvae. In the negative geotaxis assay, both the treated mutant and wild-type populations saw an increase in the time to reach the top of the vials, meaning they got slower. In the courtship assay, when treated, the amount of time to exhibit the mating behaviors decreased or stayed the same for both treated mutant and wild-type populations. Overall, we saw that the treatment did work for some assays but not for others. Further research is needed to solidify the significance of the varying results.

INTRODUCTION

FOXP syndrome is a rare disorder that leads to a variety of symptoms. Mutations in *FOXP1* result in intellectual disabilities, motor impairments, and large speech and language defects (1). Whereas *FOXP2* mutations in humans result in the lack of development of speech and language from an early age (1). These specific mutations in the FOXP transcription factors can lead to defects in motor skills and operant self-learning.

FOXP syndromes can be modeled using *Drosophila melanogaster,* as they also have *Foxp* in their genome. The FOXP subfamily has three members that are involved in brain development in humans. These three subfamily members in humans are homologous to *Foxp* in *Drosophila* (1). FOXP transcription factors are needed for operant self-learning (2). In *Drosophila*, mutations in *Foxp* also cause defects in motor skills and behavior (2). Deletions, translocations, or even point mutations in *Foxp* lead to a truncated Foxp protein. This truncation in Foxp in *Drosophila* flies leads to impairments in the capacity for operant self-learning and difficulties in coordinated movements (2). One of the main symptoms of FOXP syndrome is delays in and improper motor skills.

Tetrabenazine is a drug that could possibly be used to improve such defects (3). Tetrabenazine interferes with the transmission of nerve signals and inhibits the release of dopamine. This leads to fewer motor defects and involuntary movements. Tetrabenazine is used to alleviate such symptoms as seen through its use for chorea: a movement disorder caused by Huntington's disease (4). Cyrene was used as an alternative solvent rather than DMSO (dimethyl sulfoxide) as it is known to be a safe and sustainable solvent that doesn't cause harm to the environment (3). One of the prominent symptoms of individuals with FOXP Syndrome is sudden hyperactive movements (5).

We hypothesized that tetrabenazine would improve locomotion and mating behaviors in *Foxp* mutant *Drosophila*. To test this hypothesis, we conducted three assays: larval crawling assay, the negative geotaxis assay, and the courtship assay (6). In the larval crawling assay, the number of grid lines crossed was recorded; in the negative geotaxis assay, the time it took to climb up the vials was recorded; and for the courtship assay, the time it took to exhibit mating behaviors was recorded. The results from the larval crawling assay and the negative geotaxis assay were opposite to what was expected. In the larval crawling assay, the treatment didn't seem to help the larvae cross more lines for both populations. In the negative geotaxis assay, for both populations to which the treatment was administered, the flies took longer to reach the top of the vial, which means they moved more slowly after the treatment. In the courtship assay, the treated mutants exhibited courting behaviors in less time than the untreated, supporting an improvement with the tetrabenazine treatment. Our findings revealed that further studies and larger numbers are required to reach a proper, definite conclusion on whether Tetrabenazine could be used as a possible treatment for FOXP syndrome.

RESULTS

We used wild-type (WT) and mutant (MF) *D. melanogaster* to look at the effect of tetrabenazine treatment on motor function. The mutant flies have a mutated *Foxp*, which decreases the expression of Foxp (2). Flies were treated with no treatment (control), tetrabenazine dissolved in Cyrene, or just Cyrene. Flies were then subjected to three assays: larval crawling, negative geotaxis, and courtship.

https://doi.org/10.59720/24-255

Larval crawling assay

The larval crawling assay was used to observe if the mutant larvae differed from the wild-type larvae in terms of movement and whether the treatment would help the mutant larvae travel across more gridlines when compared to untreated (7). The main trend seen through this assay was that the WT untreated (n = 15) and the WT treated (n = 35) crossed the most lines, 6.2 and 6.28 on average, respectively, among all the larvae in the six populations **(Figure 1).** Among both populations (WT and MF), the larvae treated with Cyrene crossed the least number of lines. This could be attributed to the viscosity (14.5 cp) and thickness of the Cyrene. The populations in which the differences were statistically significant were between the WT untreated (n = 15) and the MF untreated (n = 21) (F-value: 3.78 > critical value: 1.892). However, trends were still observed among the populations as seen above. Even though the WT treated larvae crossed slightly more lines than the WT untreated, this difference was not statistically significant, meaning that the treatment didn't seem to help the WT population. On the other hand, the mutant larvae that were treated with the drug crossed fewer grid lines compared to the untreated **(Figure 1)**.

Negative geotaxis assay

The negative geotaxis assay focused on observing how fast the *Drosophila* of each specific population climbed up to the top of the vial, which helps depict any differences or defects in the motor capabilities between the WT and MF **(Figure 2)** (8). Out of all the populations, the MF untreated (n = 4) reached the top of the vials in the shortest average time of 18.5 seconds **(Figure 2).** However, no official conclusions could be made solely from this data due to the small sample sizes. The WT Cyrene population (n = 5) took the longest to reach the top of the vials with an average time of 62.68 seconds **(Figure 2).** We observed the opposite of what was expected. Before the experiment, we initially expected the mutant *Drosophila* would be slower than the wild type due to the mutated gene. When the *Drosophila* were administered the treatment, the time it took for the *Drosophila* to climb up the vial increased, meaning they became slower. The difference in time to crawl up the vial was significant between the MF untreated (n = 4) and MF treated (n = 4) populations (F = 2.175, critical value = 2.01). Instead of the *Drosophila* climbing up faster when treated with tetrabenazine, the *Drosophila* began to climb up slower (**Figure 3a and b**). For the MF untreated and MF treated for the negative geotaxis assay, the F-value was calculated to be 2.175, which was greater than the critical value of 2.01, meaning that the difference was statistically significant. However, this statistical significance could be attributed to Cyrene rather than the actual drug (11).

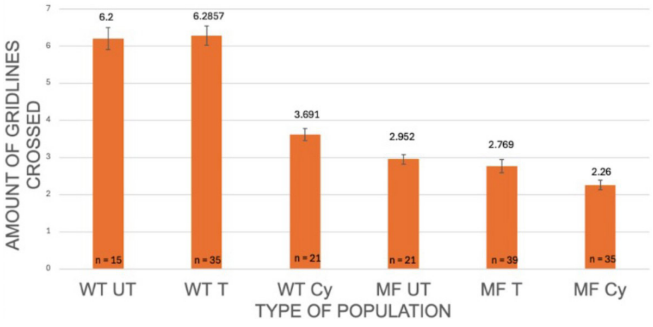

Figure 1: Average grid lines crossed by *Drosophila* larvae. WT and mutant (MF) larvae were treated with tetrabenazine dissolved in Cyrene (T), water (UT), or Cyrene only (Cy). Larvae were then placed on a grid of 0.2 cm squares, and the number of squares crossed was counted. Error bars represent the standard error of the mean. There is statistical significance between the WT UT and the MF UT (F-value: 3.78)

Courtship assay

The courtship assay was used to see the differences that the mutant *Drosophila* would exhibit in their movement. The main mating behaviors looked at during this courtship assay were the orientation of males towards the females, tapping, the curling of the abdomen, and wing song. The mutant *Drosophila* treated population performed the mating behaviors in the shortest average time of 2.8 seconds **(Figure 4a)**. On average, the WT untreated (n = 4) and WT Cyrene *Drosophila* (n = 10) took the longest to perform the specific mating behaviors, with times of, respectively, 5.58 and 4.77 seconds **(Figure 4a).** We observed that males treated with tetrabenazine showed faster orientation behavior for both mutant and wild-type flies, with average times of 1.25 and 1.83 seconds, respectively **(Figure 4b)**.

Tapping behavior is the courtship behavior performed by males when they touch the female's body with their forelegs. The population that exhibited the tapping behavior in the least amount of time was the MT population, with an average time of 1.75 seconds **(Figure 4c)**. The population that exhibited the tapping behavior after the longest amount of time was the WT untreated population with an average time of 7 seconds **(Figure 4c).** When the WT *Drosophila* were given the treatment or Cyrene, the amount of time to exhibit the tapping behavior was observed. This time was noticeably longer than that of the untreated WT populations. For the MF population, compared to the untreated population, the treated mutants exhibited the tapping behavior at a shorter average time of 0.75 seconds **(Figure 4c)**.

We next looked at the curling behavior, which is when males and females curl their abdomens to attract (males) or repel (females) one another. None of the WT untreated population showed the curling behavior. The curling behavior was exhibited in the least amount of time in the MF treated (n = 8) population, with an average time of 2 seconds **(Figure 4d).** However, it took the most time for the curling behavior

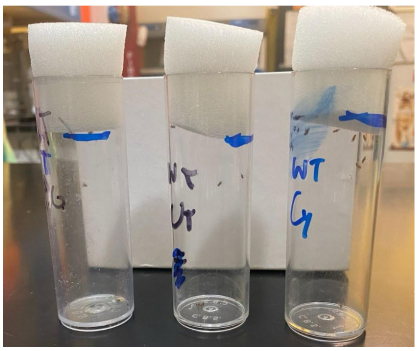

Figure 2: Negative geotaxis assay. This picture was taken right after each vial was knocked down three times. The time taken to reach the bottom of the plug (blue line) was recorded for each fly and each population.

https://doi.org/10.59720/24-255

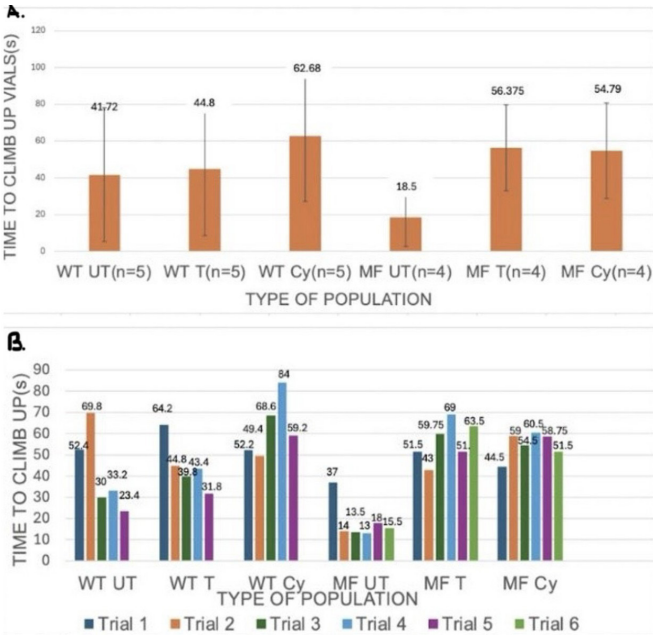

Figure 3: Negative geotaxis assay. MF = Foxp mutant flies, UT = untreated, T = tetrabenazine dissolved in Cyrene, Cy = Cyrene. **A) H**ow the climbing ability of the *Drosophila* from both populations is affected when different treatments are given. There is statistical significance for the differences between MF UT and MF T as seen with the F test. (F-value: 2.175 > critical value: 2.01: statistically significant). **B)** Time taken to climb up the vial for different *Drosophila* populations for each separate trial. The data shown represents the time it took for the *Drosophila* from each treated or untreated population to climb up the vial, separated by trial.

to be observed in the WT Cyrene (n = 10) population, with an average time of 6.7 seconds **(Figure 4d)**. The time to exhibit the curling behavior was observed between the treated and untreated mutants. The treated mutants noticeably performed this behavior faster than the untreated.

Lastly, we looked at wing song behavior, which is when males vibrate one of their wings to produce noises that attract the females. The MF treated population exhibited the wing song behavior in the least amount of time of 2 seconds (**Figure 4e**). The WT Cyrene population exhibited the wing song behavior after the longest amount of time, with an average time of 5.8 seconds **(Figure 4e).** For both treated populations (MF and WT), the time it took to exhibit the wing song behavior decreased compared to the untreated. For the WT untreated and WT treated populations for the wing song, the F-value was calculated to be 49.638, which was greater than the critical value (6.60), meaning the difference between the populations was statistically significant (10).

DISCUSSION

This overall experiment focused on the behavioral differences and effects of a *Foxp* mutant in *Drosophila*. We hypothesized that the behaviors of all the mutant *Drosophila* flies would become faster, or more efficient, when treated with the drug, tetrabenazine, compared to when no treatment was given. These behavioral differences were revealed through the three main assays: larval crawling assay, negative geotaxis assay, and courtship assay.

For both the larval crawling assay and the negative geotaxis assays, the opposite of what was expected occurred. In the larval crawling assay, the treatment didn't seem to help the larvae cross more lines for both populations. In the negative geotaxis assay, for both populations to which the treatment was administered, the flies took longer to reach the top of the vial, which means they moved more slowly after the treatment. On the other hand, the results from the courtship assay aligned with our hypothesis. In the courtship assay, the treated mutants exhibited courting behaviors in less time than the untreated, supporting an improvement with the tetrabenazine treatment.

The number of *Drosophila*/data was limited due to the drowning of *Drosophila.* This could be attributed to the viscosity and the stickiness of Cyrene, which may have caused the *Drosophila* to stick to the media and drown. A contributing reason could have been the order in which the drug was mixed. In a previous attempt at an experiment, when treating *Drosophila*, the drug dissolved in the Cyrene was added to the water initially and then poured onto the media. In this experiment, to observe which order would work the best, the dissolved drug in the Cyrene was added straight onto the media after the water was poured. One new idea that future researchers could use is instead of placing the *Drosophila* straight into media, a form of netting with small pores could be placed on top of the media. These small pores would prevent the *Drosophila* from completely falling through the netting and getting drowned in the media. These same pores would allow the *Drosophila* a pathway to feed and ingest their food. This idea would be feasible and easy to implement, and it could work very well. This new idea addresses the main issues in the experiment: the limited *Drosophila* in the assays due to drowning.

MATERIALS AND METHODS

Growing *Drosophila*

Wild-type *Drosophila* (Carolina Biologics: Item #172100) and the mutant *Foxp Drosophila* (Bloomington *Drosophila* Stock Center: Indiana University: Item #26774) were reared to a total of 1,000 flies for each population. The *Drosophila* for both populations were transferred eight different times, with a gap time of two weeks between each time, giving time for the *Drosophila* to develop. To transfer the *Drosophila*, new vials (Carolina Biologics (Item #: 173080) were prepared. Similar to the assays, the vials were provisioned with 17 g of media (Carolina Biologics: Item #73210), 9 mL of water, and 5-15 kernels of yeast. There was also netting (Carolina Biologics: Item# 173090) for the *Drosophila* to climb up on. On the eighth time, a third of the WT population was treated with tetrabenazine (Sigma-Aldrich: Item #T2952) dissolved in Cyrene (Sigma-Aldrich: Item #807796), another third was treated with Cyrene only, and the last third was left untreated (11). The same was performed with the MF population.

Preparing drug

For the treated *Drosophila*, 10 mg of tetrabenazine was dissolved in 1 mL of Cyrene. Since the appropriate amount of tetrabenazine to use was unknown, the total amount available was used. Ideally, a dose-response trial would have been conducted, but time and resources were limited. 300 µL was added to the media of the WT and mutant populations. This was used for both the courtship and negative geotaxis assays.

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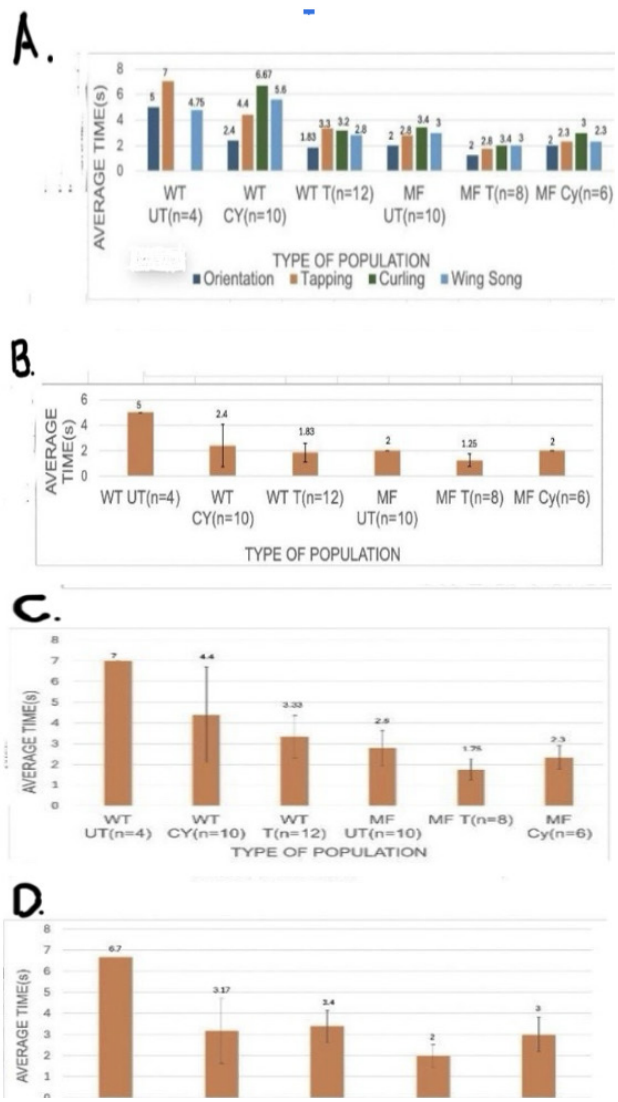

Figure 4: Courtship assay. A) Time to exhibit all the separate mating behaviors for each population. The data above represent the time it took for the *Drosophila* from each population to exhibit specific mating behaviors. The different mating behaviors were associated with the colors as specified by the legend. One male and one female were taken from each population and put in a tissue culture flask, placed under a microscope, and mating behaviors were observed. **B)** Orientation behavior for each population. The data above represent the time it took for the *Drosophila* from each population to exhibit orientation behavior. The orientation behavior was defined as the male moving towards the female. **C)** Tapping behavior for each population. The data above represent the time it took for the *Drosophila* from each population to exhibit the tapping behavior. The tapping behavior was defined as the male tapping the female. **D) C**urling behavior for each population. The data above represents the time it took for the *Drosophila* from each population to exhibit the curling behavior. The curling behavior is defined when the male curls its abdomen under itself. **E)** Wing song behavior for each population. The wing song behavior is defined when the male flaps/moves one wing back and forth. There was statistical significance for the wing song behavior, specifically between the WT UT and WT T using the F-test. (F-value: 49.638 > critical value: 6.60 (11): statistically significant).

For the larval crawling assay, 100 µL of the drug Cyrene solution was used. For the courtship and negative geotaxis assays, 300 µL of tetrabenazine was added to the media for both the WT and mutant treatment groups, with 300 µL of Cyrene added to the media of the control groups. Around 70-100 *Drosophila* were added to each vial to be treated. There were six main populations: WT and mutant flies treated with no treatment, Cyrene only, or tetrabenazine dissolved in Cyrene. The Cyrene only populations were to show that the solvent did not affect the *Drosophila*.

Courtship assay

Once all the *Drosophila* were treated, the *Drosophila* were separated based on their sex. The *Drosophila* were placed in the freezer for two minutes to immobilize them, and then on an index card for easy observation while separating by sex. The segregated vials were placed in an incubator at 25°C for five days. After the five days of incubation, one male and one female from each population were added to a tissue culture flask (culture area: 175 cm^2) (**Figure 5**). This flask was then observed to see when the specific mating behavior between the male and female occurred.

Rapid iterative negative geotaxis assay

After the *Drosophila* were treated for the negative geotaxis assay, the *Drosophila* present for each population were added into a separate vial to perform the assay. The WT populations consisted of 5 *Drosophila* each, and the trial was performed 5 times with the same 5 *Drosophila* to yield 25 data points. The mutant fly populations had 4 *Drosophila* each, and the trial was performed 6 times with the same 4 *Drosophila* to yield 24 data points. To conduct the assay, the vial to which the *Drosophila* for the assay were transferred was knocked down three times, and the time it took for each *Drosophila* to reach the top of the vial, or the “plug”, was noted.

Larval crawling assay

The larvae used in this assay were grown in 18 total vials (4” H x 1-1/4” in diameter, three vials for each population). Around 20 *Drosophila* were transferred to each vial, and a week later, larvae were seen. To allow the larvae to float up in their respective vials, 40-50 mL of 20% sucrose was added to each vial. These larvae were removed from the top of each vial with a transfer pipette and placed on a gauze wrapped over a 50 mL beaker to drain out the liquid and keep the larvae (**Figure 6a**). Over the gauze, the larvae were washed and rinsed with deionized water. This rinsing was done using a squirt bottle of deionized H2O, which was closely placed over the larvae. The larvae were either untreated, Cyrene only, or treated with tetrabenazine dissolved in Cyrene. The larvae were placed in a 10 mL beaker with the treatments/solutions so that they could feed (**Figure 6b**). For the untreated larvae, 5% sucrose was combined with water, and for the Cyrene larvae, 5% sucrose was combined with Cyrene. After the larvae were fed, they were placed in a petri dish with 2% agarose gel on top of a graph paper, and the number of grid lines crossed was observed (**Figure 6c)**. From the assay, the WT untreated population had 15 larvae that were observed, the MF untreated population had 21 larvae, the WT treated population had 35 larvae, the MF treated population had 39 larvae, the WT Cyrene population had 21 larvae, and the MF Cyrene population had 35 larvae.

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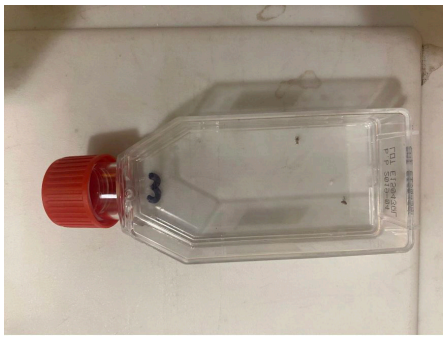

Figure 5: Courtship assay. One male and one female were added to the tissue culture flask. Using a stopwatch, the time it took for the specific mating behaviors to be exhibited by the flies was recorded.

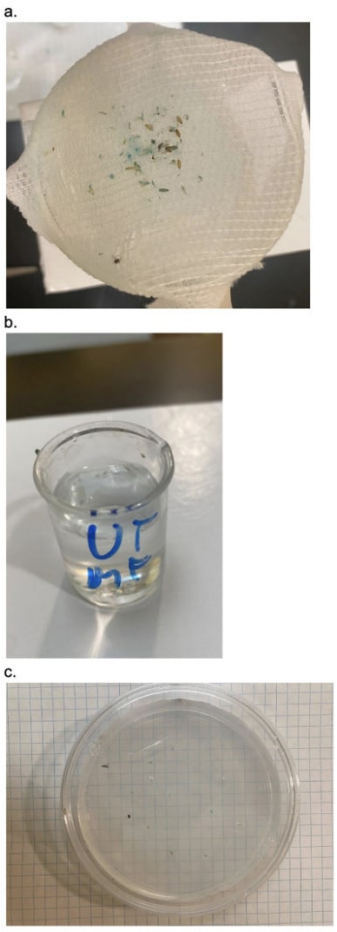

Figure 6: Larval crawling assay. A) Obtaining/rinsing the larvae. In this step, the larvae were taken from the respective vials and placed on a gauze on top of a 50 mL beaker. This gauze was used to keep the larvae from falling through and draining out the liquid. On top of this gauze, the larvae were rinsed with deionized H_2O. Once rinsed, the larvae were transferred to their specific treatments. **B) T**ransferring larvae to respective beakers with specific treatments to feed. The larvae were transferred from the gauze into a small 10 mL beaker filled with 5% sucrose + drug or just 5% sucrose if untreated, as seen in the image. The larvae remained in these beakers for 15 minutes so they could feed and ingest the treatment. **C)** Larvae placed in a petri dish on top of graph paper. After the larvae were placed on the agarose gel inside a petri dish, it was observed how many gridlines on the graph paper were crossed by each larva.

ACKNOWLEDGMENTS

We would like to thank Mrs. Schmahl and Schmahl Science Workshops for providing us with the resources to make this project possible. This paper is dedicated to the first author's cousin, who has dealt with a similar neurobiological disease all her life, known as Rett Syndrome.

Received: August 15, 2024
Accepted: March 10, 2025
Published: January 13, 2026

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