

The effect of the human *MeCP2* gene on *Drosophila melanogaster* behavior and p53 inhibition as a model for Rett Syndrome

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SUMMARY

The purpose of this study was to explore the effect of introducing the human methyl CPG binding protein 2 gene (*MeCP2*) into the *Drosophila melanogaster* genome, examine how it would affect protein levels of p53, and test how inhibiting p53 with pifithrin-alpha would affect behavioral and physical patterns in fruit flies. One goal was to observe if the symptoms of Rett Syndrome, a neurodegenerative disease in humans, are reflected in *Drosophila melanogaster*. This was achieved by differentiating the behavior and physical aspects of wild-type flies from flies expressing the full-length *MeCP2* gene and the mutated *MeCP2* gene (R106W). Differences in eye structure, climbing (the use of locomotor skills), geotaxis, as well as differences in the flies' abilities to fly were analyzed. In addition, after these series of tests, the *MeCP2* protein and the p53 protein, endogenous to *Drosophila*, were extracted and their levels were compared in each of the populations of flies. Then, after the *Drosophila* consumed pifithrin-alpha hydrobromide, their behavior, physical appearance, and protein levels were again monitored. After conducting these experiments, the effects of full-length and mutant *MeCP2* on *Drosophila melanogaster's* psychomotor skills were analyzed, along with their effects on how the treatment of pifithrin-alpha affected the level of *MeCP2* and p53 in the various populations of flies. In conclusion, some of the Rett Syndrome symptoms were recapitulated in *Drosophila*, and a subset of those were partially ameliorated by the introduction of pifithrin-alpha.

INTRODUCTION

Rett Syndrome, a neurodegenerative disease, is associated with a loss-of-function mutation on the *MeCP2* (methyl-CpG-binding protein 2) gene in humans. Rett syndrome patients are almost exclusively females because unfortunately males with this mutation do not survive past their infancy (1). This specific syndrome leads to autism and impairs learning abilities such as basic coordination and cognitive skills. Most of those diagnosed with Rett Syndrome

are unable to walk, speak, help themselves, or even recognize their loved ones (1).

MeCP2 protein is found in high levels in the brain, particularly neurons, or nerve cells (1). *MeCP2* is responsible for the process of alternative splicing of messenger RNA, which contains the instructions to make protein; *MeCP2* also is responsible for maintaining synapses, or connections, between neurons (1). If synapses are broken, then neurons cannot communicate and cannot carry out functions necessary for life. Mutations in the *MeCP2* gene cause a variety of disorders and syndromes. Most relevant to this study, insertions and deletions in the amino acid sequence as well as changes in a single base pairs are mutations that are responsible for changing the structure of the protein or decreasing the amount of *MeCP2* protein produced, leading to Rett Syndrome (1).

p53 is a protein that is very closely associated with neurodegeneration and apoptosis (2). The activation of p53 induces malfunction in dendritic branching in neurons, which is also a predominant phenotype of Rett Syndrome. A decreased level of *MeCP2* protein in the brain, as a result of a change in structure of the protein, has been shown to induce p53 (3), which, as previously mentioned, leads to neuronal damage (2). It has also been proven that inhibiting the induction of p53 restores the reduced *MeCP2* protein levels associated with Rett Syndrome, therefore improving neuronal morphology. In this study, we explore if inhibiting the p53 protein and increasing expression of the *MeCP2* protein can possibly reverse the effects of Rett Syndrome.

We chose pifithrin-alpha hydrobromide as the main p53 inhibitor, or *MeCP2* stimulator, of the reversed pathway. Pifithrin-alpha is a small molecule known to be a potent inhibitor of the p53 protein (3). Using this molecule, we asked whether p53 protein production would be interrupted, whether that would increase the production of *MeCP2* protein and whether it would reverse Rett Syndrome behavior.

We used *Drosophila melanogaster* in this study because these fruit flies have a 60% sequence homology to the human genome (7). Although *Drosophila* do not have an ortholog of the full *MeCP2* protein, they do have a gene that encodes the methyl-CpG-binding domain (4). Since Rett Syndrome impairs a variety of cognitive functions in humans, one way

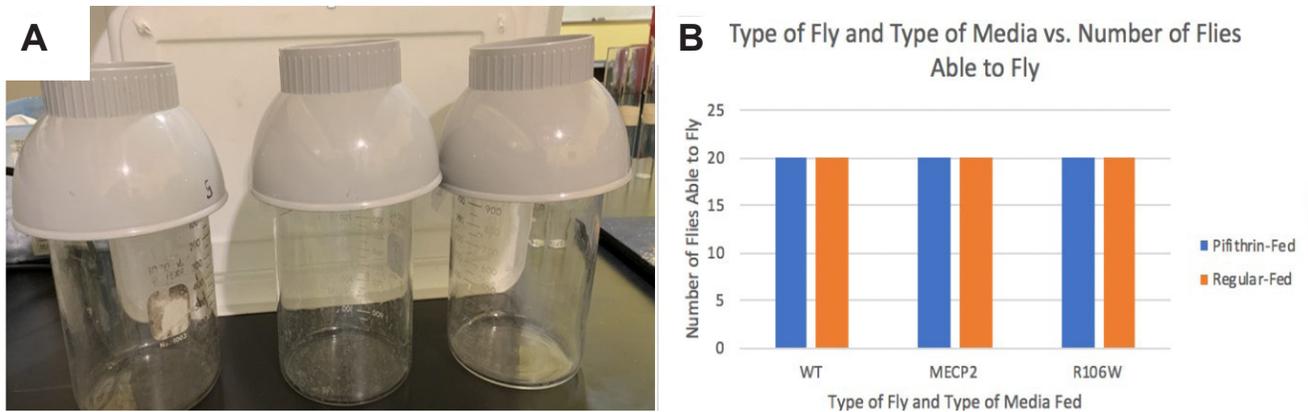


Figure 1. Ability to fly apparatus and results. **A)** To see if the variously treated flies still retained their ability to fly, three beakers, each covered with bowls, were used as an apparatus. **B)** The number of flies from each group that were able to fly is displayed; the orange bars show the normally fed flies and the blue bars show the pifithrin-fed flies. As seen, there are no error bars for standard deviation since the data for the groups is the same.

to test those cognitive functions in *Drosophila* is by studying their physical behavior. The relationship between cognitive and physical capabilities is established through the concept of psychomotor skills in *Drosophila* (5). We measured differences in psychomotor skills and eye appearance between wild-type flies, flies expressing full-length MeCP2 (FL MeCP2) and flies expressing the mutated MeCP2 (R106W). This was done to detect if the symptoms of Rett Syndrome in humans would be reflected in the flies. The flies were not sexed; this was on the basis that Rett Syndrome is sex-linked in humans and since *Drosophila* do not possess the *MeCP2* gene, there would be no reason to believe it was sex-linked in *Drosophila*. To confirm this, we counted the number of male and female flies in each of the populations and observed them to be equal, before conducting the experiments.

In this project, we compared the physical behavior as

well as eye morphology of *Drosophila melanogaster* that consumed regular media to those that consumed pifithrin-alpha infused media. Along with these differences, we measured differences in protein levels of p53 and MeCP2 among the different populations through an ELISA.

RESULTS

Since Rett Syndrome impairs the ability to walk in a human, we tested whether the flying ability in *Drosophila* would be impaired, especially because the *MeCP2* gene is not endogenous to the flies (**Fig. 1A**). Both groups of flies fed with pifithrin-alpha and those not fed the drug for all three of the populations were able to fly (**Fig. 1B**). Throughout the duration of the experiment, we noticed that the wings of the FL MeCP2 flies were positioned higher than that of the other populations, as if electrocuted. They also clearly were moving

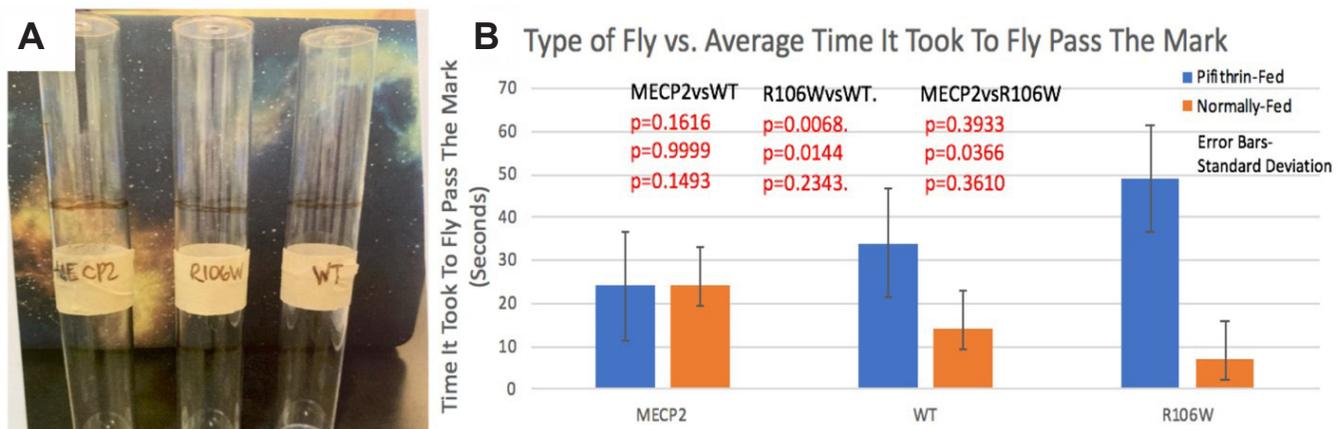


Figure 2. Geotaxis apparatus and results. **A)** Three of the geotaxis apparatuses are shown. Each one is labeled with its respective group of flies. Each of the apparatuses comprise one upside down vial on top of a right side up vial, taped where the openings meet. The brown mark is 8 cm from the table and is representative of the mark where the flies aimed to pass. **B)** The results of the geotaxis behavioral test are depicted in a bar graph. This data was collected from flies that were fed regular media (orange) and pifithrin media (blue). Ten flies were placed in each of the three apparatuses. The test was repeated for three trials for each apparatus. The mean of the trials is what is displayed on the bar graphs. The same applies for the pifithrin-fed flies. The error bars for standard deviation are shown in black while the resulting *p*-values for the three ANOVAs are shown in red.

at a faster speed than the mutant and the wildtype fly. This was observed subjectively; for example, the FL MeCP2 flies were moving at a pace such that they flew back and forth in the jar three times in the same time that the other flies flew back and forth one time. This is consistent with the hypothesis that decreased levels of MeCP2 protein in the mutant and wildtype fly lead to a slower speed due to a delayed psychomotor ability, similar to how a decreased level of MeCP2 protein in humans impairs walking ability.

To test if the flies still retained their innate ability of negative geotaxis, we performed the geotaxis test (Fig. 2A). The pifithrin-fed flies moved slower overall than the flies fed regular food; the pifithrin-fed mutant fly took the longest to regain its negative geotaxis ability (Fig. 2B). This is inconsistent with the hypothesis that pifithrin would help the fly enhance one of its psychomotor skills. The three *p*-values from the ANOVAs that were run on this data set are less than 0.05 (5%), which means the null hypothesis can be rejected for those groups: the difference between the normally fed and pifithrin fed groups, the difference between the mutant and wildtype groups, and the difference between the mutant and FL MeCP2 groups are all significant.

To test the flies' gap-climbing methods and abilities, we observed the technique that the fly utilized to climb across a U-shaped gap, through a camera (Fig. 3A). The pifithrin-fed flies used both their front and hind legs in a variety of positions, while the normally fed flies simply used their legs in an upright, simple position while crossing the ledge (Fig. 3 B-D). While each pifithrin-fly was traversing the gap individually, their bodies were more spread out and facing different directions, while the legs can be seen to be in various positions, not just upright; this was seen among all the flies (Fig. 3E-G). This difference in the way the flies crossed the ledge shows how the pifithrin-fed flies resorted to less traditional, yet more creative ways to gap-climb than the regular-fed flies.

To see if a more physical aspect of the fly differed among the variously affected flies, we assessed eye structure. The pifithrin-fed flies (Fig. 4D-F) had a darker eye color as compared to the normal-fed flies (Fig. 4A-C). The eye structure of the pifithrin-fed FL MeCP2 fly was glossier and smoother (Fig. 4E) instead of having a defined shape like the other flies, in which the hexagonal, pixel-like pattern of the eye is seen.

To compare the varying MeCP2 and p53 levels in each population, we conducted an ELISA (Fig. 5). The data shows that the MeCP2 protein expression starts out at a very low level in the normally fed R106W fly and spikes up with the application of pifithrin, consistent with the hypothesis. The MeCP2 level for the pifithrin-fed mutant fly was the highest, as hypothesized (Fig. 5B). We cannot conclude anything about the relationship between p53 and MeCP2 since the p53 ELISA failed (Fig. 5A). Since the MeCP2 ELISA did work, three sets of two-way ANOVAs without replication (comparing mutant to wild type, FL MeCP2 to wild type, and FL MeCP2 to mutant, all with respect to pifithrin and normal media) were

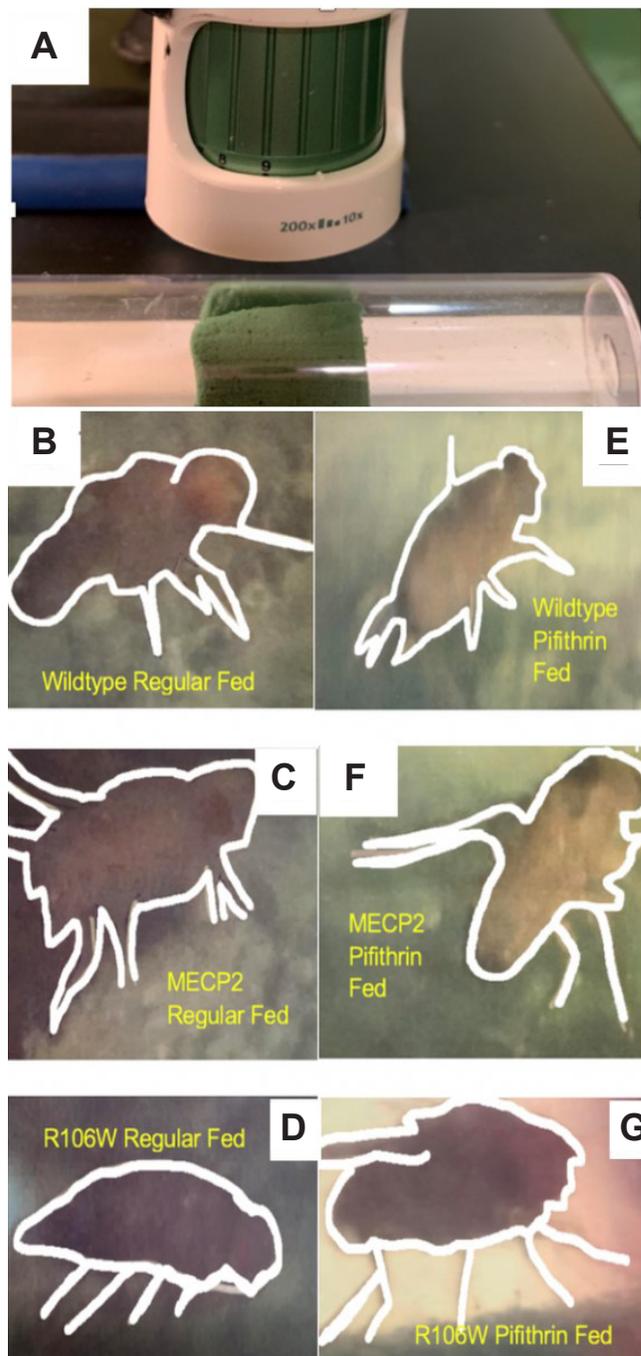


Figure 3. Gap-climbing apparatus and results. A) The apparatus used for the gap-climbing behavioral test is shown. The high-speed camera is held in place with a ring clamp and ring stand. It is situated on top of a vial lying sideways inserted with a piece of green foam with a U-shape cut-out (the gap the flies had to climb to reach the other side of the green foam). (B-G) Pictures taken of the regular-fed and pifithrin-fed flies using a high-speed camera are shown. B) A wild-type fly fed with regular media is shown. C) A MeCP2 fly fed with regular media is shown. D) A R106W fly fed with regular media is shown. (E-G) there are wild-type, FL MeCP2, and R106W flies, respectively, fed with pifithrin, instead of regular media. Outlines of the flies' head and legs were digitally drawn to identify the flies' methods and their choice of back or front legs to climb the gap.

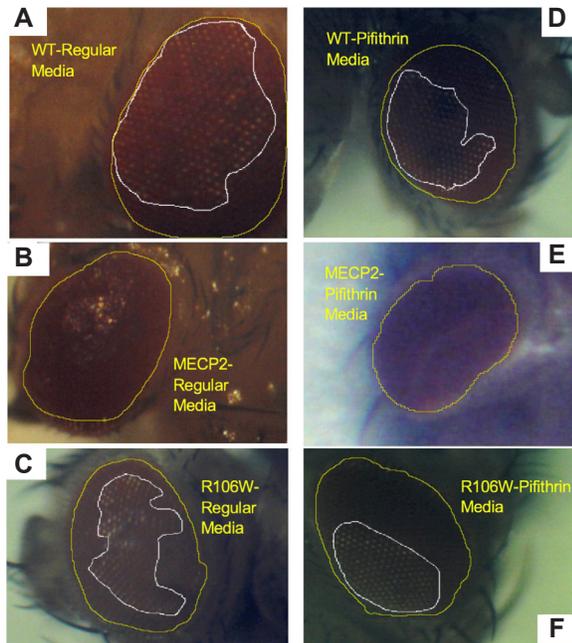


Figure 4. Eye structure results. In this figure, pictures of a zoomed-in view through a microscope of the flies' eye structures are shown. (A-C) Once again, in the first column, wild-type, FL MeCP2, and R106W flies respectively are pictured; these were fed with regular media. (D-F) Wildtype, FL MeCP2, and R106W flies fed with pifithrin are depicted. The flies' eyes were digitally outlined in white to show the actual eyeball and then outlined in yellow to accentuate the area where the color and pattern of the eyes stands out.

run on the data. The resulting *p*-values for sample, column, and interaction are displayed in red. In this case, none of the *p*-values were less than 0.05 (5%), which doesn't allow the null hypothesis to be rejected; there is no statistically significant difference between the mutant, wildtype, and FL MeCP2 (normally or pifithrin fed). However, within the graph of the MeCP2 protein amounts, the amounts are visually distinct for each group.

For the MeCP2 ELISA, the MeCP2 levels are consistent with the hypothesis that MeCP2 levels would increase with the insertion of pifithrin-alpha. The protein levels seem to have increased when the flies were fed with pifithrin-alpha, according to what the assay displays. It can be safely concluded that pifithrin-alpha is affecting the level of MeCP2 inversely. We could not identify p53 as the protein affecting MeCP2, since the p53 ELISA did not seem to work. Together, the protein and physical tests respectively confirmed the hypothesized claim that pifithrin-alpha would increase the amount of MeCP2 protein and disproved the prediction that *all* Rett Syndrome symptoms are reflected and reversed when *Drosophila* consume pifithrin, since the flies displayed behaviors that deviated from the expected ones in some of the physical tests.

DISCUSSION

From the data, we safely concluded that pifithrin-alpha is, through some way, affecting the level of MeCP2. However,

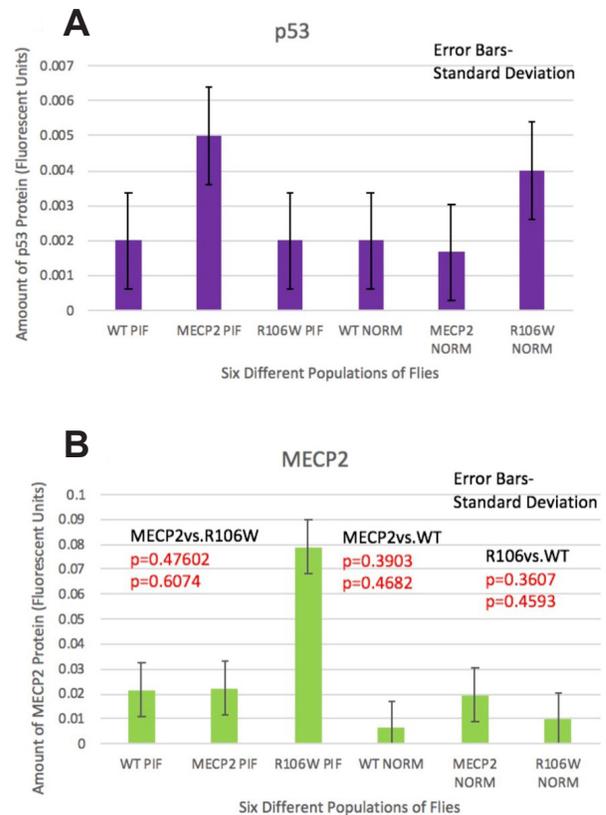


Figure 5. ELISA results. This figure displays bar graphs of the data gathered from the ELISA. **A)** The purple bar graph shows the amount of p53 protein, in fluorescent units, extracted from each of the six different populations of flies. **B)** The green bar graph represents the amount of MeCP2 protein, also in fluorescent units, extracted from each of the six different populations of flies. The error bars for standard deviation are displayed in black. Three sets of two-way ANOVAs with replication (comparing wild type to mutant, wild type to FL MeCP2, and FL MeCP2 to mutant, all with respect to pifithrin and normal media) were used to analyze this data and the resulting *p*-values for sample, column, and interaction are displayed in red.

the p53 ELISA did not work and the reason behind it was explored. Because the range of the fluorescent units is so much larger for MeCP2 than that of p53 (Fig. 5), and the p53 signals were lower than the amounts expected for the extracted p53 protein, we propose that the p53 ELISA did not work. The standard curve for the p53 ELISA also did not work as the R^2 value was not close to 1. The p53 ELISA did not function appropriately; in other words, the p53 antibody did not bind with the p53 protein and the amounts of protein displayed on the bar graph therefore cannot be used to draw any conclusions. Therefore, since the results were inconclusive, p53 may or may not be the source through which the pifithrin is affecting the MeCP2. Optimizing the p53 antibodies, testing the assay with different p53 antibodies, and testing other proteins on that pathway to see if they might be responsible for the relationship between pifithrin and MeCP2 are possible projects that could be pursued in the future. As for the MeCP2 ELISA, the ANOVAs proved that there was

no difference between the groups compared. However, when the assay is analyzed by eye, there is clearly a difference between each of the groups. To avoid this discrepancy with the ANOVA in the future, a good addition to the experiment would be to gather multiple replicates for each group in the assay; this may allow for the ANOVA to result in a rejection of the null hypothesis. The reason for the lack of difference could possibly be because of the noisy signal of the ELISA.

The restoration of MeCP2 protein levels repairs the communication between neurons allowing more synapses to be made and therefore prevents dysfunctions of motor skills in a human (4). However, the increase of MeCP2 levels observed in the various flies has proven to enhance and deteriorate certain skills such as their response to negative geotaxis. The statistical analysis for this test proved that there is a difference between the mutant and FL MeCP2 fly, when fed pifithrin, and that the difference is not by chance or sampling error. This difference supports the hypothesis that the mutated fly would be slower than the FL MeCP2 fly when fed with regular media. When we observed the data holistically, the insertion of pifithrin worsened the flies' responses to negative geotaxis, instead of improving it (inclusive of the statistically significant difference between the two groups). In addition, the flies' abilities to fly were also retained, even in the normally fed mutant fly, which we expected to have an impaired flying ability. Meanwhile, the gap-climbing and eye structure test results display binary evidence of the augmentation of certain other motor skills of the *Drosophila* treated with the pifithrin. While the success of these two tests confirm that *Drosophila* may be the ideal species to explore the symptoms of Rett Syndrome through a physical lens, the failure of the other two physical tests point out that different, more specific physiological tests are required to explore the behavior at a more intricate level.

Drosophila do not have an ortholog to MeCP2 (2), so why the pifithrin seems to increase the expression of MeCP2 in the wildtype flies remains unclear. The flies might have gotten mixed up and/or crossed when breeding. It seems that if the amount of protein as indicated by the ELISA assay is correct, there is some kind of low-level cross reactivity with the MeCP2 antibody and the wild-type *Drosophila*. The data shows that the p53 antibody may not have bound to the *Drosophila* p53 homolog, as the ELISA assay for the p53 did not work. Future projects can possibly test various concentrations and dilutions of different p53 antibodies for the one that will allow for it to bind to the protein.

Overall, the results woven together along with the statistical analysis demonstrate that the reverse pathway model was effective and accurate with regards to MeCP2 and pifithrin. If the p53 ELISA had worked, the model would be even more compelling. There is no confirmation that pifithrin-alpha is an inhibitor of the p53 protein, but there is confirmation that the pifithrin-alpha is a stimulator of MeCP2 expression. Testing the reversed pathway did seem to work, as inserting pifithrin did somehow cause the level of MeCP2

to increase, but by what means and if it specifically improved the neural activity by restoring synapses in the flies remains unclear. As mentioned previously, conducting more specific physiological tests on the flies might provide more insight into neural activity. The MeCP2 protein is found at reduced levels in humans diagnosed with Rett Syndrome, so this project is a contribution to the ways that MeCP2 level as well as cognitive abilities can be restored.

MATERIALS AND METHODS

Physical Study

The flies used for these tests belong to three different populations: the wildtype *Drosophila* (control group), the *Drosophila* inserted with the full length MeCP2 gene (experimental group), and the *Drosophila* inserted with the mutated MeCP2 (R106W) gene (experimental group). These strains were provided by Dr. Botas, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas. These three populations were fed with regular Formula 4-24® Instant *Drosophila* Medium, Blue. There were two vials for each of the three populations. Each vial contained one tablespoon of the media, nine mL of water, several yeast kernels and ten flies, which means there were 20 flies for each population in total. The flies were age-synchronized in their respective vials; the adults were shifted to new vials after consuming media and breeding for one week. The larvae that were produced in that week were given 12 days to grow (6) before they were tested. The experiments were performed in the span of two hours.

Test 1 (Ability to Fly)

20 flies from each population were placed into separate beakers (Fig. 1A) and covered as soon as they were let in. It was observed to see if they took flight to move around the vast space inside the beaker. Flying is a basic locomotor skill for flies, just like how walking is one for humans. Rett Syndrome impairs the ability to walk, which is why this behavioral test is suitable.

Test 2 (Geotaxis)

One empty vial was labeled with a mark around the circumference of the vial 8 cm from the bottom. 10 flies from each population were added to the bottom vial and the second vial (upside down) was quickly taped to the first one, so that no flies escaped (Fig. 2A). The apparatus was quickly turned upside down and back to disrupt the flies' center of gravity and then, the flies were tapped down to the bottom. The number of flies that flew above the six cm mark after ten seconds with a timer were measured (Fig. 2B). This whole experiment was repeated three times for each population. The purpose of flipping the apparatus over and back is to test the flies' innate ability to ascend the vial as an escape response to being drastically shifted in an environment.

Test 3 (Gap-Climbing)

A piece of foam cut out to shape a U was obtained and placed inside a vial (**Fig. 3A**) (3). A fly was directed to the top-left ledge of the foam and photos were taken using a high-speed video camera (DLite USB Microscope, 5 MegaPixels STR5MP-1213T) as the fly climbed from the left side to the right side of the structure (**Fig. 3B**). Whether the fly used its front or hind legs (or both) to climb across the structure was noted.

Test 4 (Eye Structure)

The eye structure of four flies from each population was individually observed with an OMAX 10X-20X-30X-60X Binocular Student Stereo Microscope with USB Camera. Eye structure is an effective phenotype that is used to identify genetic differences in models for neurological diseases (4). Differences in appearance of the texture of flies' eyes were assessed. Different patterns or different textures of the eye are indicative of a difference in phenotype due to the level of MeCP2 protein (2).

Insertion of Pifithrin-Alpha

Five mg of pifithrin-alpha hydrobromide (Sigma Aldrich) was dissolved in DMSO and water to a concentration of 20 µg/mL. Nine milliliters of this solution were seeded into tubes containing a level scoop of 1 tablespoon of Carolina fly media. For 12 days, the flies consumed the pifithrin-laced media. All four of the behavioral tests were conducted again, using the flies that had consumed the pifithrin for one week.

Protein Study

Protein Extraction

The flies were placed in the freezer for 10 minutes. Twenty flies from each group were used: 20 wild-type normally fed flies, 20 FL MeCP2 normally fed flies, 20 R106W normally fed flies, 20 wild-type pifithrin-fed flies, 20 FL MeCP2 pifithrin-fed flies, and 20 R106W pifithrin-fed flies. Prior to protein extraction using the Protein Purification Kit (Invent Biotechnologies, Inc. 8) the buffers were chilled in the collection tube on ice. Twenty frozen flies were placed in the filter and 200 µL of Buffer A was added to the filter. The frozen flies were ground with a plastic rod 50-60 times with twisting motions. 200 µL of Buffer B was added to the filter and the flies were ground for another 30-60 times. The filter was capped and centrifuged in a microcentrifuge at top speed for one minute. The flow through contained total protein extract. The clear supernatant was transferred to a fresh tube. This was repeated for each of the six groups of flies.

ELISA

The extracted protein from the flies as well as the respective purified MeCP2 or p53 protein (Aviva Systems Biology) were diluted to a final concentration of 20 µg/mL in PBS. 50 µL of the diluted protein solution from the flies was plated in a PVC microtiter plate. Standard curves of the purified MeCP2

protein and the purified p53 protein were made by preparing six 1:5 serial dilutions, with 50 µL in each well. The purpose of including a standard curve on the plate was to extrapolate the yields of the extracted proteins. The plate was covered with an adhesive plastic and incubated at 4°C for three days. The coating solution was removed, and the plate was washed twice by filling the wells with 200 µL PBS. The solutions or washes were removed by flicking the plate over a waste container. The remaining drops were removed by patting the plate on a paper towel. The remaining protein-binding sites in the coated wells were blocked by adding 200 µL of blocking buffer, 5% nonfat dry milk in PBS, per well. The plate was covered with an adhesive plastic and incubated overnight at 4°C. The plate was washed twice with PBS. 100 µL of the respective anti-MeCP2 antibody (Sino Biological) or anti-p53 antibody (Antibodies-Online) was added, diluted at a 1:1000 concentration in the blocking buffer. The plate was covered with an adhesive plastic and incubated over two days at 4°C. The plate was washed two times with PBS. 100 µL of the Anti-Rabbit IgG, HRP-linked Antibody (Cell Signaling Technology) diluted at 1:1000 in the blocking buffer was dispensed into each well. The plate was covered with an adhesive plastic and incubated overnight at 4°C. The plate was washed four times with PBS. 100 µL of TMB Substrate (BioLegend) was added to the wells. It was incubated for 30 minutes and then 50 µL of TMB Stop Solution (BioLegend) was pipetted into the wells. The absorbance (optical density) at 540 nm of each well was read with a microplate reader (9).

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