

The Effect of IMS on Cognitive and Behavioral Abilities of Mutant *Drosophila*, Causing Neuroplasticity

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ABSTRACT

The goal of this experiment was to determine the effect of the Intermediate Metabolic Switching (IMS) diet on the short and long-term memory of mutant *Drosophila* due to neuroplasticity. Neuroplasticity creates new neural networks and can be measured in ketone count. The IMS diet includes two components: fasting and a low-carbohydrate, high-fat diet. The Phenol-Sulfuric Acid Test and the Hanus Iodine Solution Test were both used to quantify two ratios, a 2:2 ratio carbs to fats, and the 4:1 ratio fats to carbs. It was hypothesized that if fruit flies are fed the IMS diet, leading to plasticity, then their overall memory and behavioral health will increase because of the amount of ketones released, increasing memory formation. Mutant *Drosophila* tested, which lacked the Amyloid-Precursor Protein-like gene (APPL), exhibited similarities to early-stage Alzheimer's patients. Assays include the Aversive Phototaxic Suppression Assay to measure short term memory, the Aversive Pavlovian Olfactory Assay to measure long-term memory, the *Drosophila* Activity Monitor software to track movement of *Drosophila*, the *Drosophila* Stress Odorant (dSO) Assay to measure mood changes, and the β -Hydroxybutyrate Ketone Quantification assay to measure ketone levels and ensure the IMS diet worked. Results were all conclusive, establishing that the flies in the 4:1 diet were more sensitive but retain short and long term memory, showing IMS could be used as a preventative mechanism for Alzheimer's.

Introduction

Synaptic plasticity is a form of neuroplasticity in which the brain modifies neural circuit function modifying subsequent thoughts, feelings, and behaviors. It includes the ability of synapses to strengthen and weaken over time, and even create new neuronal networks. There are numerous forms of short-term and long-term synaptic plasticity, causing an accumulation of calcium in nerves (Citri, 2008).

G-to-K switches occur when the dietary transition from high glucose and carbohydrates to relying on ketones and fatty acids for nutrition. Intermittent metabolic switching (IMS) is the practice of switching an individual's dieting and exercise patterns from G-to-K switches. The amount of ketones produced shows the efficacy of the IMS diet - a higher amount shows the diet is effective. Exercise has been proven to accelerate the G-to-K switch as well. After fasting, the consumption of glucose and carbohydrates leads to a rapid K-to-G switch. While the metabolic switch is on, ketone levels are elevated, specifically the beta hydroxybutyrate ketone (BHB), promoting short bursts of synaptic neuroplasticity. Whenever an organism consumes carbohydrates or sugars, liver glycogen is replenished. When liver energy stores are reduced and circulating glucose levels are depleted, adipose cells release fatty acids, which convert into BHB ketone bodies. These ketones are used as neuronal energy substrates as they are released into the bloodstream. Recent research has found that a G-to-K diet leads to increased resistance to stress and better health (Mattson et. al, 2018).

The IMS diet has two factors pertaining to it: fasting and avoiding carbohydrates and sugars. Common IMS methods include intermittent fasting (IF), which includes alternate-day fasting (ADF) and time-restricted feeding (TRF). ADF is the process of restricting an organism's food for 24 hours every other day. TRF has two forms of measurements, including the deprivation of food for a certain time period (16 hours is optimal, following the 16/8 diet trend), and caloric restriction - which occurs when organisms are provided daily amounts of food that is 30-40% below their customary caloric intake.

Modern research has shown that overweight and sedentary individuals have at least a 39% more chance to experience neurodegenerative disorders like dementia and Alzheimer's. Many obese individuals consume a high carbohydrate and glucose diet. These individuals can switch to an IMS diet if the short and long-term effects of IMS and fasting prove successful. A low glucose and ketone diet could act as a possible deterrent to neuronal diseases by maintaining the plasticity longer. If there are no drastic long-term effects of neuroplasticity, this practice could eventually be applied to humans.

In fruit flies, IMS diet can be recreated through fasting for varying periods of time, as fasting before training has been proven to enhance 1-day memory. Additionally, the ketogenic diet (KD) is a high-fat, low-carbohydrate diet (4:1 ratio) that has shown effectiveness on fruit flies behavior, especially fruit flies prone to epilepsy and other similar neuronal diseases. The control diet (*ad libitum*) has proportions of 48 carbohydrates: 7.5 protein: 1 fat (Li et. al, 2017). The effect of a KD, which allows a G-to-K switch to occur, has not been tested on memory, however.

Long-term memory (LTM) and short-term memory (STM) patterns lead to the long-term potential phenotype (LTP). Researchers at Johannes Gutenberg University Mainz (JGU) have shown that the fruit fly *Drosophila melanogaster* develops a very stable long-term memory for its own body size and the reach of its extremities after it has hatched from the pupal case (Hirano, 2013). LTM requires protein synthesis, through the activation of the cyclic adenosine monophosphate protein (cAMP) acting as a second messenger molecule that relays external events to a unique location to initiate an action. The cAMP messenger response element-binding protein (CREB) is an intracellular protein and transcription factor that regulates gene, dopamine, and neuronal expression. LTM formation occurs after multiple single-cycle trainings due to a response in the cAMP. Both cAMP and calcium-dependent signaling pathways underlie learning and memory through information flowing through the neuronal networks. This leaves an imprint of cellular and synaptic changes on the pathways, also known as memory retention. The CREB increases intellectual levels (measured through comprehension and retention) of cAMP and calcium, leading to CREB-activated transcription being required for LTM. In appetitive memory formation, formation based on the introduction of new rewards as motivation, the neurohormone octopamine is active, while it is not in aversion LTM, pairing behavior with an unpleasant symptom. Additionally, appetitive LTM cannot form without fasting. Standard/ canonical aversion LTM, which requires multiple rounds of spaced training, differs from appetitive LTM, which can be formed by a single-cycle training. Fasting dependent LTM (fLTM) and spaced training-dependent LTM (spLTM) are both dependent on CREB activity, but spLTM requires two active CREB neuronal populations, in both mushroom neurons and dorsal-anterior-lateral (DAL) neurons, while fLTM only requires CREB mushroom bodies. Appetitive LTM is not impacted by DAL neurons. fLTM has been proven to be blocked by a repressor isoform of CREB, CREB2-b. With higher amounts of CREB2-b, there were flaws in LTM formation. STM refers to memory which develops in a few seconds or minutes and lasts for hours, whereas LTM forms slowly and lasts at least 24 hours. Recent studies have shown plasticity due to cAMP-regulated transcriptional coactivators (CRTC) by promoting CREB-dependent gene expression in the nucleus. CRTC has been shown to repress appetitive LTM, but not STM (Hirano, 2013).

Drosophila melanogaster is the preferred organism in this experiment due to their high number, a large amount of offspring, short generations for long-term testing, and providing an ease of testing (Li et. al, 2017). They are grown at 25 degree celsius, around room temperature. Females are photoperiod-sensitive, so they hatch earlier in the day. The common life span of a fruit fly is around 20 days, including around 7 days spent

in larvae stage. By day 11, the adult emerges from the pupa and becomes a mature adult. The *Drosophila* mutant line *Appl^d w** lacks the Amyloid Precursor Protein-like (APPL) gene, which is an ortholog of the Amyloid Precursor Protein (APP) gene (Geiger). These genes are often used in memory retention and provide information to the Amyloid Protein. Without this gene expression, *Drosophila* expresses symptoms similar to those found in early stages of Alzheimer’s Disease (AD) and other memory loss disorders. The development of AD is often associated with the loss of neurons, synapses, and the formation of amyloid plaques (Penserg, 2018). Many of the plaques and deposits are formed from an agglomeration of neurotoxic A β -peptides, which are generated through APP processing. The APP genes found in a human brain mirrors the APPL genes found in *Drosophila* (Goguel, 2011). However, *Drosophila* only expresses one APPL, while mammals tend to express three APP genes. Flies lacking the APPL gene have difficulty retaining LTM and also display other signs of neuronal difficulties, specifically in peripheral nervous systems. APPL is heavily identical to APP, and overall, the brain functions of the *Drosophila* are related to ~70% of all human neurons and genes. APPL is essential for axol development and the development of the enteric nervous system (ENS) (Cassar, 2016). Dietary choices were based on modeling different nutritional balances in *Drosophila*, specifically carbohydrate and fat-rich diets.

The research question was if the duration and type of IMS diet methods (IV) on *Drosophila* varies for differing time periods, what are the short term and long term effects of neuroplasticity on the cognitive and behavioral abilities (DV) of the organism? There was a three part hypothesis in this experiment, which was: If fruit flies are fed IMS diet on a 16:8 fast, leading to synaptic plasticity, then:

Both mutant and wild-type long term memory will increase due to the amount of ketones released, increasing memory formation

Their short term memory will increase in both mutants and wild-type

There will be positive behavioral effects on the *Drosophila*

The null hypothesis was that there will be no significant increase in long-term and short-term memory or any behavioral changes in both mutant and wild type *Drosophila* due to the IMS diet and synaptic plasticity. The purpose of this experiment is to determine the effect of brief synaptic plasticity and the effect it has on short term and long term memory. Additionally, this project researches the effect of neuroplasticity on the earlier stages of Alzheimer’s disease, by utilizing a mutant strain. A huge symptom of Alzheimer’s is synaptic plasticity loss, so enabling brief synaptic plasticity could be a possible deterrent of Alzheimer’s and other similar neurodegenerative diseases.

Methods

The IMS diet requires a 4:1 ratio of fats to carbohydrates. In order to create this ratio, avocado oil was chosen to be the medium. To create a 2:2 ratio of carbohydrates to fats, the standard diet for fruit flies, banana puree will be used. These choices are influenced by their repeatedly high amounts of carbohydrate and/or fat level per 100 grams of food - there are 23 grams of carbohydrates per 100 grams in bananas, and 15 grams of fat per 100 grams of avocado (Watanabe et. al, 2019).

Materials

Aversive Phototoxic Suppression Assay: 1. Quinine	Beta Hydroxybutyrate Measurement Assay: 1. β -Hydroxybutyrate	Social Avoidance Assay: 1. T-Maze 2. Maze	Aversive Pavlovian Olfactory Assay: 1. T-maze 2. copper	Drosophila Activity Monitor (DAM) 1. PSIU9 Power Surface Inter
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Hydrochloride (0.1M stock)	(Ketone Body) Colorimetric Assay Kit	3. Vortexer	mesh	2. LC4 Light Controller
2. T-maze	2. Cold micro-tube centrifuge	4. CO2 Sensor	3. 4-methylcyclohexanol (1:65)	3. Monitor Cables
3. White LED light	3. Micropipette (20-200 µl)		4. 3-octanol (1:100)	4. DAMSystem3 Software
4. Red LED lamp			5. 4,000 Volt Electric Zapper	5. DAM2 Drosophila Activity Monitor
5. Filter paper				

Trials

	IMS+16:8	Regular (2:2)	IMS Diet (4:1)	Control (blue food)
Appl ^{-d} Mutants	x	x	x	x
Standard	x	P	x	N

Procedures

Growth

The *Drosophila* was grown in preferred environmental conditions, including a temperature of 25° C. Larva were fed using grapefruit agar plates and yeast paste to synchronize age. All fruit flies were stored in different sterile food bottles with blue food, separated for testing with foam plugs as a stopper. The negative control was fed the standard diet of Nutri Agar and banana (2:2 carbs and fats), and the positive control and experimental group were fed a diet consisting of avocados (a 4:1 ratio of fats to carbohydrates). The experimental group was fed on a 16:8 time-restricted feeding method or every other day.

Diet Standardization

Phenol-Sulfuric Acid Method

Using the glucose standard solution (100 mg glucose/L) and distilled water as indicated in the table below, stand curve tubes were created by pipetting aliquots of the glucose standard into clean boiling tubes (duplicates for each concentration) so that the tubes contain 0-100 µl of glucose, using a 1000 µl mechanical pipette.

Glucose levels	0	20	40	60	80	100
ml glucose stock	0	0.2 (200 µl)	0.4	0.6	0.8	1.0

ml distilled water	1.0	0.8	0.6	0.4	0.2	0.0
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Figure 1. Glucose standard concentrations

Then, 1 ml of 5% Phenol and 5 ml of 96% Sulfuric Acid was added and vortexed for 10 seconds. After 10 minutes, the tubes are placed in a water bath at 25-30°C for 15 minutes. Blank was set with 1 ml of distilled water and O.D. of each tube was taken at 490nm with the spectrophotometer. Steps were repeated with 0.2ml of food sample.

Preparation of Sample: 100 ml of sample is filtered and placed in a boiling tube and kept in a water bath for 3 hrs, then removed and cooled at room temperature. Sodium carbonate is added until effervescence ceases and then water is added until a final volume of 100 ml is reached. Following centrifugation, steps 2-5 are repeated with the sample. Percentage of total carbohydrate present is determined by the following method:

$$\begin{aligned} \text{Absorbance corresponds to } 0.2 \text{ ml of test sample} &= X \text{ mg of glucose} \\ 100 \text{ ml of sample solution contain} &= X/0.2 \times 100 \end{aligned}$$

Hanus Iodine Value Test

After weighing 0.25g of avocado oil and 0.25 of olive oil into an iodine flask, samples were dissolved in 10 mL of chloroform. 25 mL of Hanus iodine solution was added using a pipette, draining it in a definite time. After mixing well, allow it to stand in dark for exactly 30 minutes with occasional shaking. 10 mL of 15% KI and 100mL of freshly boiled and cooled water is then added, washing down any free iodine on the stopper.

After titration against 0.1N sodium thiosulphate, the yellow solution turned almost colorless. A few drops of starch as indicators are added and titrated until the blue color completely disappears. Toward the end of titration, the flask was shaken vigorously so that any iodine remaining in solution in CHCl_3 is taken up by potassium iodine solution. A blank was run without the sampler and the amount of oil needed to have a 4:1 ratio of fats to carbohydrates was found (the standard IMS ratio).

Aversive Phototaxis Assay

6 hours before testing, flies are into food bottles and are properly starved for the next 6 hours so they are more perceptive to the aversive taste. Quinine hydrochloride is dissolved to prepare an active solution in a 0.1M stock (1.98g in 50mL of distilled water), stored at -20° C. To prepare a working solution of 1 μM , dilute the stock with distilled water.

T-Maze Assembly: The T-maze consisted of a central column with a trap door and two side independent chambers, a dark and a light chamber. At the 2mL mark, a manual saw was used to cut off the closed end of two 15mL plastic centrifuge tubes and adaptors are inserted at each end and sealed with parafilm. The adapters at the end serve as holding locations for the gooseneck light source.

One chamber is connected to the light source (light chamber). The other chamber is wrapped in aluminum foil (dark chamber). Then, 180 μM of water solution is added to the filter paper, and each tube is screwed into the center of the t-maze with the trap door closed.

Testing: Each fly is transferred from a group of 10 to an empty 50 mL centrifuge tube and capped tightly to prevent escape. A single fly is placed into the dark chamber and quickly added back to the maze. Lights are turned off and red light is turned on to prevent eyesight. Following a 30 second adjustment period, the trap door is raised and the light source is turned on. If the fly walked to the light, it was considered positively

phototactic and ready to be trained.

Once tested, the fly was transferred back into the holding chamber with nine other flies and the light was subsequently turned off. During the 30 second adjustment period, the filter paper with water was removed and then replaced with 180 μ M of quinine solution. The trap door was then opened and the light was turned on, allowing the flies to walk into the light chamber. After one minute, the flies were transferred back into the dark chamber. A total of 10 conditioning trials were completed.

Immediately after training, 5 test trials were completed. If the fly did not walk into the lighted chamber during 10 seconds, it is a pass. This was PC0. After testing 5 times, each fly group was placed back into its food vial and placed back for 6 hours. 6 hours after training, 5 more test trials were conducted as earlier. This measured short term memory and was PC6.

β -Hydroxybutyrate Colorimetric Assay

Sample Preparation: 20 flies are separated from group and placed in a glass homogenizer. 8 ml of PBS wash is added into the glass homogenizer and mixed until the solution turns red, and then yellow. Once the solution is yellow, it is poured into centrifuge tubes and centrifuges at 10,000 for 2 minutes. Following that, flies were re-homogenized and centrifuged again. Fly remains concentrated at the bottom of the tube, while yellow serum has risen to the top. This serum was stored at -80°C .

Reagent Preparation: β -Hydroxybutyrate Standard was reconstituted with 1 ml of β -HB Assay Buffer and was for 2 hours on ice. β -Hydroxybutyrate Enzyme solution was reconstituted with 2.4 ml of β -HB Assay Buffer and is also stable for 2 hours on ice. 100 μ l of the β -HB Colorimetric detector is added to the reconstituted Enzyme solution and stored on ice, as it was stable for one hour.

Standard Preparation: Eight clean centrifuge tubes were cleaned and labeled A-H. They were then diluted according to the following table below.

Tube	β -HB Stock Solution (μ l)	Assay Buffer (μ l)	β -HB Concentration (mM)
A	0	200	0
B	5	195	0.025
C	10	190	0.05
D	20	180	0.1
E	40	160	0.2
F	60	140	0.3
G	80	120	0.4
H	100	100	0.5

Figure 2. Concentrations of HB Standard

In order to create β -Hydroxybutyrate Standard Wells, 50 μ l of each Standard (tubes A-H) is added to two or three wells. For the Sample wells, 50 μ l of the sample was added to four consequent wells. The reaction was initiated by adding 50 μ l of the Developer Solution to all wells being used, and then incubated at 25°C in the dark for 30 minutes. The absorbance was read at 445-455 nm using a plate reader.

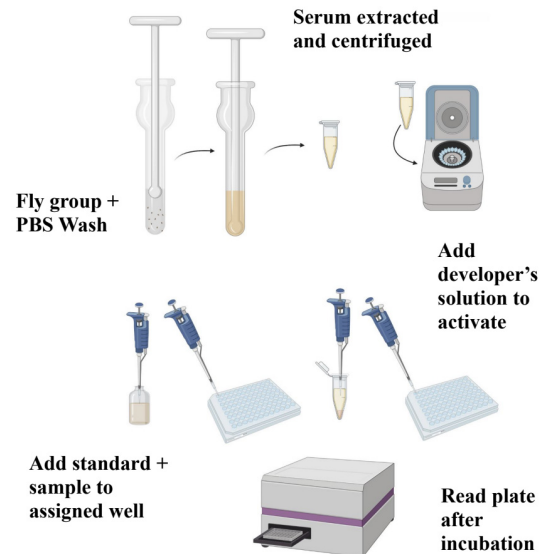


Figure 3. Assay Set-Up and Procedure is shown.

Aversive Pavlovian Olfactory Assay

T-Maze Assembly: A custom-made Perspex T-maze is created with copper grids inside the training tubes. The grids are connected by weaving copper wire through it and then, using crocodile clips, are connected to a restrictor.

Preparation: 4-methylcyclohexanol is diluted at a 1:65 ratio and 3-octanol at a 1:100 ratio using mineral oil. Then, 30 mL of the diluted odor is pipetted onto filter paper (cut 8x8 squares).

Shocking: A Fly Zapper is reverse engineered and then rewired into a shocking system used to attach to the copper wire in the T-maze. A voltmeter was applied to ensure the apparatus was delivering the required shock. Then, a 750,000 resistor is attached to limit the amperage and volts traveling through the circuit system. Flies were periodically shocked for one second every 3.5 seconds, twelve times.

Testing: All experiments were conducted under a red light, so fruit fly vision was impaired. Flies were placed in T-maze for 90 seconds before testing. Then, odors are introduced with a 60 volt shock for 12 one-second pulses and 3.5 second interpulse intervals, for a duration of 60 seconds. Then, after 30 seconds, OCT is introduced without a shock for a minute. Following a 30 second rest period, the flies were moved back into the central chamber and were simultaneously exposed to both odors for 120 seconds. Flies were counted in each tube and then these training trials were repeated for five cycles with 15 minute intervals in between. The test was conducted 24 hours later to measure long-term memory.

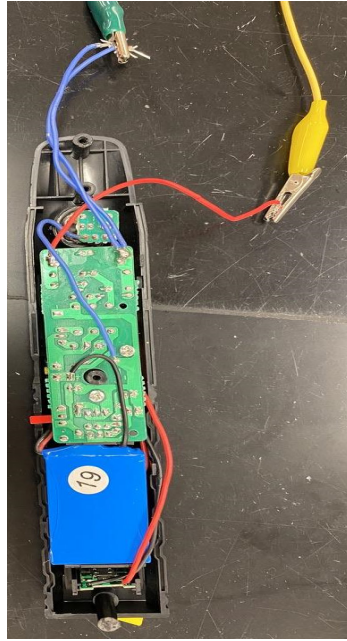


Figure 4. Reverse engineered fly-swatter; open circuit.

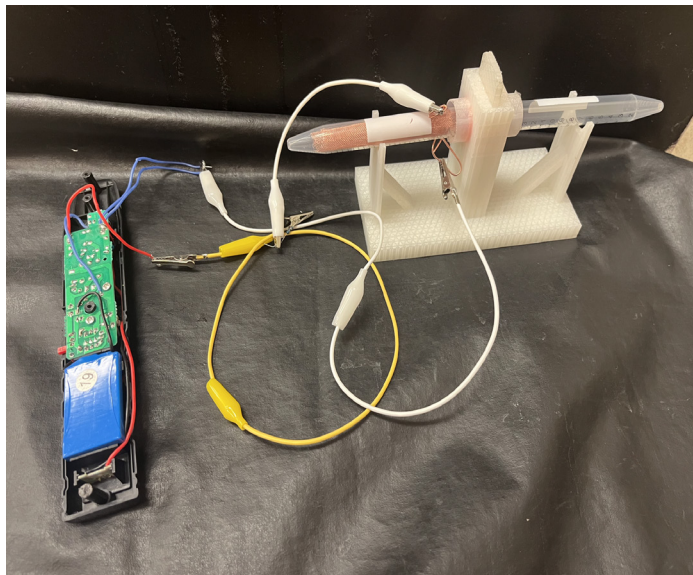


Figure 5. Attachment of circuit board to t-maze used for the assay.

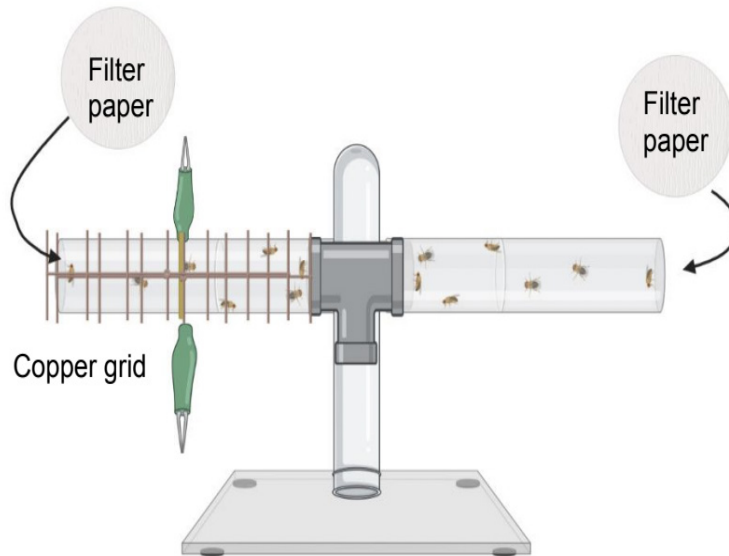


Figure 6. Construction of T-Maze for assay.

Social Avoidance Assay

Control flies were placed in the left side of the t-maze, and emitter flies were mechanically agitated using a vortexer by vortexing for 15 seconds, then removed for 5 seconds, repeated 3 times for a total of 55 seconds. The new emitting flies were placed into the right side of the t-maze, and introduced a third, unaffected group of flies into the maze. Flies were allowed to choose which fly vial to associate with, and then counted. Following that, stressed flies had CO₂ content measured in order to compare stress levels to regular levels.

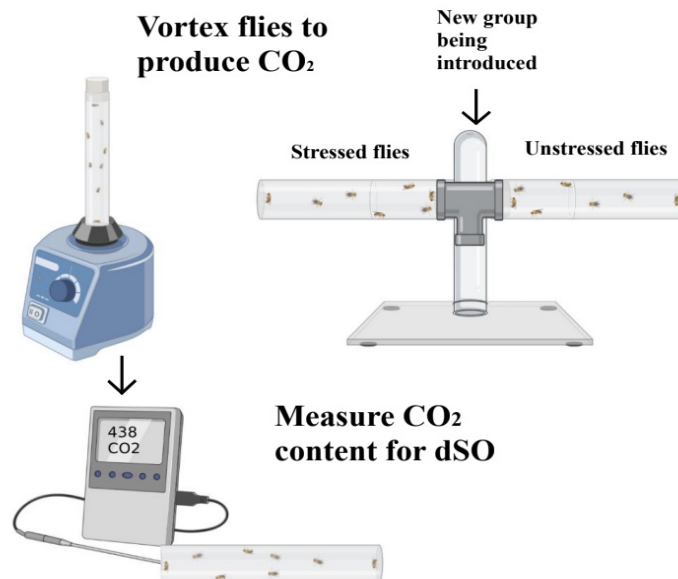


Figure 7. Set-up of the Social Avoidance Assay and Drosophila Stress Odorant (dSO) Assay

Drosophila Activity Monitor (DAM)

The DAM2 activity monitors were set up by filling each row with 5 mm tubes containing one fly, nutri agar, and a cotton swab at the top.

Software: The USB Driver zip archive was downloaded from Trikinetics on a Windows 8 desktop. After determining the bit size of the computer (64 bit), the USB Port was installed and then the USB Zip was connected to the computer following the steps prompted by the computer. DAM Systems3 was extracted from the ZZip File and saved into a folder on the desktop.

The PSIU9 (Power supply interface unit) was then connected to the desktop by plugging the cable into the computer and ensuring the light was green. DAMSystem3 was launched and Serial Port was selected in Preferences, specifically Monitor 1, PSIU9. Data collection was verified by ensuring that the data collection was being complete and recording numbers properly.

Hardware: The monitor was then plugged into the PSIU9 using monitor cables. Then, the PSIU9 was connected to the computer using a USB drive. The PSIU9 was then plugged into the wall for power. The software system then counted the amount of times a fly moved across the vial per vial.

IMS Diet Preparation: After measuring $\frac{1}{2}$ (78 grams) of pre-packaged nutrient agar and pouring it into 500 mL of water, the solution was split into 2 beakers and stirred. Once boiling, 2 mL of preservative solution and 135 mL of avocado oil was added for a 4:1 fats to carbohydrates ratio. Then 7 mL of nutri agar was poured into 50 vials and cleaned. The remaining half of the pre-packaged nutri agar was used to make another batch of nutri agar, this time adding 75 mL of avocado oil and 50 g of banana purée for a 2:2 fats to carbohydrates ratio.

Safety Precautions

The main risks in this experiment included the use of corrosive and flammable chemicals including Sulfuric Acid, Hanus iodine solution, and Carbolic Acid. Safety precautions included proper protective equipment (PPE) being worn, including a lab coat, goggles, and gloves, working in a fume hood, and having an advisor supervise the hood while Sulfuric acid was being used. A pipette was used to transfer the chemical, and it was kept at arm's length to prevent spills. In order to neutralize the sulfuric acid, basic ammonium tablets were used to neutralize the acids, creating a pH of 6.5.

Additionally, there was an open circuit used in the Aversive Pavlovian Assay, where there was the risk of being shocked. Protection included proper PPE and the supervision from engineers and those specialized in robotics.

Data Analysis

For the Phototaxis Assay, the average pass rate is the pass rate of each fly in a group of 10 added up, and then divided by 10. The average pass rate is referred to as PC0 (directly after training), or average pass rate, PC6 (6 hours after training). The P-value is then taken from the pass rates using a two-way ANOVA, and then Tukey's Honestly Significant Difference Test as a follow-up. For the β -HB Colorimetric Assay, a standard curve was created and values were compared to the curve. A two-way ANOVA was run, followed by Tukey's Honestly Significant Difference Test. For the Social Avoidance Assay, CO₂ Levels were measured and compared using

a two-way ANOVA. Data for this assay is still being analyzed. For the Aversive Pavlovian Olfactory Assay, the Performance index was calculated to determine the measure of memory. Calculate this as the number of flies avoiding the shock-paired condition (CS⁻) minus the amount of flies who chose the shock-paired order (CS⁺) divided by the total number of flies (CS⁻ + CS⁺). (# CS⁻ flies - CS⁺ flies)/(total # of flies). Calculate the final PI by averaging the statistic when MCH was the shock-paired odor and when OCT was shock-paired. This removes preferential bias. The *Drosophila* Activity Monitor Data, which had over 1,000 inputs for each run, was analyzed using a two-way ANOVA and then T-Tests.

Discussion

Results

Aversive Phototaxis Assay

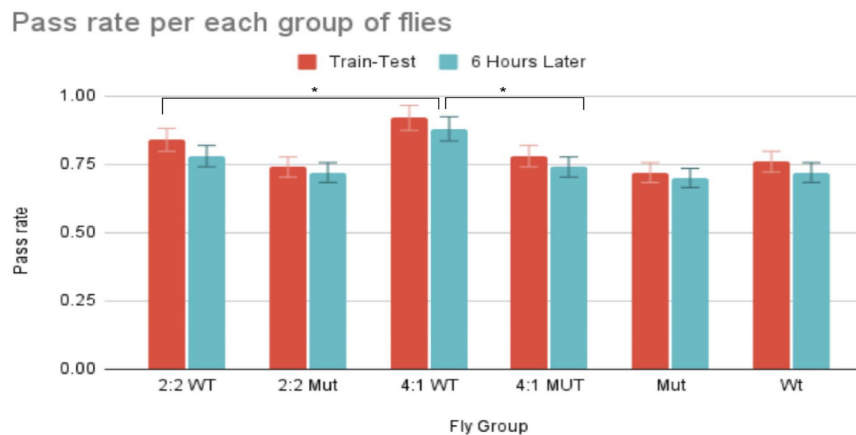


Figure 8. The pass rate of each test group, averaged from trials and then divided by amount of flies in trial (10). The Aversive Phototactic Suppression Assay measures short term memory.

Compared to the negative (untreated flies) and positive control (2:2 WT), the flies fed the 4:1 diet had much higher memory retention. Overall, the 4:1 wild type group had higher percentages of remembrance, with their average pass rate being nearly 20% more than both the positive and negative control group. The 4:1 wild type group had the highest memory retention overall, and the highest rates across the board. Each group's pass rate did lower as 6 hours passed, but each 4:1 group (mutant and wild type) only decreased by 4%, the 2:2 mutant group only decreased by 2%, while the positive control group decreased by 6%. The P-value of this data set was 0.02 for flies tested immediately after training, and 0.03 for flies tested 6 hours after, both significant values, as shown by the ANOVA tests.

Drosophila Activity Monitor (DAM)

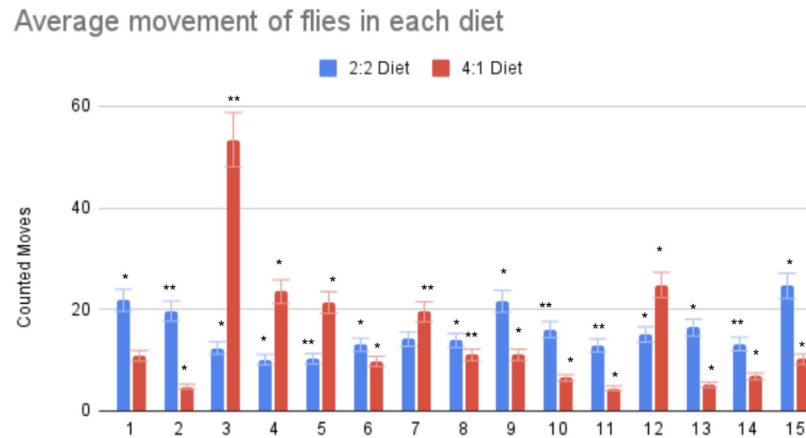


Figure 9. Average of fly movement for 15 flies fed each diet, averaged from over 1,000 data points collected over 48 hours in the drosophila activity monitor.

Overall, the flies that were fed the 4:1 diet were much more active sporadically, while those fed on the 2:2 diet were much more active consistently. While the 4:1 diet flies had high levels of activity at specific time periods, 2:2 flies showed much less variability. P-values were both significant, with values of 0.02 and 0.04 respectively.

β-Hydroxybutyrate Colorimetric Assay

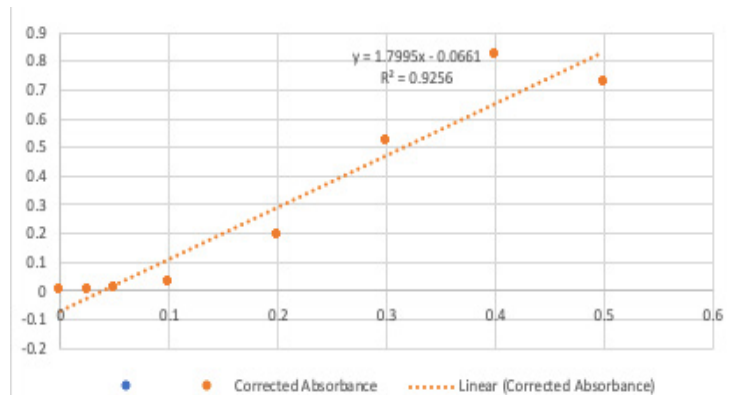


Figure 10. The standard curve, a linear regression comparison of absorbance to hydroxybutyrate concentration per fly group.

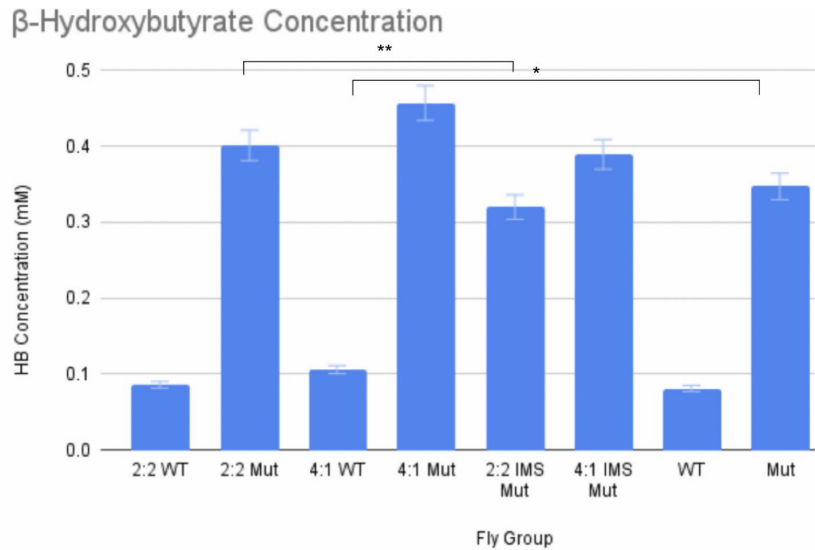


Figure 11. The concentration of beta-hydroxybutyrate ketones (mM) in each fly group.

After performing this assay twice, the p-value has lowered to 0.02 for overall statistics, and the ANOVA has determined that the results are statistically significant. Overall, mutants did release a higher amount of ketone bodies. The 4:1 WT flies had 17% increase in ketone count, while the 4:1 mutant flies had a 74% increase. Overall, 4:1 flies and mutants had higher ketone counts overall, showing the impact of neuroplasticity forming new neuronal networks in the fly. Fasting flies had ketone counts 10% higher than wild type control, while non-fasting flies had a 20% increase. Flies fed the diet of 2:2 ratio, which was a much lower ratio of the IMS diet, did have subtle increases in their ketone body count. Flies on a fast did have higher ketone body count compared to their control (untested flies) by about 10%, but flies that stayed consistently feeding on the diet had the highest rates overall (by nearly 20%). There was a larger variance in ketone body count in mutants versus wild type flies as well, which is worth noting.

Aversive Pavlovian Olfactory Assay

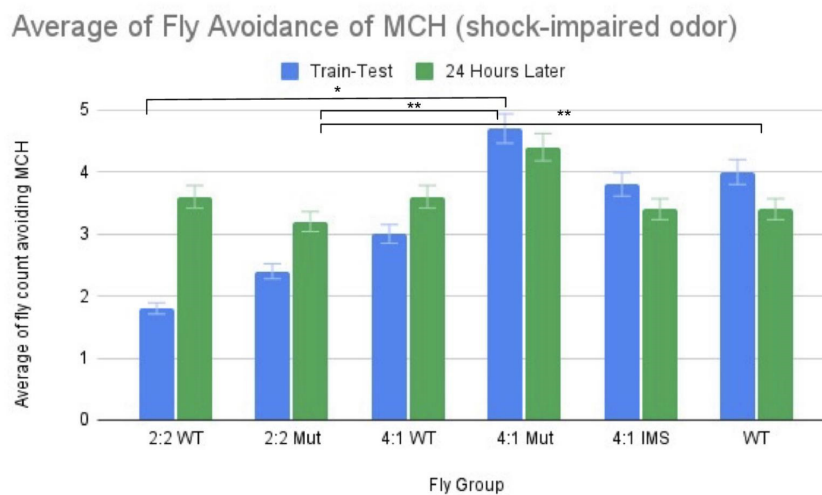


Figure 12. The average of flies that avoided the shock-impaired odor (MCH) in the Aversive Pavlovian

Olfactory Assay. Shows results from flies tested immediately after trials and 24 hours later (for long term memory).

The Aversive Pavlovian Olfactory Assay measures long-term memory (LTM) and compared to the WT control, which after 24 hours, retention rates decreased by 6%, all other diets only changed in their long term memory from 2-4%. The 4:1 flies all had much higher memory retention rates compared to other groups. 4:1 WT and 2:2 WT actually increased memory retention.

Social Avoidance Assay

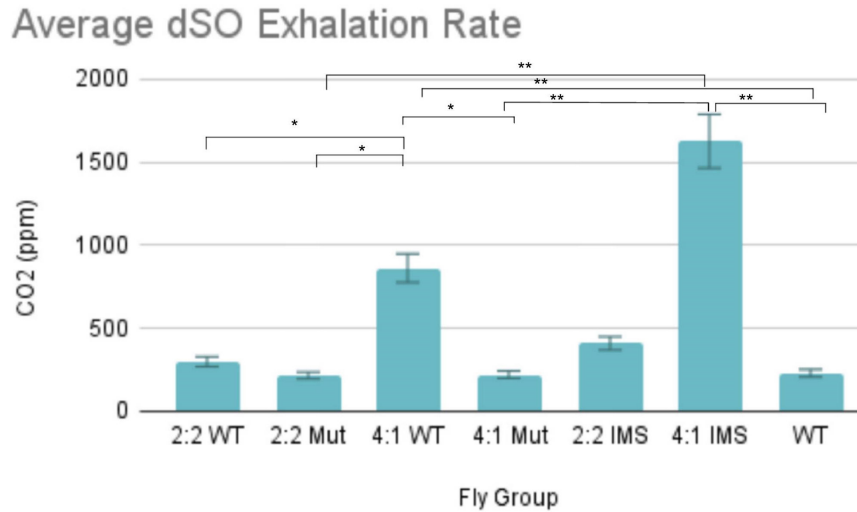


Figure 13. Average dSO exhalation rate, measured by CO₂ exhalation (in ppm) due to a direct correlation between CO₂ and dSO.



Figure 14. The average pass rate of flies that avoided stressed flies (10 flies per trial).

Sensitivity is determined from the behavioral assays run, one of them being the Social Avoidance Assay. Each diet group was higher than the control, but the 2:2 diet only increased by 22%, while the 4:1 groups, both wild type (WT) and IMS, increased. The 4:1 WT flies exhaled 73% more CO₂ compared to the negative control group (untreated wild type). As CO₂ corresponds directly with Drosophila Stress Odorent (dSO), 4:1 flies were more stressed when provoked, as they also exhaled 66% more CO₂ compared to the positive control (2:2 WT flies).

Phenol-Sulfuric Acid Testing

Con. in mg/ml = X/0.2100

Sample	O.D. Average	Con. in mg/ml = X/ 0.2100
Avocado A	1.82	0.05
Avocado B	2.24	0.2
Banana A	2.26	0.53
Banana B	2.30	0.68

In the phenol sulfuric acid test, it was found that the label on the food products are accurate, there is around 22 g of carbohydrates in 100 g of banana puree, and 0.5 mL of carbohydrates in 15 mL of avocado oil.

Conclusion

Two of the three hypotheses were supported, as both the long term memory and short term memory increased in flies experiencing plasticity, but the flies under the 4:1 diet had more sensitive behavior compared to the flies under the 2:2 diet and the control, which can be seen as negative behavioral effects, instead of positive.

Sensitivity is determined from the behavioral assays run, one of them being the Social Avoidance Assay. Each diet group was higher than the control, but the 2:2 diet only increased by 22%, while the 4:1 groups, both wild type (WT) and IMS, increased. The 4:1 WT flies exhaled 73% more CO₂ compared to the negative control group (untreated wild type). As CO₂ corresponds directly with Drosophila Stress Odorent (dSO), 4:1 flies were more stressed when provoked, as they also exhaled 66% more CO₂ compared to the positive control (2:2 WT flies).

The other behavioral assay was the Drosophila Activity Monitor (DAM). The 4:1 flies showed 98% more variability than the 2:2 diet flies, meaning they were more active sporadically. While the 2:2 WT only had a variability of 3.32, the 4:1 WT had a variability of 433.21. This variability could account for the sensitivity of 4:1 flies. This sensitivity could possibly be due to changes induced in the flies due to neuroplasticity.

The Aversive phototaxis assay shows the short term memory of the 4:1 diet group (both wild type and mutants) had the highest retention rates overall, with the 4:1 WT pass rate being 21% more than the wild type control. Pass rates were determined by averaging data points and dividing by the number of flies in trial. Each group's pass rate did lower as the 6 hours passed, but both mutant and WT 4:1 groups memory retention decreased by 4%, while the 2:2 WT (positive control) decreased by 6%.

After measuring ketones using the hydroxybutyrate colorimetric meter, the 4:1 WT flies had a 17% increase in ketone count, while the 4:1 mutant flies had a 74% increase. Overall, 4:1 flies and mutants had higher ketone counts overall, showing the impact of neuroplasticity forming new neuronal networks in the fly.

Fasting flies had ketone counts 10% higher than wild type control, while non-fasting flies had a 20% increase.

Limitations

Errors in this experiment included mechanical and human error. Additionally, flies tend to stay in the middle chamber, which could delay research as touching the chamber would then skew results in assays such as the Social Avoidance Assay and Aversive Pavlovian Olfactory Assay. In the Aversive Pavlovian Olfactory assay, the copper grid often got caught in fly wings, which also delayed testing. Sometimes, the voltage in this assay also jumped at sporadic times, which meant re-running the trial to maintain a constant voltage of 60v. Also, the nutri agar with the dietary treatments added in was less firm and consistent than standard nutri agar, so after three days passed without refrigeration, the agar started to decay, limiting the time a nutri agar treatment bottle could be used.

Applications

This project can be extremely applicable to those facing neurodegenerative diseases and are looking for preventative mechanisms. As this project has shown, inducing neuroplasticity on someone has proven to increase both short and long-term memory, which can help those battling these diseases. As mutant flies exhibited traits similar to those exhibited by Alzheimer's patients, and neuroplasticity was able to increase their memory retention by a significant difference, then this could further be applied in this field of study.

Future Research

Future research is highly recommended for this experiment, further ways of inducing neuroplasticity which deal with less variability could be researched, as well as ways to apply the concept of neuroplasticity to other neurodegenerative diseases and see whether it has an impact of later-staged patients. Additionally, recent research has suggested that neuroplasticity could be used as an anti-aging agent, which is relevant to this project and could be further studied. A long-term solution to these neurodegenerative diseases should be continued to be worked on, with the final goal of finding a cure.

Acknowledgments

I would like to thank my advisor for the valuable insight provided to me on this topic.

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