NF1 MUTATIONS IMPAIR MEMORY RELATED PLASTICITY IN *DROSOPHILA MELANOGASTER* MUSHROOM BODIES

by

Brandon Gilliland

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Gregory Macleod, and has been approved by the members of her/his supervisory committee. It was submitted to the faculty of The Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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ABSTRACT

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In neurofibromatosis type 1 (*Nf1*), cognitive deficits have been reported. Calcium imaging and olfactory classical conditioning were used to measure activity of postsynaptic response of the neurons in the mushroom body. During olfactory classical conditioning, large subsets of dopaminergic neurons are activated, releasing dopamine across broad sets of postsynaptic neurons. However, it is unclear how the diffusion of dopamine causes highly localized patterns of plasticity required for memory formation. Knocking down *Nf1* disrupts cAMP-dependent plasticity in the gamma lobes. Dopaminergic neurons drive compartmentalized elevation of postsynaptic cAMP, and this cAMP elevation drives postsynaptic plasticity in the mushroom bodies.

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INTRODUCTION

Dopaminergic neurons are an important part in learning and memory. In humans, these neurons involve less than one percent of the total number of neurons in the brain. In *Drosophila melanogaster*, these neurons play an important role in dopamine dependent processes such as learning and memory, motor control, arousal, motivation, addiction and obesity, and salience-based decision making. During olfactory classical conditioning, the dopaminergic neurons respond to the electric shock. Ethyl butyrate causes an influx of calcium to elevate cAMP, which suggests that dopamine is involved in learning (3).

The dopaminergic neurons make up multiple circuits in the brain, and they each have a specific role in learning and memory. There are subsets of mushroom body neurons in *Drosophila melanogaster* that receive conditioned stimulus and unconditioned stimulus information, and these neurons are able to express biological molecules associated with the stimuli. This phenomenon allows the dopaminergic neurons to generate dopamine/ cAMP-dependent plasticity in the mushroom bodies of *Drosophila melanogaster;* however, there are only some subsets of dopaminergic neurons that are involved in supporting memory following conditioning. The primary basis for this research was to discover the specificity in how spatial patterns of plasticity are generated during conditioning through olfactory classical conditioning in *Nf1*.

One of the main objectives of this project was to examine whether or not memory related plasticity was impaired with an *Nf1* mutation. A mutation in the *Nf1* gene can cause a disease known as neurofibromatosis type 1. This particular disease is recognized in humans by cutaneous phenotypes such as café-au-lait spots, axillary and inguinal freckles, and subcutaneous or cutaneous neurofibromas. In addition to these phenotypes, studies have shown that humans can also show signs of cognitive impairments (29). Neurofibromatosis type 1 is inherited in an autosomal dominant pattern of inheritance, and it occurs in approximately 1 in 3,500 individuals. In humans, the *Nf1* gene codes for the making of the neurofibromin protein, which acts a tumor suppressor. A mutation in this gene allows for abnormal cell proliferation. As a result, tumors such as neurofibromas can form.

In the *Drosophila melanogaster* model, phenotypes are displayed differently. The *Nf1* gene is a 30 kb DNA segment between the *bride of sevenless* gene and the *Enhancer of split* complex (2). This gene codes for five transcripts, and it produces five unique polypeptides. The *Nf1* gene in *Drosophila melanogaster* is expressed in most cell body regions of the adult central brain. The gene expression is seen in cell body regions near the antennal lobes, lateral horn, protocerebrum, and the mushroom body calyces.

The *Nf1* gene has been observed to play a part in many functions such as determination of adult lifespan, locomotor rhythm, negative regulation of synaptic growth at the neuromuscular junction, perineurial glial growth, long-term memory, response to oxidative stress, response to heat, and regulation of RAS protein signal transduction. Although the functions listed previously are important, the purpose of this project was to focus on short-term memory, olfactory learning, and cAMP-mediated signaling.

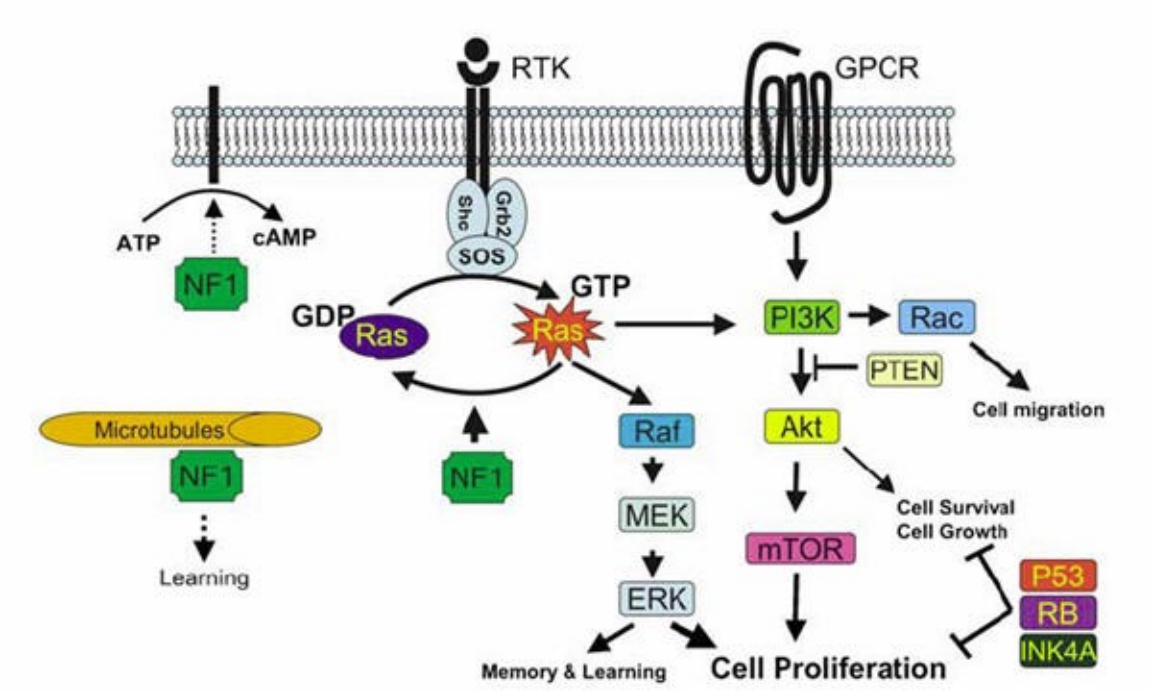

Figure 1: Proposed Pathway for Nf1

The proposed pathway shown in Figure 1 for *Nf1* involves RAS protein signal transduction. Although this pathway is not fully understood, RAS is believed to be influential in the effects of *Nf1* in both humans and *Drosophila melanogaster*. Because the dopaminergic neurons have a role in mediating memory related plasticity, studying control versus *Nf1* mutants would help provide insight into whether or not this mutation causes cognitive impairments in *Drosophila melanogaster*. In addition to cognitive impairments, other phenotypes such as fragmented circadian rhythm are observed. A publication in 2013 from the Journal of Child Neurology showed that sleep disturbances and fragmentation of circadian rhythm was associated with *Nf1* in children (14).

In order to find the cognitive effects of the *Nf1* mutation, a GAL4-UAS system was used in both groups to drive expression of the 238Y driver, which helps drive expression in all three classes of mushroom body neurons: α/β, α′/β′, and γ. The RNAi knockdown group of *Nf1* was the *Nf1* mutant group. GCaMP6f, a fluorescent calcium sensor, was used to allow for expression of fluorescence upon excitation of photons in confocal imaging. This calcium sensor is made from green fluorescent protein, calmodulin, and a specific sequence from myosin light chain kinase. The different regions of the mushroom body were analyzed individually through confocal imaging, and they were quantified according to the different areas of afferent dopaminergic neurons. These dopaminergic neurons have distinct roles in memory acquisition.

“Multiple subsets of MB neurons receive CS and US information and express molecules associated with coincidence detection, making them theoretically eligible to generate dopamine/cAMP-dependent plasticity. Yet only some subsets are required to support memory at any given time following conditioning, leaving open the question of how spatial patterns of plasticity are generated during conditioning (3).” Because only some subsets were essential for memory following conditioning, it was necessary to examine the postsynaptic neuronal pathway effects in the dopaminergic neurons. This was accomplished through olfactory classical conditioning.

Olfactory classical conditioning experiments were used as a way to examine the pre versus post conditioning calcium responses for the control group and the *Nf1* mutant group. The olfactory classical conditioning experiments were accomplished through *in vivo* microsurgery to the brain of the *Drosophila melanogaster* followed by administering forskolin in specific timed intervals with a conditioning period to analyze the distinction between presynaptic and postsynaptic neural activation.

METHODS

The *Nf1* project consisted of a few procedures to effectively analyze whether or not memory related plasticity was impaired with an *Nf1* RNAi. These experiments involved specifically timed intervals of odor and air stimuli to the fly. The air stimuli served as the controlled stimulus, as it was able to give a baseline in the response graph. The conditioned stimulus for olfactory classical conditioning was ethyl butyrate.

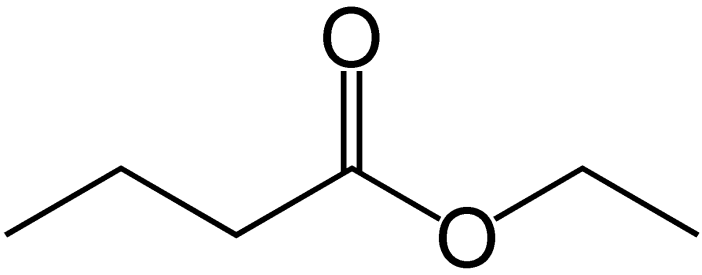

Figure 2: Ethyl Butyrate Molecular Formula

Olfactory Conditioning Baseline Methods

Ethyl butyrate, an ester known for its fruity smell, evokes a positive odor response in *Drosophila melanogaster* because of its ability to stimulate olfactory pathways. A training period was established to analyze the differences between pre-conditioning and post-conditioning in both groups. This training period involved administering 100 μM of forskolin, which served to elevate cAMP in the mushroom body neurons by adynylyl cyclase activation (25).

The specific intervals used for the experiment are shown in Figure 3. Following this experimental procedure, a proper baseline was established for use in the comparison of pre versus post conditioning to examine the odor evoked calcium transients in the mushroom body.

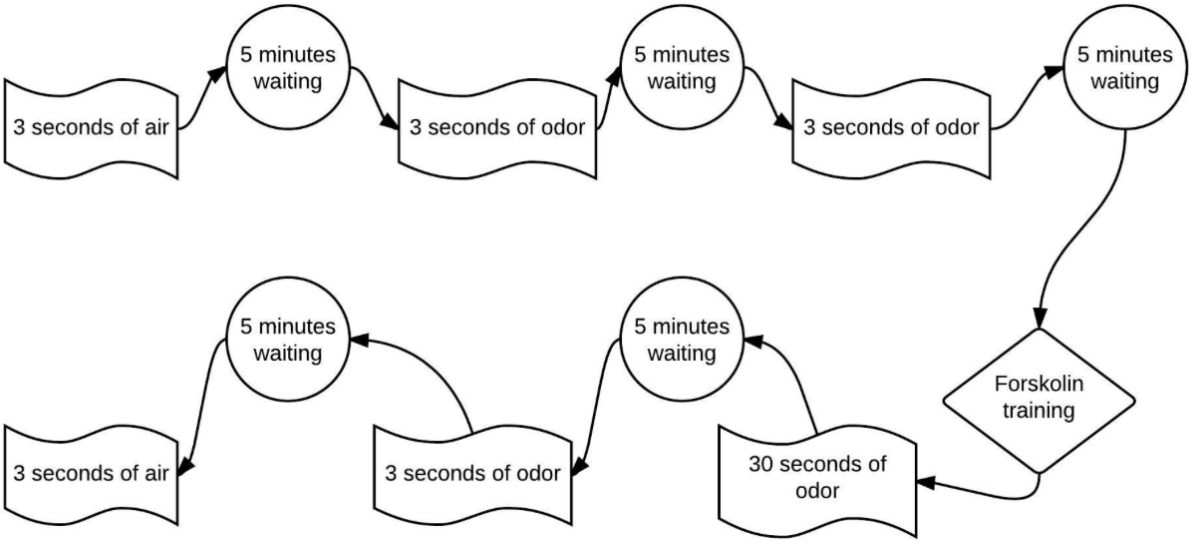

Figure 3: Flowchart of Olfactory Experimentation

In Vivo Microsurgery

Before the olfactory experiments could be conducted, it was necessary to properly expose the brain for confocal imaging. This was accomplished via *in vivo* microsurgery to the head of the fly. The chamber pictured in Figure 5 was used to properly position the fly for imaging and olfactory experimentation. To position the fly successfully, it is anesthetized by using CO_2. Once positioned appropriately in the chamber, myristic acid was used to glue the fly's eye to the side of the chamber, so the head would remain fixed for confocal imaging.

Myristic acid ($C_{14}H_{28}O_2$), also known as tetradecanoic acid, is unique because it is a solid at room temperature. The melting point is 54.4 °C, so an electrode was used to cause the myristic acid to change from the solid phase to the liquid phase. Once the electrode was taken away from the area, the myristic acid changed back to a solid.

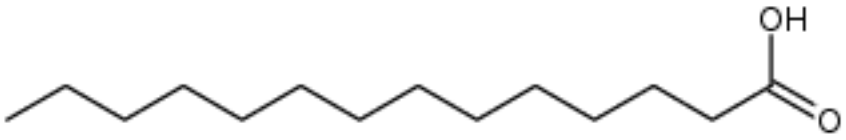

Figure 4: Structure of Myristic Acid

Once the fly was positioned with the myristic acid and saline was poured into the open area of the chamber, *in vivo* microsurgery was performed on the fly's head. Small incisions were made in the cephalic region to create a square, exposing the brain. Fat bodies were carefully picked out of the fly head with forceps to avoid interference during confocal imaging. Once the microsurgery procedure was completed, the fly in the chamber was placed under the confocal microscope, and saline was pumped through the chamber at consistent velocity of approximately 1 ml/min. The position of the fly with respect to the saline and confocal laser is demonstrated in Figure 5.

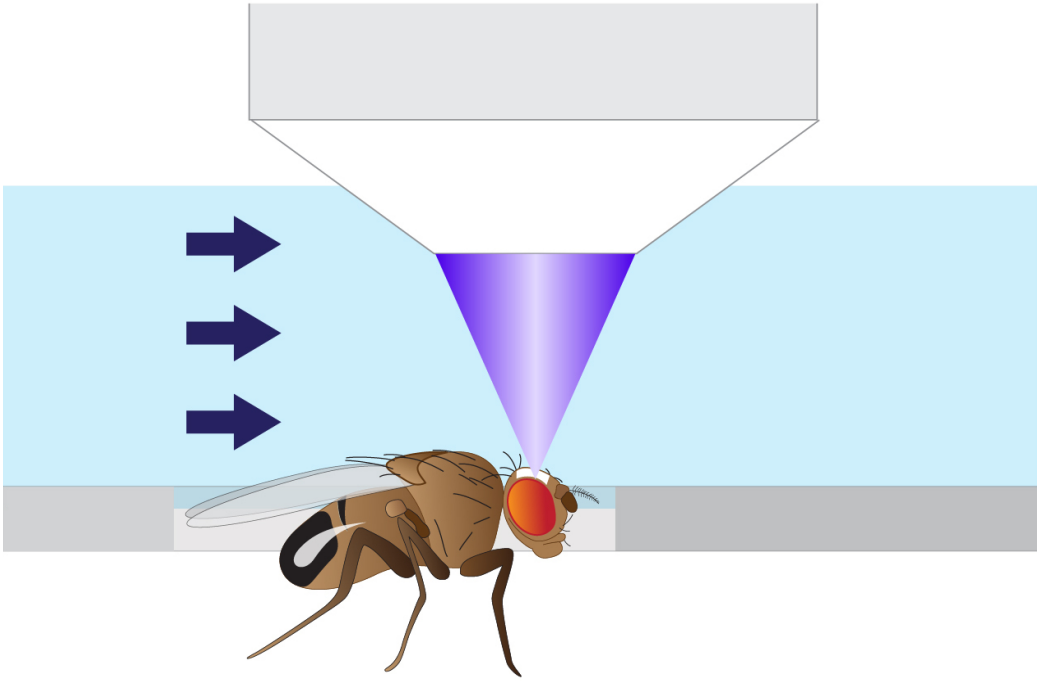

Figure 5: Fly Placed in the Chamber for Confocal Imaging

In order to establish consistency for comparison, only female flies were used. In addition, twelve flies from each group, both the control and the RNAi knockdown group, were used for comparison. It is also important to note that the flies used in the experimentation were no older than two weeks. This is because flies older than two weeks do not learn as well as the younger flies. It was also found that flies older than two weeks did not fluoresce as well with the GCaMP6f fluorescent calcium sensor compared to the flies within the two week range.

Olfactory Training With Forskolin

The 100 μM forskolin training period stimulated adynylyl cyclases to allow for the elevation of cAMP in the mushroom body.

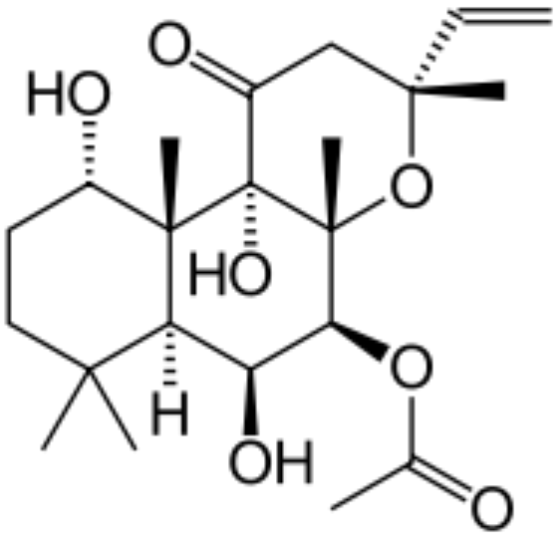

Figure 6: Structure of Forskolin

Forskolin is a natural bicyclic determine molecule that is derived from the Indian Coleus plant (*Coleus forskohlii*). The role of forskolin is to stimulate adenylyl cyclases. The sensitivity to stimulation is determined by the *rutabaga* gene, which is a gene that codes for calcium-sensitive dependent adenylate cyclase. The concentration of forskolin used to elevate intracellular cAMP in the experiment was calibrated to be a half log unit above threshold.

The 100 μM forskolin concentration was found to produce the most conclusive results for use in comparison between the control and Nf1 RNAi groups.

Importance of Forskolin in Pre Versus Post Conditioning

The training period with forskolin in olfactory experimentation served to elevate intracellular cAMP. When cAMP is elevated intracellularly through adynylyl cyclase stimulation, mushroom body neurons drive the compartmentalized elevation of postsynaptic cAMP. In return, this cAMP elevation drives postsynaptic plasticity in the mushroom body of the fly. Thus, comparing pre versus post training levels of calcium would allow for analysis in the role of Nf1 in learning and memory. The pre-training versus post-training odor responses can be represented in Figure 7 below.

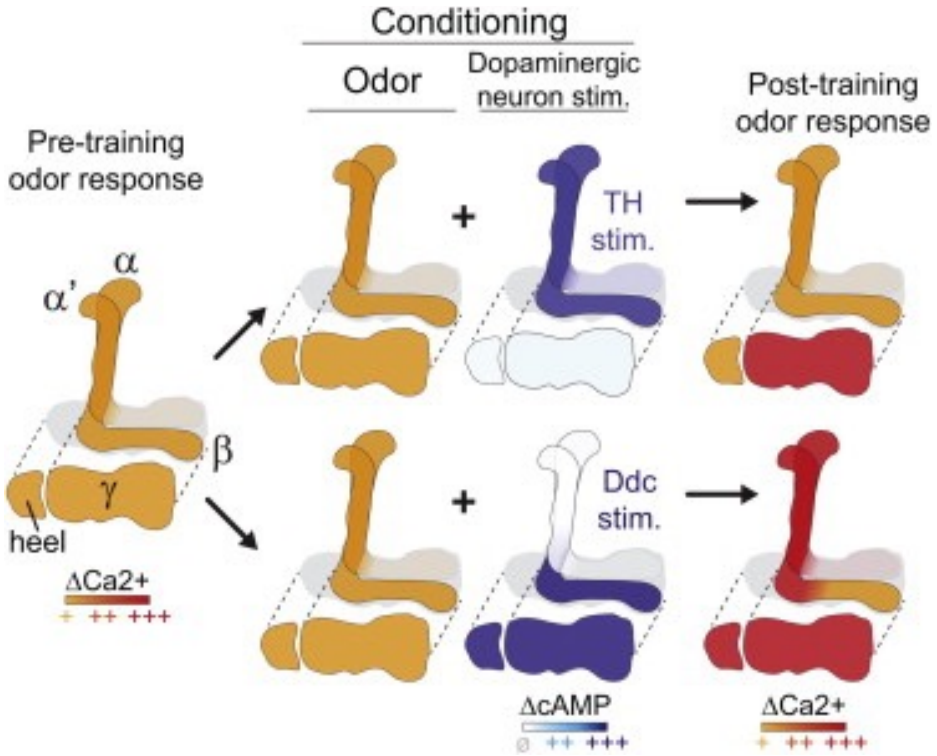

Figure 7: Pre versus Post Training Odor Response

The pre versus post training odor response figure shows odor presentation paired with stimulation of dopamine release from tyrosine hydroxylase (TH) or dopa decarboxylase (Ddc) expressing neurons. Dopaminergic neuron stimulation was with odor presentation according to the experimental procedure outlined in Figure 3. During the training period, bath temperature was increased to 32 °C to thermogenetically activate the Ddc or TH positive neurons. The Ca^{2+} imaging responses for pre-conditioning versus post-conditioning were analyzed. "It was determined that pairing odor with stimulation of TH-GAL4-positive dopaminergic neurons produces aversive behavioral memory and pairing odor with Ddc-GAL4-positive neuron stimulation produces appetitive memory (3)."

It is important to note that there was no effect in control experiments, where heat was omitted or flies lacked either the GAL4 or UAS element. Therefore, it can be concluded that the Ca^{2+} response plasticity in the γ lobe was due to coincident reception of odor and stimulation of dopaminergic neurons via TRPA (3).

The synthesis pathway of dopamine can be demonstrated in Figure 8.

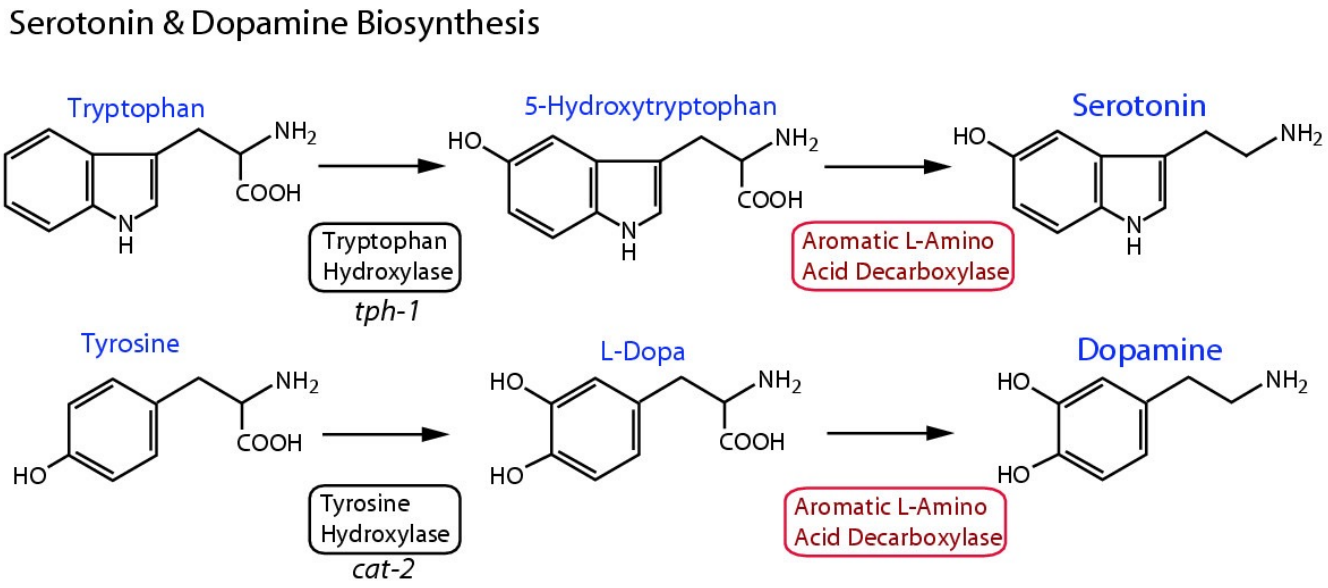

Figure 8: Dopamine and Serotonin Synthesis Pathway

In the sets of experiments, three different areas of the mushroom body were investigated: β-lobe, lower-stalk, and γ-lobe. These areas of the mushroom body can be demonstrated in the graphic developed by the Ron Davis lab at The Scripps Research Institute (37) found in Figure 9.

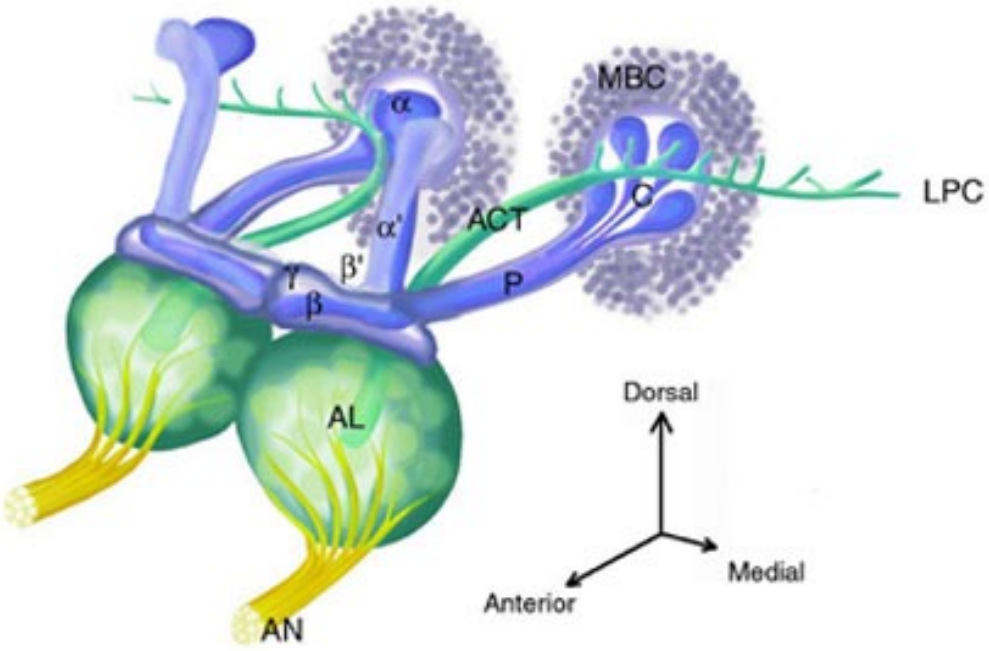

Figure 9: Different Lobes of the Mushroom Body

RESULTS

When data from the olfactory classical conditioning experiment were quantified, it was found that knocking down *Nf1* disrupts memory-related plasticity in the mushroom body. The exact regions of the mushroom body were speculations until after data analysis.

Due to each area of the mushroom body each serving a different purpose, it was important to examine whether dopamine causes uniform or differential effects in the different spatial regions of the dopaminergic neurons in the mushroom body. To accomplish this, the GAL4-UAS expression system (1) was used to drive expression of a reporter in the expression of α/β, α′/β′, and γ mushroom body neurons. The distinct regions of the mushroom body were examined during confocal imaging of the olfactory experiments.

It was important to analyze the dopamine-induced plasticity of the mushroom body in the lower-stalk, β, and γ regions. The odor stimulation produced a positive Ca^{2+} response change in GCaMP6f fluorescence that could be seen through confocal imaging. These values were recorded and quantified using MATLAB software.

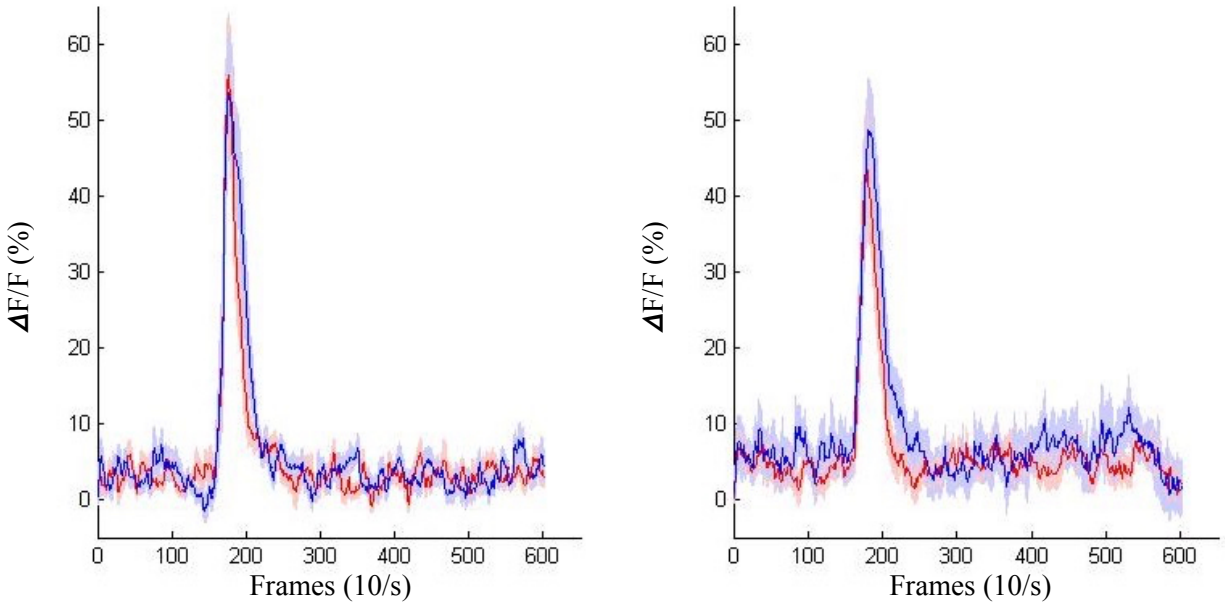

Figure 10: Pre versus Post Conditioning Ca^{2+} Response in Beta Lobe

The control group in the graph is show in blue, and the red is the *Nf1* RNAi group. It can be seen in the graph that there is no significant change in the levels of calcium response after the elevation of cAMP pre versus post conditioning for both groups.

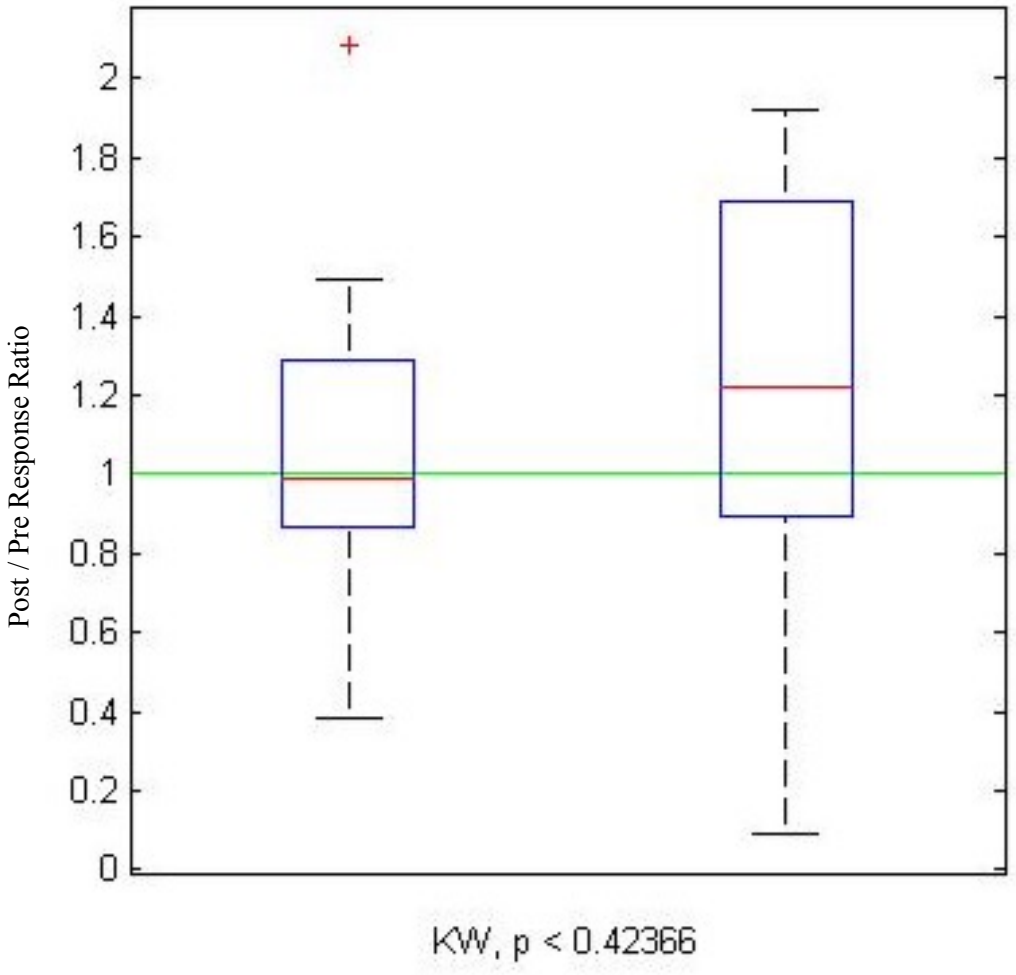

Figure 11: Kruskal-Wallis Statistical Test for Beta Lobe Ca^{2+} Response

The p value of 0.42366 from the Kruskal-Wallis statistical test confirms no significant odor induced calcium response pre versus post conditioning in the beta lobe of the mushroom body.

After finding that there was no significant change in response in the beta lobe pre-conditioning versus post- conditioning, the data from the lower-stalk region of the mushroom bodies in the control and *Nf1* RNAi group were analyzed.

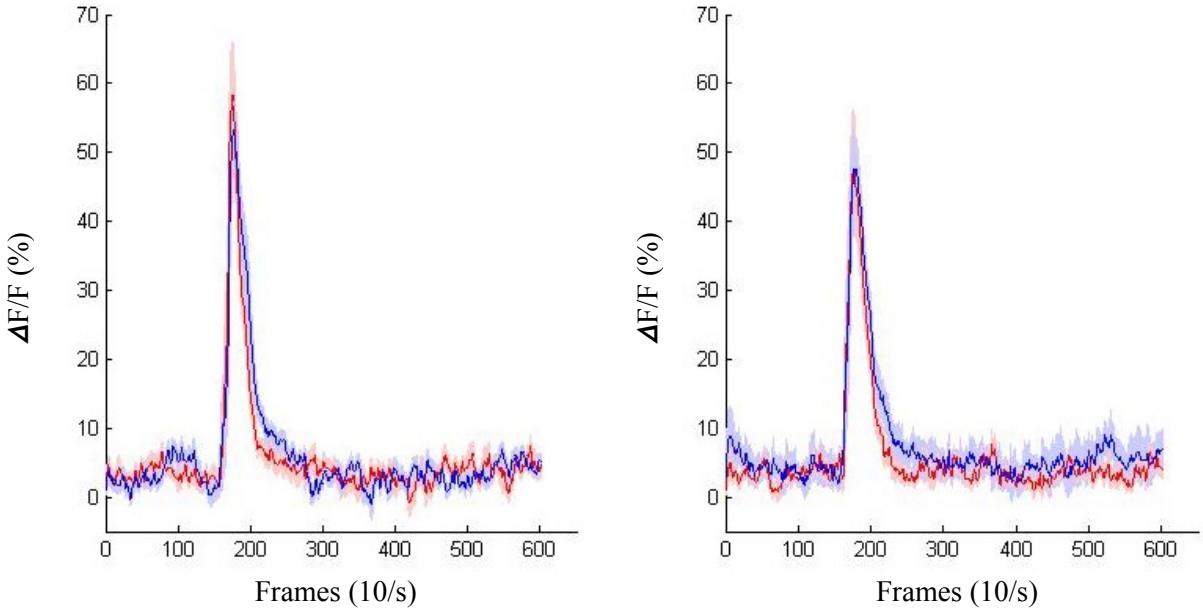

Figure 12: Pre versus Post Conditioning Ca^{2+} Response in Lower-stalk Region

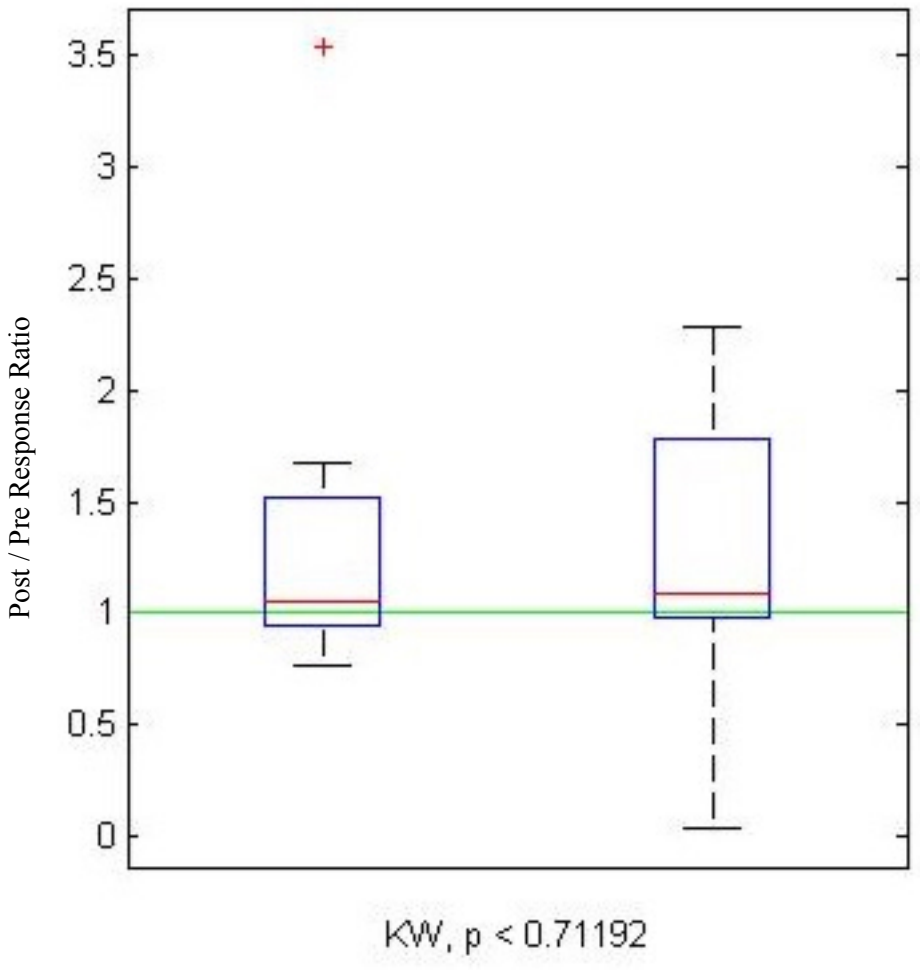

Figure 13: Kruskal-Wallis Statistical Test for Lower-stalk Ca^{2+} Response

The p value of 0.71192 from the Kruskal-Wallis statistical test confirms no significant odor induced calcium response pre versus post conditioning in the lower-stalk region of the mushroom body.

The data from the γ lobe of the mushroom body was quantified, as represented in Figure 14.

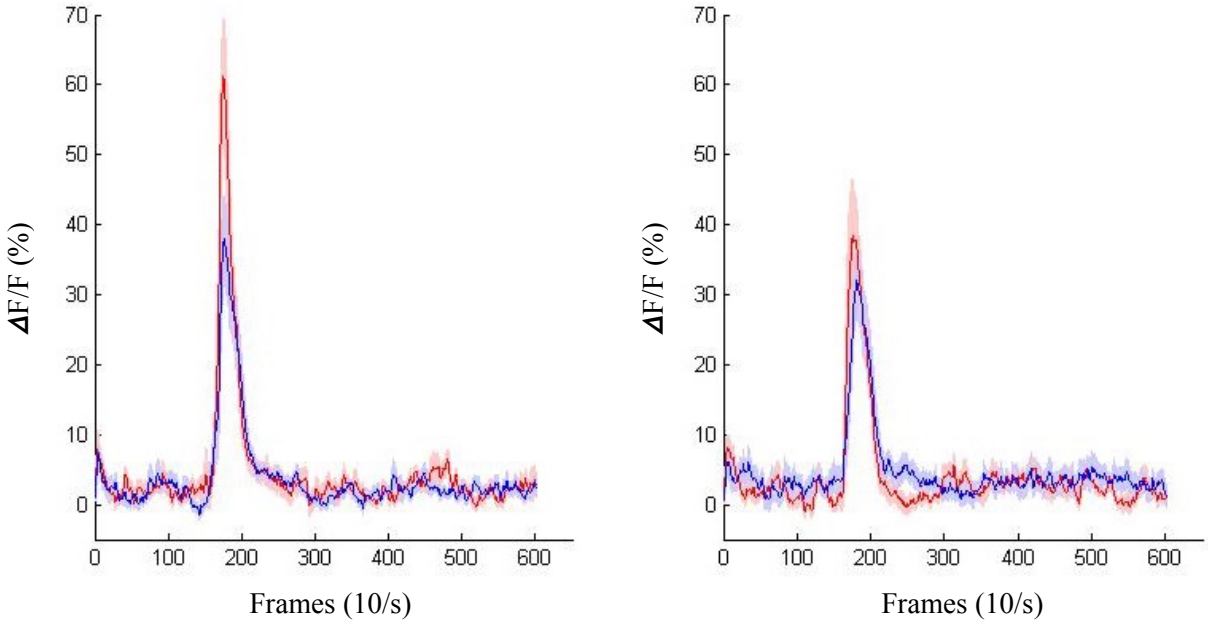

Figure 14: Pre versus Post Conditioning Ca^{2+} Response in γ lobe

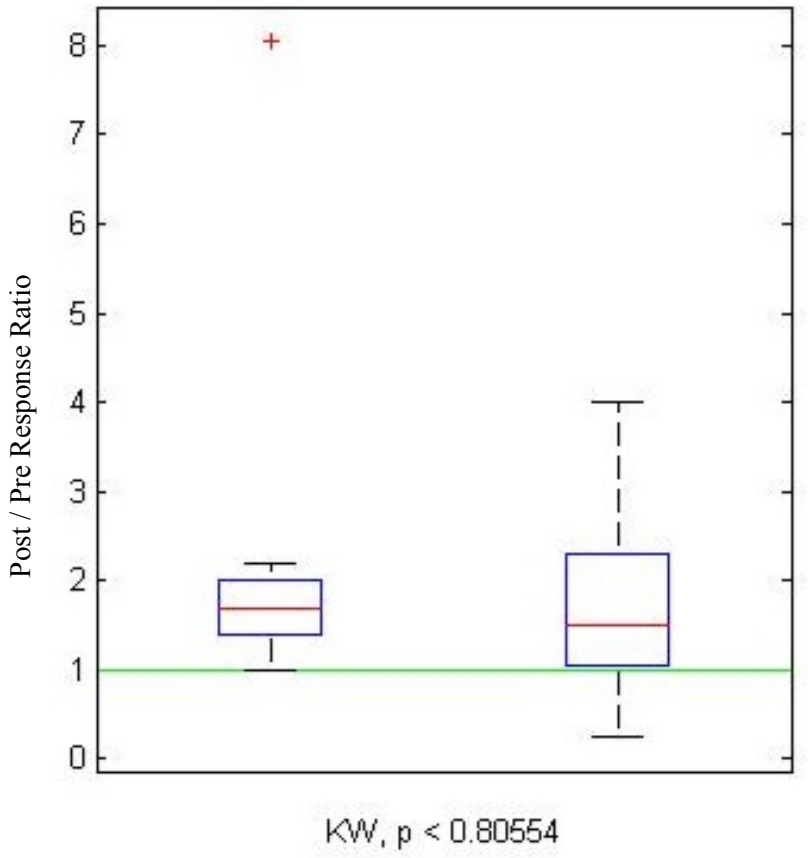

Figure 15: Kruskal-Wallis Statistical Test for γ lobe Ca^{2+} Response

The Kruskal-Wallis statistical test for the γ lobe calcium response data analysis shows a p value of 0.80554. This high value was a result of outliers that were still used in the data sets.

Therefore, it can be seen in Figure 14 that the control group, represented in blue, shows little change in the level of calcium response pre versus post conditioning. In contrast, the *Nf1* RNAi group, represented in red, shows a significant decrease in the level of calcium after conditioning in the γ lobe.

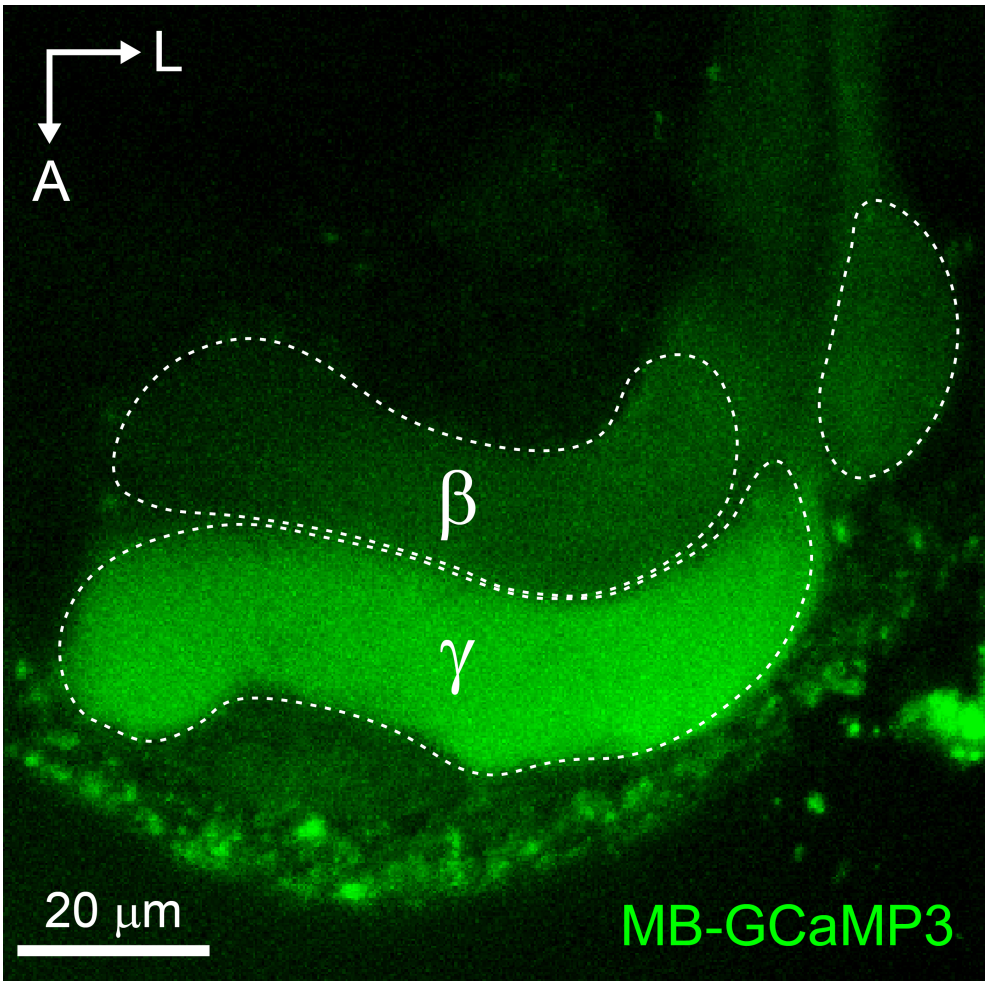

Figure 16: Confocal Fluorescence Microscopy Image of Gamma and Beta Lobes

DISCUSSION

Dopaminergic Neurons Drive Compartmentalized Elevation of cAMP

The results from the experimentation and data analysis revealed that the stimulation of mushroom body neurons using ethyl butyrate in olfactory conditioning caused elevation of cAMP in the *Drosophila melanogaster* mushroom body. In addition, the broad stimulation of dopaminergic neurons causes the broad postsynaptic elevation of cAMP in the mushroom body. The calcium response plasticity that was measured for comparison of pre versus post conditioning in the β lobe, lower-stalk region, and γ lobe of the mushroom body was seen to occur in spatial regions (Figures 10, 12, 14).

Sensitivity of *γ* Lobe Dopaminergic Neurons to cAMP

It can be seen from the calcium response data (Figure 14) that the γ lobe exhibited sensitivity to cAMP caused by the administration of forskolin. This suggests that the spatial pattern of plasticity is directly related to the cAMP sensitivity in the γ lobe dopaminergic neurons. Based on these findings, it can be suggested that a potential mechanism for localization of short-term, learning-related plasticity exists. This phenomenon is caused from synaptic vesicle release in or from the mushroom body γ lobe neurons (11).

A study in 2013 showed that “blocking γ lobe output led to severe deficiency of aversive early memory retrieval and partial impairment of appetite early memory retrieval (15)”. In the γ lobe neurons, training decreases synaptic vesicle release elicited by the unpaired conditioned stimulus, while leaving presynaptic activation by the paired conditioned stimulus unchanged (11).

It is important to note that the direct elevation of cAMP caused specific regional and concentration dependent calcium response plasticity. The effect was not acute because the forskolin was washed out of the chamber with saline before confocal imaging for calcium responses. This shows that the γ lobe is the most sensitive to the elevation of cAMP. According to the data findings, the γ lobe is responsible for short-term learning and memory in the mushroom body. There is also known to be different roles for the other lobes of the mushroom body in regards to learning and memory such as the role of β lobe neurons in long-term memory acquisition (15).

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