Non-Genetic Manipulation of Serotonin in Central Nervous System of Drosophila Melanogaster through Ingestion of 5HTP-Containing Food

An Honors Thesis (HONR499)

By

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Abstract

The Serotonergic System is a major component in the function and integration of brain circuitry. Serotonergic neurons have long cable-like structures called axons. The developmental process of axon elongation and branching is fundamental to the function of the nervous system (Gaspar et al., 2003). Studying the morphology, structure, and function of axons has been difficult due to limitation in traditional staining and imaging technologies. Expansion Microscopy and Brainbow imaging pose a new avenue to study these detailed axon morphologies. The protocol implemented through this experiment was developed to better enhance the advantages of these innovative technologies. This protocol is a non-genetic manipulation to increase serotonin, 5HT, in the Central Nervous System (CNS) of *Drosophila melanogaster (Drosophila).* Genetic approaches to manipulating serotonin exists, but do not allow either concentration or temporal variances to be studied. This goal of this experiment was to show that administering a 5HTP-infused food to the *Drosophila* larvae would increase the levels of serotonin in the 3rd instar *Drosophila* larvae Central Nervous System. In this protocol, a serotonin precursor, 5-hydroxytryptophan (5HTP), was added to the food during the 2nd instar larval stage to increase 5HT levels in the 3rd instar *Drosophila* larvae CNS. Through both immunohistochemistry and imaging analysis of pixel intensities, quantification of 5HT in the CNS of *Drosophila* was obtained for experimental (5HTP-infused food) and control groups. The results indicated this protocol increased the 5HT levels in the CNS of 3rd instar *Drosophila* larvae of the experimental groups with statistical significance. These results show that this protocol was successful and could possibly be used for further experimentation in Brainbow *Drosophila*. This allows for detailed study of changes in brain circuitry at varying levels of serotonin.

Acknowledgements

I would like to thank Dr. Douglas Roossien for the countless hours of mentorship, lab space, and overall commitment to the success of this thesis. I would like to thank the Honors College Fellowship Program that funded this project, alongside Start-Up funds provided by the Vice Provost for Research to Dr. Roossien.

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PROCESS ANALYSIS STATEMENT

As a STEM student, I have taken many lab classes and conducted a multitude of different experiments. In classes, these experiments have been done repeatedly and were designed to obtain a desired outcome. I joined the Roossien Lab in October of 2019 feeling ready, prepared, and excited to conduct novel research. This is where I found that the "Experimental Method" I studied in every science class was not as simple as it seemed in textbooks. I was put on my first project, which was adding 5HTP, a precursor of the neurotransmitter serotonin, to *Drosophila Melanogaster* larvae. My initial thoughts were along the lines of, "This is going to be easy," and, "This is kind of boring." That was not the case, not even in the slightest. My first academic skill I had to learn to master was reading, a lot, of scientific literature on a subject I was very new at; developmental neuroscience. The reading was dense and hard, but I began to love what I was doing and truly understand the importance of my "easy" project. I was creating a protocol, which meant that I needed to combine methods of different papers with similar projects and modify them to fit serotonin. This was way harder than I expected it to be. I began by copying a protocol used for dopamine through a Neckameyer 1996 paper. This failed, and I had to begin dissecting what was going wrong and why. The biggest issue initially, was that my experimental group and control group were identical. Were the flies not eating the 5HTP within the food? Was there not enough 5HTP in the food? The next skill I began to master was the skill of problem solving. In textbooks, the experiments have one variable, and you simply change that variable to obtain a new outcome. When starting from scratch everything was a "new" variable, therefore intense research in the literature, along with trial and error was the only way to dissolve the problems. The first successful solution came about when I discovered that, unlike dopamine, serotonin does not successfully suspend in water and requires an organic solvent. This raised a new problem despite it solving the

previous problem. *Drosophila Melanogaster* is a fruit fly and, although simple organisms, they were quite temperamental when it came to their food. DMSO, an organic solvent, was at the top of the solubility list. The flies did not like DMSO in their food and would either die from starvation or die from consuming the DMSO. I ran a multitude of side experiments trying to find what solvent the serotonin would successfully suspend in and the flies would eat and thrive. Roughly 5 experiments later, I found a food combination that worked! A huge step in the right direction, but only solved my second of many other problems. At this point in the process, I had felt defeated, frustrated, and all other emotions that accompany failure. This was where I think I learned the most about myself. I learned that I loved science, and I wasn't just doing it because it was decent career choice. I woke up in the morning and thought about my project; what I could do, what I think could work, and what I should try. I learned perseverance. Science is not simple and it's not easy. Once I found the food that worked, I added the 5HTP, and ran the experiments, and although in previous the food trials the larvae survived, this time they were incredibly too small. An important part of the protocol was dissecting the *Drosophila Melanogaster* brains, staining them with immunofluorescence, and imaging these samples on a confocal microscope. The larvae are tiny when they are the appropriate size, so it was impossible to dissect these larvae when they were incredibly smaller than normal. This lead back to the food, I hypothesized that the food I had tested was edible but lacking proper nutrition for the *Drosophila Melanogaster* to thrive. The moment in which I was able to trouble-shoot and propose a solution to this problem on my own was a highlight of this project that I will never forget. I proposed that the larvae be raised on an agar plate, which is used for the first half of the protocol, and then add the 5HTP food to the agar but with a colored food dye. The dye was safe for the flies and did not alter any concentration differences in the food and allowed me to select the larvae that had ate the food as dye stains their digestive tract. I ran

the experiment and it worked! I was able to obtain healthy size larvae that ingested the 5HT-treated food, dissect, stain, and image them for analysis.

This project gave me a true and insightful look into what it means to be a scientist. I discovered a spark inside of me that was lit by research. I had originally joined the lab, in part, as a resume booster for medical school. I was very interested in neuroscience and I knew having research experience would make me a more competitive medical school applicant. What I didn't know is how much I would fall in love with the work. As I finished my junior year, I took the MCAT, started my medical school application, and couldn't figure out why I couldn't send the applications in. It was because I was passionate about research. This project was literally life changing. I changed my career, I applied to graduate school, and got accepted into Vanderbilt University Doctor of Philosophy in Biological Sciences Program. I have been on cloud nine ever since. I learned so many biotechnological skills, problem-solving skills, research techniques, but the most vital and treasured insight I gained from this project was finding my home in science. This thesis embodies the work that I did and expertise I gained through this process. It's the outcome of one of the best journeys I took in my undergraduate career.

INTRODUCTION

Serotonin (5HT) is a prevalent neurotransmitter and is a fundamental component in neurological development. Serotonin is produced by a class of neurons in the nervous system called serotonergic neurons. Serotonergic neuron function and integration within brain circuits is achieved through long cable-like structures called axons. Therefore, the developmental process of axon elongation and branching is fundamental to the function of the nervous system (Gaspar et al., 2003). In mammalian nervous systems, serotonin neuron cell bodies are located in very distinct clusters along the midline of the brainstem, while their axons innervate nearly every area of the Central Nervous System (CNS). Developmental signals from serotonin neurons modulate a multitude of different developmental events such as neuronal migration, cell differentiation, cell division, and synaptogenesis (Daubert & Condron, 2010). Yet, previous pre-clinical models demonstrate targeted deletions of the 5HT receptors do not cause gross abnormalities in neurological development (Daubert & Condron, 2010), suggesting that the role of serotonin during development is ancillary to its function as a trans-neuronal neurotransmitter.

Serotonergic neurons express serotonin receptors (serotonin autoreceptors) on their own plasma membranes. This provides an intrinsic feedback mechanism allowing serotonin neurons to sense extracellular serotonin levels through autoreceptor activation and downstream signaling cascades. This process, called serotonin autoregulation, can influence axon development in serotonergic neurons. For example, genetic deletion of the serotonin catalytic enzyme dopadecarboxylase in *Drosophila* causes an increase in axon length and an overly branched morphology of a serotonergic neuron in the peripheral nervous system (Budnik et al., 1989). *Drosophila* larval brains treated with external serotonin show a decrease in pre-synaptic

varicosities (Sykes & Condron, 2005). Previous reports demonstrate that genetically increasing the amount of serotonin increases axon branching. Together these observations suggest serotonin autoregulation limits axon growth, branching and the formation of pre-synaptic varicosities. Defects in serotonin autoregulation during development could therefore be problematic, as they create aberrantly developed axons. However, previous experimental approaches have been limited in their ability to observe complete serotonergic systems in a developing brain. As serotonergic axon innervations have recently been implicated in neuropsychiatric diseases and disorders (Hornung, 2003) developing a direct link between serotonin autoregulation and changes in axonal morphology in complete serotonergic systems can help us understand novel aspects of these diseases and disorders.

Most studies on serotonin autoregulation have relied on the model organism *Drosophila*. *Drosophila* as a model for brain development offers many advantages such as a short life cycle, large numbers of offspring, diverse manipulation of genetic techniques, a well-known anatomy, and a variety of stable mutants. The *Drosophila* life cycle is 13.5 days at 25 degrees Celsius. The flies have six distinct life stages: embryo (1 day), first instar larvae (1 day), second instar larvae (1 day), third instar larvae (2.5-3 days), pupae (3.5-4.5 days), they go through metamorphosis and become adult flies. The flies are in each stage for a known amount of time allowing for detailed analysis of development. Additionally, studying molecular mechanisms in *Drosophila* is advantageous as it can be studied and understood in comparison to human brain disease since many fundamental molecular pathways are conserved (Frazer & Hensler, 1999). Previously, both genetic and pharmacological tools have been utilized to manipulate serotonin levels in the brain paired with fluorescent visualization of axonal morphologies (Budnik et al., 1989; Gaspar et al., 2003; Sykes & Condron, 2005). While these have provided fundamental insights into the process of

serotonin autoregulation, they relied on the use of traditional labeling and imaging technologies. The highly branched and convoluted morphology of individual serotonergic neurons are difficult to resolve using these traditional methods. The problem is compounded by the number of serotonergic neurons in close proximity in the nervous system; it becomes near impossible to distinguish one axon from the other. Therefore, how the serotonin manipulations affect the complete network of serotonergic axons is still unclear.

A novel and innovative solution to this current problem is to use a multispectral three-dimensional labeling and imaging approach called Brainbow. Brainbow is a genetic cell-labeling technique where hundreds of different hues can be generated by stochastic and combinatorial expression of a few spectrally distinct fluorescent proteins (Li et al., 2020). Recently, a novel transgenic *Drosophila* line was developed that utilizes cell-type specific promoters to express these fluorescent proteins selectively in the desired cell type (Li et al., 2020; Veling et al., 2019). We can thereby label each serotonergic neuron in the *Drosophila* nervous system a unique composite color. These brain samples can be further processed for Expansion Microscopy (Tillberg et al., 2016), a technique that allows for super-resolution microscopy and resolving highly convolved structures. Altogether this approach allows us to digitally reconstruct and analyze the complete serotonergic system in a single *Drosophila* brain. While a broad objective is to manipulate serotonin levels in developing Brainbow *Drosophila*, existing methods for these manipulations need to first be adapted to become amenable to Brainbow labeling, imaging, and Expansion Microscopy.

There have been previous studies done to manipulate serotonin in *Drosophila*, such as genetically modifying the fly to over or under express serotonin (Budnik et al., 1989; Daubert et

al., 2010). While beneficial this does not allow for "dose control" nor precise temporal control. We know through these studies that serotonin axon behavior is different at incredibly high and incredibly low concentrations, but we don't know at what level do these disruptions begin. Another approach that has been used in Sykes and Condron 2005, is to perform explant culture of the *Drosophila* brain and then treat them with externally applied serotonin. In the interest of this study, the Sykes and Condron approach doesn't allow for selectivity of the serotonergic neurons because any neuron with a serotonin receptor will respond to the serotonin in the cultured brain. This potentially confounds interpretation of data by influencing more processes than just serotonin autoregulation.

In order to specifically isolate and test serotonin autoregulation, manipulations of serotonin need to be selectively within the serotonin neurons themselves. Following this, Neckameyer administers 5-hydroxytryptophan (5-HTP), a serotonin precursor, to the flies' diet (Neckameyer, 1996). In cells, tryptophan is enzymatically converted to 5-HTP by L-tryptophan-5-monooxygenase. Following this, l-amino acid decarboxylase converts 5-HTP to 5-HT, 5-hydroxytryptamine, also known as serotonin. This synthesis can only occur in the serotonin neurons because the enzymes required for synthesis reside only in serotonergic neuronal cell bodies (Fraser, 1999). While convenient and effective, the Neckameyer protocol relied on high performance liquid chromatography (HPLC) to measure the amount of serotonin in pooled samples of brains. This limits the ability to directly correlate serotonin levels to anatomical changes in a single brain. Moreover, HPLC is not amenable with the paraformaldehyde fixation required for Brainbow imaging.

Here we sought to develop a convenient and reliable method for manipulating serotonin in the developing *Drosophila* brain that is compatible with Brainbow imaging, Expansion Microscopy and allows for independent serotonin quantification in each sample. Our protocol was adapted from Neckmeyer's diet administration approach. In brief, 5HTP was administered to first instar larvae by suspension in liquid *Drosophila* food with a dye indicator for consumption. Next, larvae identified to have ingested the liquid food were dissected and immunolabeled using an antibody targeted to serotonin. Serotonin quantity was estimated using fluorescent confocal microscopy and showed an increase in serotonin in serotonergic neurons from larvae grown on liquid food with serotonin compared to negative controls. Since Brainbow labeling and imaging relies on the same immunolabeling protocol these methods can easily be combined in future experiments. The ability to collect quantitative serotonin data at the point of Brainbow image

acquisition will enable direct correlation between serotonin quantity and changes in serotonergic neuron morphology and circuit structure in each brain. This will ultimately allow us to examine the effects of serotonin autoregulation on the development of the serotonergic system.

MATERIALS AND METHODS

Egg lay and 5HTP Administration

Fly Husbandry. Wild type w^{1118} flies were reared at 25°C on standard fly food (NutriFly powder cooked with MiliQ water and propionic acid at a 533g:2L:14.4mL dilution). Egg lay dishes were prepared using 29.49 % v/v(mL/mL) of grape fruit juice, 2.95 % m/v (g/mL) of agar, 1.01 % v/v (mL/mL) of ethanol, 69. 50 % v/v (mL/mL) of DI water and 0.05% w/v (g/mL) of Methly Paraben (methyl 4-hydroxybenzoate)per plate. This mixture of agar and water was mixed and then

autoclaved. The methyl paraben and grape-fruit juice was added after the mixture was autoclaved, and then the entire media was poured into a petri dish and placed in 4C let to solidify until needed for egg lay. Yeast paste was added to the egg lay media plates prior to starting the egg lay.

Egg lay. At time zero, t=0, flies were placed into an egg lay chamber with an egg lay plate for 4 hours at 25C. At t=4 hours, the egg lay was stopped, and the adult flies were placed back into the bottle, and the egg lay dish containing the embryos was placed back into the 25C incubator. At t = 24 hours, embryos were age matched by removing any hatched larvae.

5HTP Administration. Preparation of Liquid Food was 5mL of DI water, 0.5g yeast extract, 0.5g glucose, 0.375 g of sucrose. 5HTP was suspended in ethanol and mixed with liquid food at a 1:3 ratio with final concentrations of 10 mg/mL. At t=60 hours 10 second instar larvae were placed on each condition with a total of 30 instar larvae. Condition 1 contained 200 µL of Condition 1 food, Condition 2 contained 200 µL of Condition 2 food, and Condition 3 had 200 µL of Condition 3 food. The larvae were left on these dishes for 12 hours, and at t=72 hours the third instar larvae were dissected, fixed, stained, and imaged.

Dissection and Fixation of Larval Brains

Drosophila brains from the larvae at t=72 hours were dissected in PBS at room temperature (RT). All tubes used for the storage of the brains and all pipette tips used for transferring the brains were coated with 2.5 % BSA. Dissected brains were fixed in 4% PFA (Sigma #P6148, diluted in PBS) at RT and then gently rocked for 30 minutes. Prior to transferring the brains to blocking buffer (PBS+1% Triton X-100), the brains undergo three quick PBS washes for 5min x 3.

Immunohistochemistry

Brains were incubated in blocking buffer overnight at 4C rocking gently. All steps were done with gentle rocking. After blocking, the brains were incubated with rabbit anti-5HT (Immunostar 20080) primary antibodies at a 1:500 dilution in PBS with 0.3% Triton X-100 for 2 days at 4°C. Three PBS washes 15min x 3 were done, before the brains were incubated with donkey anti-rabbit Alexa488 secondary antibodies diluted 1:500 in PBS with 0.3% Triton X-100 for 2 days at 4°C. Finally, PBS washes for 15min x 3 were done. The brains were then mounted using Vectashield.

Confocal microscopy

To ensure accuracy in quantitative fluorescent image analysis, all brains were imaged using identical imaging conditions and in a single imaging session. Mounted brains were imaged on a Zeiss LSM 5 using a 20x NA 1.2 objective. Alexa488 was excited using an Argon laser set to 75% power through a 220 nm pinhole. Emission was filtered using a 515-560 BP filter and collected at 360 gain. Images were collected at 2 µm Z-intervals, 300 nm x 300 nm resolution in x, y, and stored as 16-bit with no binning.

Image analysis

Image analysis was done through the Fiji application through NCBI. Each file contains a Z-Stack with 2 micrometer intervals. Twenty-five neurons were labeled per brain, and all Z-stack images of each neuron were traced. The free hand tool through the Fiji app was used and on one single neuron, each frame where signal was present the neuron was traced and measured. The measurements taken per frame were the area, maximum, minimum, mean, integrated density and

raw integrated density. The raw integrated density and integrated density were used for analysis, as these quantities provide a sum total of the pixel intensity or an average intensity, respectively, in each cell. This is important as fluorescent signal is indirectly related to the amount of serotonin. For each neuron there was roughly 7-11 measurements taken. These different numbers pulled from the images measured different aspects of pixel intensity. For each neuron, all of the raw integrated densities were summed and this number was recorded. The average of the integrated densities per neuron was recorded. This was done for 25 neurons per brain. Further analysis was done by taking the average of the sum of the raw integrated densities per cell body and the averages of the averaged integrated densities per cell body.

RESULTS

5HTP Administration on a combination of solid and liquid food

We chose the Neckameyer 1996 protocol for our initial attempt at 5HTP administration, which raises larvae from 1st through 3rd instar stages (72 hours) in a mesh-bottomed chamber set in a liquid food source containing 5HTP. We found that larvae were small, showed developmental delays, and had a low survival rate. This suggested the larvae were malnourished, likely from the limited access to nutrients available in the solution in the mesh chamber. We therefore excluded the mesh chamber in subsequent experiments in favor of a 12-well dish. The larvae were noticeably small for their stage of development, and were sedentary in comparison to wild type. The larvae were compared on a gridded sheet and the larvae living in the mesh chamber were roughly 1/3 the size of the wild-type larvae. It became quickly apparent that the larvae were not thriving in the mesh and 12-well plate environments. We found a modest improvement in larval survival, but development through larval stages still lagged. Moreover, when we quantified serotonin we found

no significant difference between brains from larvae raised on food with 10 mg/mL 5-HTP and brains raised on food without 5-HTP (data not shown). Altogether this suggested access to adequate nutrition with substantial 5-HTP remained a barrier to increasing serotonin levels in the serotonergic system.

We implemented two further modifications of the protocol to overcome this barrier. We first reasoned that dissolving 5-HTP in an organic solvent, rather than water, would increase the amount available for ingestion. We additionally reasoned that including a solid complete food source in addition to the liquid food would increase access to adequate nutrition to support normal larval development. We therefore performed food trials with either dimethyl sulfoxide or ethanol. We found that larvae raised on Liquid Food with 5mL of DI water, 0.5g yeast extract, 0.5g glucose, 0.375 g of sucrose, and 10 mg/mL 5-HTP on top of an egg lay plate developed to 3rd instar and were of normal size, suggesting the larvae had adequate access to nutrition. Whether the larvae consumed only solid food or a mixture of solid and liquid was unclear. We therefore added food coloring to the liquid food, which can be observed in the larval gut to confirm liquid food ingestion prior to brain dissection. Altogether this new protocol allowed us to raise healthy and developmentally normal larvae with access to 5-HTP through their diet.

Administration of 5HTP through Diet Increases Serotonin in the Larval Brain

Having developed a suitable protocol for 5HTP administration, we next asked whether this increased serotonin levels in serotonergic neurons in the larval brain. After 72 hours on food plates containing 5-HTP or ethanol control (no 5-HTP), brains from 3rd instar larvae were dissected and probed for serotonin using immunofluorescence. After 3D confocal imaging, fluorescent emission was used as an indirect measurement of the amount of serotonin in serotonergic neurons (Fig. 1A-

D). For our first quantitative comparison we chose to measure the raw integrated densities in every plane of focus for each serotonergic neuron and sum these together, resulting in a "total serotonin" measurement in arbitrary fluorescent units (AUs). We found the average total serotonin in neurons from larvae raised on 5-HTP food to be 3.02 times higher ($p < 0.01$ by unpaired two-tailed t-test) than in ethanol control brains (Table 1; Fig 1E). While *Drosophila* serotonin neurons are approximately the same size, it is possible that size differences between neurons under different experimental conditions can arise. To account for this, we also analyzed the integrated pixel densities, which gave us an average pixel intensity per neuron. When these were averaged together for each experimental condition, we also found a 2.6-fold increase ($p < 0.01$ by unpaired two-tailed t-test) in serotonin in neurons from larvae raised on 5-HTP food compared to ethanol controls (Table 1; Fig 1F). Therefore, we can conclude that our protocol for 5HTP administration caused an increase in serotonin levels in the *Drosophila* larval brain.

DISCUSSION

Here we sought to describe a reliable means to pharmacologically increase the amount of serotonin in the developing *Drosophila* larval brain by administration of the serotonin precursor 5-HTP through diet. By using immunohistology, confocal microscopy, and quantitative fluorescent analysis, we showed the protocol produces a robust increase in serotonin level in serotonergic neuron in 3rd instar larval brains.

A vital aspect in the success of this protocol was the use of primary and secondary antibody staining. This staining uses a single primary antibody that places a tag on 5HT. The use of a specific rabbit anti-5HT (Immunostar 20080) primary antibodies isolate the 5HT within the fly brain. The secondary antibody, donkey anti-rabbit Alexa488, has an immunofluorescent protein that attaches

to the primary antibodies. The secondary antibody creates signal with the 488 laser in the confocal microscope. The use of a single and specific chemical antibody tagging system ensures that the signal produced in the images is only from the 5HT within the CNS of the fly (Lidov & Molliver, 1982). When analyzing the images, one can safely assume that more serotonin means more binding of the primary antibody and more binding of the secondary antibody, thus, producing more fluorescent emission from the sample to be detected by the digital microscope. This emission is coded as pixel intensities in the two-dimensional image space, which we used for analysis in this study. Therefore, pixel intensity is an indirect but reliable way to quantify serotonin in the brains for use in statistical analysis. The staining regime is well-established ensuring reliable and accurate data collection. Nonetheless, there are still some discrepancies that may occur through this type of data collection.

Two sources of discrepancies arise from the nature of confocal fluorescent imaging. First, convoluted signal may be produced as the brain tissue samples are 3-dimentional, and out-of-focus signal may be detected and placed in the measurements taken. However, our use of integrated density measurements minimize this, and the results were consistent with our total serotonin measurements per neuron (Fig 1). Second is the potential of background signal. Precision and accuracy of the measurements could be increased by the use of calculating the background noise and incorporating that value into the analyzed data sets. While consistent imaging conditions theoretically average this error out across samples, further analysis would benefit from this calculation to decrease potential error in comparing data sets from different experimental runs.

The largest obstacle that needs to be approached for furthering the success of this protocol is to repeat with varying concentrations of 5-HTP, and administration at different developmental

time points. After such, we will have a tunable and temporal means for selectively increasing serotonin levels in serotonergic neurons in the developing *Drosophila* brain. Understanding the development of axon morphology, structure and function of serotonergic neurons can give insight and avenues to answer some large questions in the field of neuroscience. By applying this protocol to *Drosophila* expressing Brainbow in serotonergic neurons, we can develop novel insights into the mechanisms of serotonin autoregulation of serotonergic axon morphology.

FIGURES AND TABLES

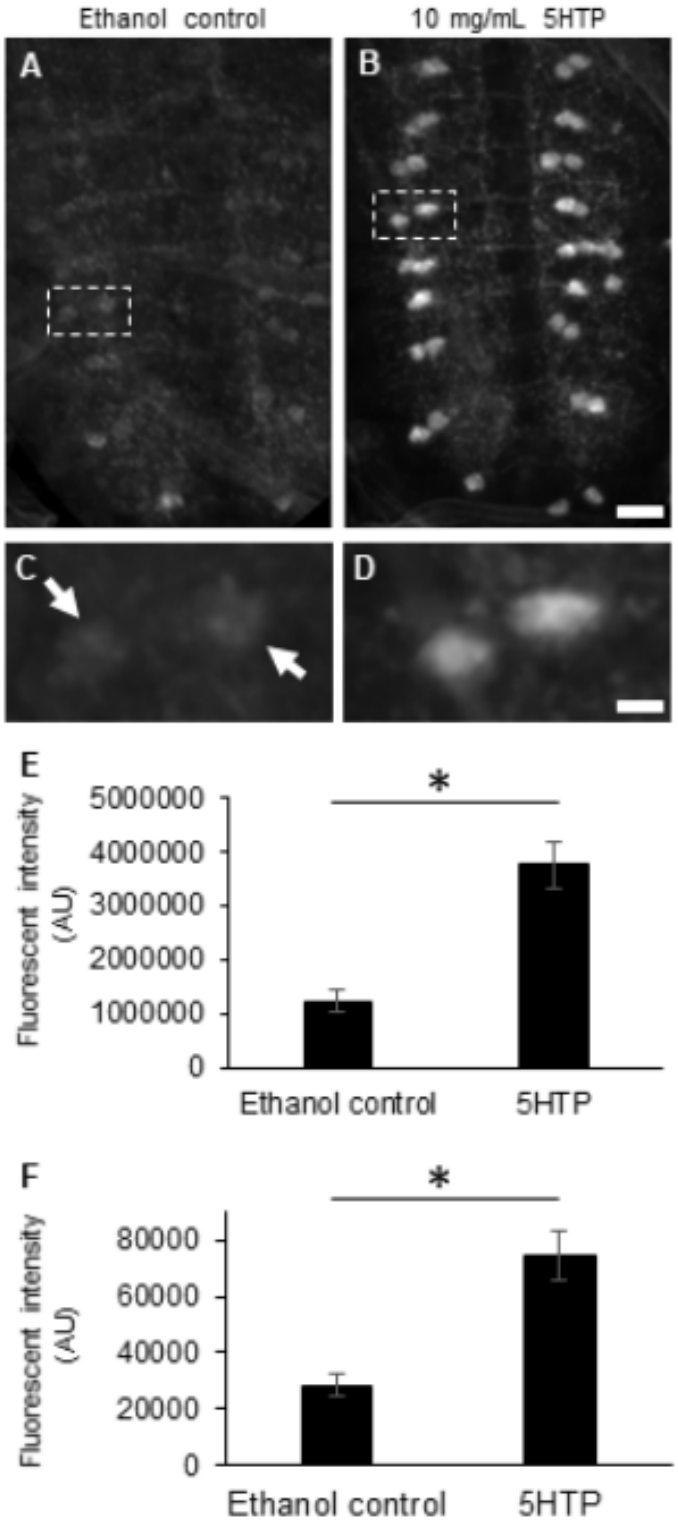

Figure 1: 5-HTP administration in larval diet increases serotonin in the *Drosophila* central brain. (**A-B**) Abdominal regions of the 3rd instar larval ventral nerve cord after immunolabeling for serotonin and imaged under identical conditions shows an observable increase in fluorescent intensity in brains from larvae raised on 5HTP-containing food. (**A**) Negative control brain from a larva raised on food with ethanol and no 5HTP. (**B**) Brain from a larva raised on food with 10 mg / mL 5HTP. (**C-D**) Magnified pairs of serotonergic neurons from control and treated brains. Arrows in (C) identify the location of the two weakly fluorescent serotonergic neurons from the brain in (A). (**E**) Bar graph displaying average total cell fluorescence from 50 neurons per condition shows a statistically significant increase in serotonin in brains from larvae raised on 5HTP-containing food. (**F**) Bar graph displaying average cell fluorescence from 50 neurons per condition shows a statistically significant increase in serotonin in brains from larvae raised on 5HTP-containing food. * $p < 0.01$ by student's unpaired, two-tailed t-test. Scale bars in (A-B) = 40 µm. Scale bars in (C-D) = 5 µm.

Table 1: Summary of Raw Data Collection

Raw Integrated Density				
		Mean (A)	**Standard Deviation (AU)**	**n**
	Untreated Control	1243729	702935.7	50
	5HTP	3758291	1491273	50
Integrated Density				
	Untreated Control	28349.15	13567.81	50
	5HTP	74587.1	32192.03	50

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Office of Research Integrity
Institutional Biosafety Committee (IBC)
2000 University Avenue
Muncie, IN 47306-0155
Phone: 765-285-5106

DATE:	February 19, 2020
TO:	Douglas Roossien, Ph.D.
FROM:	Ball State University IBC
RE:	IBC Protocol # 1567980-1
Title:	Development of the Drosophila central nervous system
Submission Type:	New Project
Action:	APPROVED
Decision Date:	February 19, 2020
Expiration Date:	

The Institutional Biosafety Committee (IBC) has recently reviewed and approved the above protocol.

Approved Biosafety Level: [X] BSL-1 [ ] BSL-2

If human participants are involved in this research, IRB reference #: [enter IRB reference #]

Approved Animal Biosafety Level: [ ] ABSL-1 [ ] ABSL-2

If animals are used as part of the research, IACUC reference #: [Enter IACUC reference #]

Editorial Notes

PI's and their research team are required to abide by any requirements listed above, outlined in their protocol and/or by the following (as applicable):

- Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition
- NIH Guidelines for Research Involving Recombinant DNA Molecules
- 7 CFR Part 331 and 9 CFR Part 121USDA Possession Use and Transfer of Select Agents and Toxins
- Any other applicable Federal or State regulation or University policy applicable to the above research

As a reminder, it is the responsibility of the P.I. and/or faculty supervisor to inform the IBC in a timely manner:

- when the project is completed,

- if the project is to be continued beyond the approved end date,
- if the project is to be modified,
- if the project encounters problems, or
- if the project is discontinued.

Any of the above notifications must be submitted electronically to the IBC through IRBNet (https://www.irbnet.org/release/index.html). Please reference the IBC protocol number given above in any communication with the IBC or the BSU Office of Research Integrity (ORI) regarding this project. Be sure to allow sufficient time for review and approval of requests for modification or continuation. If you have questions, please contact Chris Mangelli at (765) 285-5070 or cmmangelli@bsu.edu.

Phillip Smaldino, PhD/Chair
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