Testing Nebivolol's Effects on Zebrafish Swim Patterns Using a Behavioral Assay

By

Cameron Brown

A.B. Brown University, 2021

Thesis

Submitted in partial fulfillment of the requirements for the degree of

Master of Science in the Department of Pathobiology

at

Brown University

Providence, Rhode Island

May 2022

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Date_____ _____
Robbert Creton, Ph.D., Advisor

Date_____ _____
Jill Kreiling, Ph.D., Reader

Approved by the Graduate Council

Date_____ _____
Andrew G. Campbell, Ph.D.
Dean of the Graduate School

Preface and Acknowledgements

The experiments described in this thesis were conducted as a 5th Year Master's student in the Creton Laboratory at Brown University from September 2021 to April 2022. The work was performed in collaboration with and the advice from Robbert Creton, the principal investigator for this research, Thaís Del Rosario Hernández, the research assistant, Rahma Ibrahim, an undergraduate student, Lily Zhou, an undergraduate student, Sayali V. Gore, a postdoctoral research associate, and Rohit Kakodkar, a research software engineer.

Table of Contents

List of Figures

List of Tables

Background

What is Alzheimer's?

Alzheimer's is a devastating disease that affects millions of individuals around the world. Alzheimer's is a progressive neurodegenerative disorder that is the most common cause of dementia[1]. While it is a multifactorial disease, age is the strongest risk factor for the onset of Alzheimer's. The older an individual gets, the more at risk they are for developing the disease and with our increasing aging population, Alzheimer's is becoming a major public health crisis. With that being said, aging is not the only risk factor. Other risk factors occur over our lifespan that can increase the likelihood of developing the disease. This includes genetics or family history like carrying the APOE e4 allele, psychosocial factors, high blood pressure, high BMI, diabetes, cerebrovascular disease, and environmental exposure over one's lifespan[1]. Along with causing debilitating physical and emotional effects, Alzheimer's is a financial burden as well. "The 2009 reports from Alzheimer's Association showed that in the US the annual costs for patients with AD and other dementia were estimated to be US $148 billion plus US $94 billion unpaid care service, and that AD tripled health care costs for Americans aged 65+ years"[1]. So not only do patients have to worry about how they will physically change with the disease, but they also must worry about the financial toll that this will take on them as well.

Alzheimer's is associated with the loss of function, or the death, of neurons[2]. The disease initially causes damage to neurons in areas of the brain that are associated with memory such as the entorhinal cortex and the hippocampus[2]. As AD progresses, it also causes damage to neurons in the cerebral cortex[2]. The cerebral cortex is important for language, reasoning, and social behavior, so once neurons in this area of the brain are damaged, function of these skills are also lost[2]. Due to the loss of neurons, Alzheimer's comes with a plethora of symptoms. These

symptoms include changed behavior, impaired memory, difficulty with language and cognitive functions, as well as overall impairments in daily life[1]. Tasks that were once considered simple become increasingly difficult once a patient begins to develop Alzheimer's.

There are many proposed mechanisms for the progression of AD and its symptoms. The most common cause for the onset of the disease is the loss of dendritic spines and synapses along with the accumulation of amyloid plaques and phospho-tau tangles[3]. Dendritic spines are crucial for memory, so their deterioration makes it very difficult for the patient to remember old memories or form new ones as well. Amyloid plaques develop from the breakdown of larger proteins known as amyloid precursor proteins[2]. Once those amyloid plaques break off and begin to accumulate, they collect between the neurons and disrupt the function of the cell, leading to impaired memory[2]. Phospho-tau tangles accumulate when abnormal chemical changes in the brain cause the tau to detach from microtubules[2]. The loose tau molecules then come together and form threads that eventually lead to tangles[2]. The accumulation of tau is detrimental because they block the neuron transport system and in turn disrupt the synaptic connection between neurons[2]. The loss of dendritic spines and the accumulation of amyloid plaques and tau tangles occurs in regions of the brain associated with memory and language, which is why there is serious cognitive deterioration as the disease progresses[2] .

Prevention

Alzheimer's is a major public health crisis, worldwide. Currently, the UN Aging Program and US Centers for Disease Control and Prevention project that the number of older individuals will increase from 420 million in 2000 to 1 billion by 2030[1]. With that increase in elderly population also comes an increase in patients with Alzheimer's. Knowing that the cases of Alzheimer's are going to continue to rise, there has been a push to find a way to prevent the

disease. As of right now, there is not an FDA-approved drug to treat Alzheimer's, nor is there any definitive method to prevent the onset of disease. Researchers have found that even "...modest advances in therapeutics and preventative strategies that lead to even a 1-year delay in the onset and progression of clinical AD, would significantly reduce the global burden of this disease"[1]. With this in mind, large amounts of time, money, and effort have been put into understanding the pathogenesis of Alzheimer's to develop a therapeutic that can treat or prevent the disease, and in turn finally address this growing global health issue.

There have been a few proposed drugs that can be used to treat Alzheimer's. "Currently, United States Food and Drug Administration (FDA) approved AD therapeutics target cholinergic (Aricep) and/or glutamatergic signaling (Memantine) and can improve memory and daily cognitive function," but they do not fully address the primary pathology of Alzheimer's[3]. Improving cognitive function is great, but if the sources of degeneration and impaired memory are not addressed, then there is always the chance for the impairments to come back or for the disease to progress. One of the main focuses of treatment research is dendritic spine loss. If dendritic spine loss can be slowed or stopped, then neurodegeneration could also be slowed in the process[3]. Another target for treatments is amyloid plaque accumulation. Some drugs have been used to target amyloid accumulation, and while they have shown to positively improve amyloid levels in patients with familial AD, they do have significant side effects[3]. Although amyloid levels were improved, they were not significant enough to warrant FDA approval[3]. The lack of progress with these treatments is frustrating, but it is challenging researchers to find more creative ways to treat and prevent Alzheimer's. A potential target for therapeutics that has been gaining popularity in recent years, and is an important component of this study, is calcineurin.

What is Calcineurin?

Calcineurin is a protein phosphatase that is widely expressed in the brain[3]. More specifically, calcineurin is expressed in postsynaptic dendritic spines[3]. Calcineurin has a few roles within dendritic spines and within the brain. Typically, calcineurin helps to maintain the plasticity of spines as well as promote learning, memory, and long-term potentiation[3]. It has proven to be useful for promoting brain health, but it has also shown to have devastating effects when it functions abnormally. "In vitro, animal and human studies have implicated the excessive activation of the protein phosphatase calcineurin (CN) as an early step in the pathogenesis of AD"[3]. This hyper-activation has proven to be a significant factor that can lead to the progression Alzheimer's[4].

There are a few proposed mechanisms for how calcineurin contributes to Alzheimer's pathogenesis. One of them being that through calcineurin-mediated dephosphorylation, triggered by amyloid signaling, prolyl isomerase (Pin1) is suppressed[3]. The suppression of Pin1 by calcineurin leads to the reduction of dendritic spines and synapses, which promotes the progression of the disease [3]. Pin1 has also been connected to Alzheimer's due to its role in regulating amyloid precursor protein cleavage and the hyperphosphorylation of tau[3]. Through calcineurin's suppression of Pin1, dendritic spines deteriorate, amyloid plaques and tau tangles accumulate, and Alzheimer's can take hold in the brain. With this new connection between calcineurin and the progression of Alzheimer's, inhibiting calcineurin could be the key to treating or preventing Alzheimer's.

Calcineurin Inhibitors

Inhibiting the excessive production of calcineurin could prevent the onset of Alzheimer's. tacrolimus (FK506) and cyclosporine (CsA) are two main compounds that are known to inhibit

calcineurin. Tacrolimus is an immunosuppressant that is generally used for organ transplant recipients[3]. So far, researchers have found that patients who take this medication had a much lower incidence of Alzheimer's compared to their age-matched control population[3]. Seeing the protective potential of tacrolimus, further studies were done to try and understand how this drug leads to a decreased risk of developing this disease. Researchers found that, "Blockade of CN with FK506 (tacrolimus) restored long-term potentiation, normalized behavior in AD-model mice, and prevented synapse loss associated with Aβ42 overproduction or mutant tau overexpression"[3]. As great as these findings are, FK506 is still an immunosuppressant, which can be detrimental for an individual to take on a regular basis. So, researchers are still on the hunt for a drug that can inhibit calcineurin and prevent Alzheimer's.

Another compound that has had similar effects to tacrolimus is cyclosporine. Cyclosporine is also an immunosuppressant. Initially used for kidney transplant patients, cyclosporine has remained the gold standard treatment for transplant patients of all kinds as well as treating numerous autoimmune diseases[5]. CsA, as well as other calcineurin inhibitors, inhibits calcineurin by binding to intracellular proteins called immunophilins[5]. Once this complex between cyclosporine and immunophilins is formed, they bind to calcineurin which then leads to inactivation[5]. Cyclosporine's inactivation of calcineurin, similarly to FK506, has shown to provide neuroprotection through the reduction of neuroinflammation, improving synapse function, and inhibiting cognitive deterioration and loss[6]. Patients taking cyclosporine also rarely develop Alzheimer's[7]. With that being said, the same issue with FK506 is seen with CsA. With cyclosporine being an immunosuppressant, it is not an ideal compound to give to patients wanting to treat Alzheimer's. Not only is the immune system suppressed and weakened with cyclosporine, but there is also the chance of significant damage to the kidneys[5]. With these

concerns in mind, a new goal has been put in place to find another drug that is a calcineurin inhibitor, or behaves similarly to the calcineurin inhibitors described above, without dangerous side effects.

Previous Research Done by Creton Lab

Previous experiments have tried to find compounds that have similar effects as cyclosporine. Using drugs that are already FDA-approved, researchers in the Creton Laboratory set out to test over a hundred compounds in zebrafish to see if they produce similar effects on behavior as cyclosporine[7]. In this study, zebrafish that were five days post fertilization were treated with 10µM of one of the 190 compounds from the Tocris small-molecule library[7]. After being treated with the compounds, the fish sat in the treatment in an incubator for 2 hours before being transferred to 96-well ProxiPlates[7]. Once transferred to the plates, the fish were shown a 3 hour behavioral assay and imaged in a temperature controlled cabinet[7]. The behavioral assay consisted of a 3 hour PowerPoint presentation that had a series of visual and acoustic stimuli[7]. The first hour period was without visual or acoustic stimuli, then 80 minutes of visual stimuli only, then a 10 minute period of no visual or acoustic stimuli, followed by a final 30 minutes of only acoustic stimuli[7]. During the assay, a camera took pictures of the fish every 6 seconds. ImageJ was used to analyze the behavior of the fish during the assay[7]. From there, any changes seen in the activity, excitability, startle response, habituation, excitability and optomotor responses were compared to the DMSO samples and summarized into a behavioral profile[7]. Compounds with similar behavioral profiles were then grouped together using a hierarchical cluster analysis[7].

To determine which compounds were similar to cyclosporine, the same protocol and behavioral assay was used on zebrafish treated with CsA[7]. From these experiments, they found

that cyclosporine-treated fish had increased excitability, increased activity, decreased habituation and decreased optomotor responses compared to zebrafish treated with DMSO[7]. Compounds from the small-molecule library that had these same effects on zebrafish behavior were then grouped together. These compounds were then known as 'CsA-type' drugs[7]. Some of these drugs included tetrabenazine, XL184 and nebivolol hydrochloride[7]. For this study, nebivolol is the drug of interest.

Nebivolol

Nebivolol is FDA-approved adrenergic beta-1 receptor antagonist that is used to treat hypertension[8]. In Europe, nebivolol is also used to treat heart failure[8]. As a third generation beta-blocker, nebivolol increases the bioavailability of nitric oxide[9]. Through the release of endothelial nitric oxide, nebivolol creates vasodilation, effectively decreasing peripheral blood pressure[10]. Nebivolol is not only tolerable and safe, when it comes to treating hypertension, but it has also shown to have neuroprotective effects as well. Studies have shown that nebivolol significantly reduces amyloid neuropathy in the brain[11]. Nebivolol also acts as an estrogen receptor (ER) agonist[12]. This is significant because estrogen has shown to have neuroprotective effects in acute and chronic neurodegenerative disorders like Alzheimer's and Parkinson's[12]. Previous research has shown that nebivolol has the potential to be repurposed for other diseases, including Alzheimer's[12].

This is not the first time that medications used to treat hypertension have been considered when it comes to treating Alzheimer's. Some studies have had promising results regarding the protective effects of hypertension medication. One study found, "...Substantially less Alzheimer neuropathological changes (i.e., neuritic plaque and neurofibrillary tangle densities) in the medicated hypertension group than the non-hypertensive group, which may reflect a salutary

effect of antihypertensive therapy against AD-associated neuropathology"[1]. They also found that the younger the patient was, and the more time they spent on the treatment, the better the effects of the medication[1]. Seeing the potential beneficial effect that antihypertensive medication can have on patients with Alzheimer's could provide some hope of eventually finding a therapeutic for the disease. Nebivolol's neuroprotective effects have made it a compound of interest, and the next step is to determine if it could be an alternative for cyclosporine.

Goals

Before nebivolol is approved as a therapeutic for Alzheimer's, further experiments need to be carried out to not only confirm that this drug has the same therapeutic effects as cyclosporine, but to also determine the correct concentration and dosage of this compound. During the past year, this study has set out to learn more about nebivolol, how it affects zebrafish behavior and larval swim movements. The first goal of this study was to confirm whether nebivolol behaves similarly to cyclosporine. If I can confirm that nebivolol is a CsA-like compound, the next goal is to determine which concentration of nebivolol elicited effects on zebrafish that were most like cyclosporine. Finally, after collecting thousands of images of larval swim movements, the goal was to create a protocol that allows for larval swim movements over different periods of time to be collapsed into one image to analyze and characterize the common swim patterns seen during the behavioral assay.

Methods

Note: The protocols used for treating the zebrafish, running the behavioral assay, imaging the fish, analyzing the data, and the statistical analysis were the same as the methods used in the Creton Laboratory and the study "Novel use of FDA-approved drugs identified by cluster analysis of behavioral profiles."

Approval for Animal Experiment

All experiments carried out in this study were in accordance with federal regulations and guidelines for the ethical and humane use of animals. All experiments have been approved by Brown University's Institutional Animal Care and Use Committee (IACUC). In this study I followed the PREPARE, 3R, and 3S guidelines.

Zebrafish

The animal model used in this study was Zebrafish. Zebrafish are an excellent model for Alzheimer's-related studies because their neuroanatomy and neurochemical pathways are very similar to the human brain[13]. Due to their similar brain make up, zebrafish have been used to successfully simulate AD pathology and tauopathy[13]. This animal model allows researchers to gain a better understanding of how Alzheimer's affects the brain. It also provides a way to test how different pharmacological treatments affect living models. Zebrafish are also a great model because they have a short generation time, are easy to maintain in the lab, and they can produce many embryos that only need a few days to grow before being used in experiments[13]. For all experiments in this study, wild type zebrafish larvae, that were 5 days post fertilization (dpf), were used.

Egg Collection

Zebrafish eggs were collected using Pyrex dishes that were covered with a mesh lid that contained green yarn. The green yarn was used to replicate seaweed and encourage the zebrafish to breed. The Pyrex dishes were placed in each tank and the zebrafish were allowed to breed in each dish for 2 hours. After the 2 hours, all the eggs were strained from the Pyrex dishes and moved to a new, large container that was filled with egg water. Egg water is a combination of Instant Ocean in deionized water mixed with methylene blue. The large container was labeled with the date of collection and then placed in the incubator at 28.5°C for 5 days. Periodically, the container was checked and any unfertilized eggs, seen by their white color, were removed from the container. After 5 days, the larvae were ready to be used in experiments for that day.

Pharmacological Treatments for Confirmation Experiments

For the three confirmation experiments, 5dpf zebrafish were exposed to either 10µM dimethyl sulfoxide (DMSO) or 10µM nebivolol. The nebivolol stock solution was provided by Fisher Scientific. The 10mM stock solution of nebivolol was diluted 1000x by being dissolved in DMSO and separated into smaller vials. Those vials were frozen so that they could be used in later experiments. For the nebivolol treatment, 9.6 mL of egg water and 9.6 microliters of 10uM nebivolol were added to a petri dish. For the DMSO treatment, 9.6mL of egg water and 9.6 microliters of 10uM DMSO were added to another petri dish. 96 zebrafish were placed in each petri dish. The fish were exposed to either nebivolol or DMSO for a total of 6 hours throughout the entire experiment. They were treated in the petri dish with egg water and DMSO or nebivolol and incubated for 3 hours before a zebrafish, along with the treatment, was transferred to each well of two 96-well ProxiPlates. The wells in the first row contained DMSO-treated fish and the wells in the second row contained nebivolol-treated fish. This pattern was continued until both

ProxiPlates were filled. Finally, the fish were imaged in the plates in the treatment solution for 3 hours. This procedure was repeated for all 3 confirmation experiments.

Pharmacological Treatments for Concentration Experiments.

For the concentration experiments, 5dpf zebrafish were treated in either 10uM DMSO, 5 uM nebivolol, 10uM nebivolol, or 20uM nebivolol. The nebivolol treatment groups were first split into 5mM, 10mM, and 20mM stocks. Then each stock was diluted 1000x with DMSO for a final concentration of 5uM, 10uM, and 20uM nebivolol. Again, the final concentrations were split into vials and then frozen for later use. There were 48 zebrafish for DMSO, 48 zebrafish for 5uM nebivolol, 48 zebrafish for 10uM nebivolol, and 48 zebrafish for 20uM nebivolol in each experiment. 4.8 microliters of each concentration group were added to their own petri dish along with 4.8mL of egg water. The whole process of adding the treatment and incubating the fish took 3 hours. After being treated, a zebrafish and their treatment was added to a well of a 96-well ProxiPlate. The rows alternated with each treatment group. The first row was DMSO, the second row was 5uM nebivolol, the third row was 10uM nebivolol, and the fourth row was 20uM nebivolol. This order was continued until two ProxiPlates were filled. The plates were then imaged in the treatment groups for 3 hours during the PowerPoint presentation. This procedure was repeated for all 3 concentration experiments.

Imaging Cabinet

For all experiments, a temperature-controlled cabinet was used to image the fish. This cabinet, which stayed at 28.5℃ due to a heating pad, was equipped with an 18-megapixel Canon EOS Rebel T6 with an EF-S 55-250mm f/4.0-5.6 IS zoom lens camera that was connected to a continuous power supply (Canon ACK-E10 AC Adapter). The camera was controlled by a laptop that used Canon's Remote Capture software (EOS Utility, version 3). The zebrafish, located in

the white 96-well ProxiPlates, were placed on a glass stage with two speakers (Office Tec USB Computer Speakers Compact 2.0 System) attached. The speakers were connected to the laptop through a USB, and they were set to the maximum volume. Beneath the glass stage was a M5 LED pico projector (Aaxa Technologies) that has a 900 lumens LED light source and displays the 3-hour PowerPoint presentation through the opaque bottom of the 96-well plates. The glass stage holds four 96-well plates, so the automated analysis of behavior was in a 384-well format. In this study, only two plates were filled with zebrafish, so two empty 96-well ProxiPlates were added to the stage. The plates filled with fish were placed on the top left and right corner of the stage and the two empty plates were placed on the bottom left and right corner of the stage to keep the 384-well format for the analysis.

Behavioral Assay

As mentioned before, to carry out the behavioral assay, a 3-hour Microsoft PowerPoint Presentation was shown to the larvae. The same presentation was used for all experiments in this study. The presentation contains visual and acoustic stimuli. A broader view of this presentation can be broken down into 4 sections. The first part of the presentation is an hour of no visual or acoustic stimuli, then the second part includes 80 minutes of visual stimuli, followed by a 10 minute period without visual or acoustic stimuli, and then a final 30 minutes with just acoustic stimuli. It is important to note that the larvae were never exposed to visual and acoustic stimuli at the same time. It was either one or the other, or no stimuli at all.

To get more specific, there are 18 ten minute periods that make up the entire 3 hour presentation. Periods 1-6 have no visual or acoustic stimuli. Period 7 has downward red lines and Period 8 as upward red lines. Period 9 has downward green lines and Period 10 has upward green lines. Period 11 has downward blue lines and period 12 has upward blue lines. Period 13 has

downward red lines that are now fast and period 14 has upward red lines that are also fast now. Period 15 has no visual or acoustic stimuli. Period 16 has an acoustic stimulus that includes a pulse that occurs in 20 second intervals. Period 17 has the same acoustic stimuli, except now it occurs in 1 second intervals. Finally, Period 18 has the same acoustic stimuli occurring in 20 second intervals.

The visual stimuli included a series of moving lines that were either red, green, or blue because previous studies have shown that zebrafish tend to swim in the direction of moving lines that they are presented with[7]. The behavior to swim in the direction of the lines is coined the optomotor responses or the OMR[7]. The lines in the presentation were 1mm thick and they were 7mm apart from each other. The normal moving lines moved 7mm per 8 seconds in either the upward or downward direction. For the fast red lines, the lines were the same size, but they moved at a faster speed of 7mm per 0.5 seconds, which is 16 times faster than the original set of red lines. For the acoustic stimuli, brief pulses (100ms, 400Hz) that were created in Audacity as 20 second soundtracks were used in the PowerPoint.

Image Analysis

ImageJ, or FIJI, was used to analyze the 1,800 images that were produced from each experiment. A macro (version 26rc062019) was created and used to carry out automated analysis of behavior of the zebrafish in the 384-well format. The macro can analyze images from four 96-well plates at a time that contain different treatment groups as well as all the different visual and acoustic stimuli. Before the macro is started, the users must input information about the plates and periods with different stimuli. The software begins by opening the first image and then splits the color channels. Next, it selects the channel where the visual stimuli and background stimuli have similar intensities. The images are then subtracted from each other which removes the

background and highlights the moving larvae. Next, the macro adds a threshold and then selects the first well and measures the area and centroid of the larvae. That data is then added to a results file. This process is then automatically repeated for all wells in the images and then all the images in the file. How frequently the larvae move, as well as their position, is analyzed and included in the results file. The information from the results file is copied and pasted into a Microsoft Excel template. Data from each of the 3 experiments is combined in a second Microsoft Excel template which provides a combined results file for all 3 experiments together.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel. The measurements of the larval movement and position were averaged in each well during each 10 minute period and then those values were averaged between larvae in the same treatment group. The differences between the experimental groups and the control groups were tested for significance. Then a non-parametric Chi-square test was used because larval behaviors do not follow a normal distribution.

Swim Pattern Analysis

Another macro on ImageJ was created to analyze the swim patterns of the zebrafish in each experiment. The macro allows for the individual to open a folder that contains up to 100 images. A text box was added to provide options for the line color of the images used, the number of images, and the starting image that lets the macro know where it should start. If there were no visual stimuli on the images chosen, the red color was chosen. Two stacks, shifted by one image, are then created. The macro then splits the color channels of both stacks into red, blue, and green. Then depending on the line color on the images and the color chosen in the first step, it closes out the other color channels and only leaves open the color that represents the line

color of the images. The macro then subtracts the two stacks and collapses the stack of images. Then a predetermined threshold is set and the background of the images is darkened. The stacked images that contain the larval movements are then given a red threshold and the 96 well plates are darkened and saved as a jpeg. Finally, the image of the larval movements overlaid onto a darkened image of the 96-well plates and a final image is given. The final image contains all the movements of a zebrafish in each well during the predetermined time interval. For periods 2-6, the swim patterns were analyzed in 10 minute intervals. For periods 7-18, the larval movements of the zebrafish were analyzed in 2 minute, 5 minute, and 10 minute intervals. Period 1 was done in 8 minute intervals for all experiments.

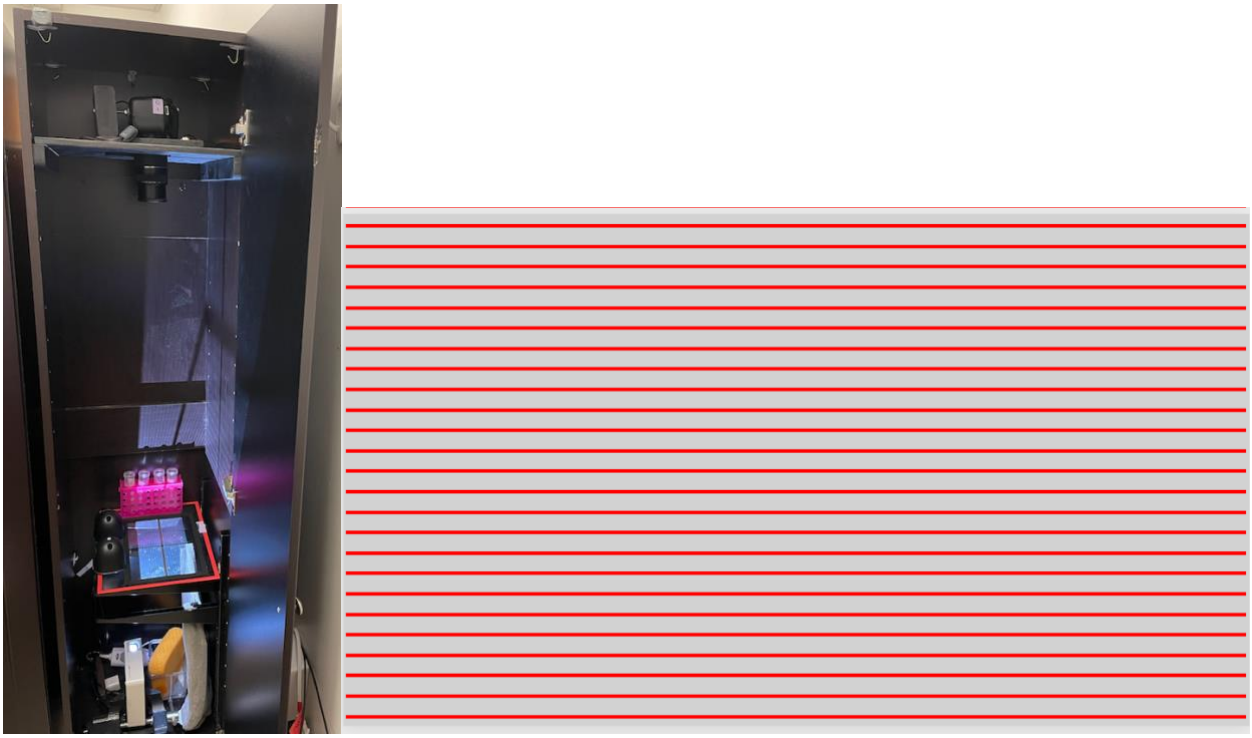

Figure 1: Left Image: Picture of the imaging cabinet used for the behavioral assay. The camera is located on the top shelf of the cabinet and takes pictures of the glass stage where the 96-well ProxiPlates sit. The projector is located underneath the glass stage. Right Image: Example of one of the periods from the behavioral assay. This is an image from period 7, which contains red lines moving in a downward motion.

Results

Confirmation Experiments

To create an overview of the changes in behavior, behavioral profiles were created by calculating the differences in behavior in comparison to the control DMSO-treated fish. This allows for the comparison of effects on zebrafish behavior between different treatment groups. An excel sheet was used that shows the different categories used to show the differences in behavior. Those categories are Difference in Activity and Difference in Vision. Within the Difference in Activity category, there is 1hr, P15, Hab, S, and E. 1hr refers to the average activity in periods 1-6 and P15 is the average activity in period 15. These two groups have no visual or acoustic stimuli present[7]. Hab is habituation to acoustic stimuli at 1 second intervals, or period 17, and S is the startle response to acoustic stimuli in 20 second intervals[7]. The habituation to acoustic stimuli is important because zebrafish without treatment can habituate to the frequent sound pulses that occur in the one second intervals. On the other hand, fish treated with cyclosporine struggle to habituate, and they stay increasingly excited during period 17. The information from habituation is used to determine if the compound is creating similar effects as cyclosporine. Finally, E is excitability in reference to acoustic stimuli in 1 second intervals[7].

The Difference in Vision category is split up into R, G, B, FR, and RGB. R is the optomotor response to moving red lines, G is the optomotor response to moving green lines and B is the optomotor response to moving Blue lines[7]. FR is the optomotor response to fast moving red lines and RGB is the combined optomotor response to moving lines of any color or speed[7]. Within the excel sheets, some of the data will be colored a red or a green color. Red represents an increase of more than 10% and green represents a decrease of more than 10%. These colors show us that there was a major increase or decrease compared to the other data points. The startle

response is not a major focus in this study, but the results are still included to aid in the overall understanding of how nebivolol affects zebrafish behavior.

The results from these experiments were not only compared to cyclosporine, but they were also compared to previous experiments on nebivolol, using the same behavioral assay, in the Creton Laboratory[7]. If the compound is to be considered CsA-Like, it must behave like cyclosporine and have similar trends within the excel sheet. Table 1 shows the results from a previous experiment on cyclosporine in the Creton Laboratory. In this experiment, cyclosporine induces hyperactivity and increased excitability, suppresses visually guided behavior, and reduces habituation to acoustic stimuli (Table 1). This is seen in the positive numbers for 1hr, P15, and E, as well as the negative, or very low, numbers in vision (Table1). The data from the same experiment, except using nebivolol now, show a similar increase in 1hr, P15, and E (Table 1). There was also a decrease in the optomotor responses as well (Table 1). These experiments show that nebivolol behaves similarly to cyclosporine and that it should be considered a CsA-like compound.

	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
Group	1hr	P15	Hab	S	E	R	G	B	FR	RGB
DMSO	0	0	0	0	0	0	0	0	0	0
CsA	10.84	21.97	-7.29	-3.23	20.73	0.43	-0.81	-7.37	-4.90	-4.49
Nebivolol	4.97	13.99	-9.34	-6.21	15.86	-16.16	-4.67	-16.62	-19.59	-16.52

Table 1: Table containing the data analysis from one of the previous behavioral assay experiments in the Creton Laboratory. The experiment tested Cyclosporine (CsA) and Nebivolol. Numbers in red represent an increase of more than 10%. Numbers in green represent a decrease of more than 10%.

Data from the first confirmation experiment only aligned with cyclosporine in the increase in activity in 1hr (Table 2). The other categories within the Difference in Activity section, including P15, Hab, and E, did not match with cyclosporine or previous experiments carried out on nebivolol (Table 2).

Group	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
	1hr	P15	Hab	S	E	R	G	B	FR	RGB
T1H9	2.6	-12.2	1.7	-6.2	-0.9	1.9	-7.3	-0.4	-1.8	-2.0
DMSO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 2: Table containing the data analysis from the behavioral analysis of the first confirmation experiment.

The effects on vision in the first confirmation experiment were similar to cyclosporine in that there was a decrease in vision (Table 2) The only exception was seen in R, and there was a slight increase for this line color (Table 2). Overall, the results from this experiment were different from cyclosporine and previous experiments done on nebivolol. They are not what one would expect from a compound that is clustered with cyclosporine.

The second confirmation experiment had results that were more around the lines of how a CsA-like compound would affect zebrafish behavior. There was an increase in excitability in 1hr and P15 (Table 3). There was a decrease in habituation, which is a major characteristic of cyclosporine behavior (Table 3). There was also an increase of more than 10% in excitability (Table 3). The vision section showed a decrease in optomotor responses for all line colors (Table 3). Again, these results all align with cyclosporine and previous experiments carried out on nebivolol.

Group	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
	1hr	P15	Hab	S	E	R	G	B	FR	RGB
Nebivolol	9.6	4.8	-9.1	3.2	12.0	-11.9	-11.2	-14.1	-17.9	-15.5
DMSO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3: Table containing the data analysis from the behavioral assay of the second confirmation experiment.

The third confirmation experiment also had results that were similar to cyclosporine and other nebivolol experiments. There was an even larger increase in 1hr and E (Table 4). The habituation in experiment 3 wasn't as negative as experiment 2, but there was still a decrease

(Table 4). The vision was decreased in all categories and was the largest decrease in vision out of all the experiments (Table 4).

	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
Group	1hr	P15	Hab	S	E	R	G	B	FR	RGB
DMSO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nebivolol	26.6	3.8	-0.9	0.8	16.4	-23.9	-12.5	-24.3	-36.2	-26.0

Table 4: Table containing the data analysis from the behavioral assay of the third confirmation experiment.

After all the experiments were analyzed, their data was combined into one excel sheet to get a general understanding of how nebivolol affected zebrafish behavior in the confirmation experiments. In this combined sheet, 1hr, Hab, and E were all increased (Table 4). There was also a decrease in all categories of vision (Table 4). There was also a decrease for P15 (Table 4).

	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
Group	1hr	P15	Hab	S	E	R	G	B	FR	RGB
DMSO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nebivolol	12.4	-3.8	-5.0	1.8	15.7	-14.4	-8.6	-14.3	-18.9	-14.9

Table 5: Table containing the data analysis from the behavioral assay of all 3 confirmation experiments combined.

Concentration Experiments

The concentration experiments used the same excel sheet template and criteria to analyze the effects of nebivolol on zebrafish behavior. As mentioned before, 3 concentration experiments were carried out using the same 3 hour behavioral assay. The images were placed into ImageJ and the data from those results were uploaded into the excel template. Once again, the 1hr, P15, Hab, and R, G, B, FR, and RGB were the main points of interest when trying to see how different concentrations of nebivolol affect the zebrafish.

In the first concentration experiment, 5uM and 10uM nebivolol were the most similar to cyclosporine. They both had a large increase in 1hr and an increase in P15, with 5uM being

higher than 10uM (Table 6). The habituation for 5uM and 10uM were both decreased, and they had similar increases in excitability as well (Table 6). In the 5uM nebivolol, there was a large increase in blue and fast red vision as well as increases for the other vision factors except for red (Table 6). With 10uM there was an increase for all vision factors, with the largest being in blue (Table 6).

For 20uM nebivolol, there was an increase in 1hr and a large decrease in P15 (Table 6). The habituation was decreased, and the excitability was increased, similarly to cyclosporine (Table 6). The vision was also off for 20uM nebivolol as there were increases in vision for red, blue, fast red, and RGB, with blue having the largest increase (Table 6). The only decreasing value was seen with the optomotor response to the green line color (Table 6).

	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
Group	1hr	P15	Hab	S	E	R	G	B	FR	RGB
DMSO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nebivolol 5uM	22.5	5.6	-5.4	1.3	24.4	-0.9	2.0	11.6	13.0	6.4
Nebivolol 10uM	23.6	0.8	-5.7	0.4	24.5	3.5	3.1	12.9	1.2	5.4
Nebivolol 20uM	0.7	-13.7	-8.0	-3.1	23.0	1.9	-7.1	13.7	9.7	4.6

Table 6: Table containing the data analysis from the behavioral assay of the first concentration experiment.

For the second concentration experiment, all three concentrations had an increase in 1hr and P15, with 5uM and 10uM being higher than 20uM nebivolol (Table 7). The habituation was also decreasing for all concentrations and 20uM had the lowest habituation out of all the treatment groups (Table 7). The excitability in all three groups increased, but the increase was not as large as the first experiment (Table 7). For vision, green, blue, fast red and RGB were all decreased (Table 7). 10uM nebivolol had some of the largest decreases in B, FR, and RGB out of all 3 treatment groups (Table 7). The red lines were still increased for all 3 treatment groups (Table 7).

Group	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
	1hr	P15	Hab	S	E	R	G	B	FR	RGB
DMSO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nebivolol 5uM	17.4	13.8	-9.6	-2.9	7.1	7.4	-8.0	-5.0	-19.1	-6.4
Nebivolol 10uM	13.4	17.2	-6.9	-3.6	3.1	2.9	-5.4	-13.9	-24.3	-13.0
Nebivolol 20uM	10.0	1.0	-13.0	-2.1	0.4	6.1	-9.5	-5.4	-23.0	-9.2

Table 7: Table containing the data analysis from the behavioral assay of the second concentration experiment.

For the third concentration experiment, all treatment groups of nebivolol have an increase in 1hr, P15, and excitability, as well as a decrease in habituation (Table 8). There is also a decrease in vision for all line colors (Table 8). Even the red color has low vision for all concentrations, which is a first in the concentration experiments (Table 8).

Group	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
	1hr	P15	Hab	S	E	R	G	B	FR	RGB
DMSO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nebivolol 5uM	20.9	5.9	1.0	9.4	18.8	-13.5	-16.1	-7.5	-18.6	-14.9
Nebivolol 10uM	25.3	12.4	-2.2	-0.3	20.1	-9.1	-10.1	-11.8	-21.8	-15.1
Nebivolol 20uM	19.5	4.6	-6.8	-0.9	12.0	-15.2	-9.1	-16.8	-18.7	-17.0

Table 8: Table containing the data analysis from the behavioral assay of the third concentration experiment.

Finally, the data from all 3 concentration experiments were combined into an excel sheet. The 1hr and E were increasing for all 3 treatment groups (Table 9). The P15 was increasing for 5uM and 10uM but decreasing for 20uM nebivolol (Table 9). The habituation was decreased for all 3 experiments as well (Table 9). As for the vision, all the line colors and speeds were decreased (Table 9).

Group	Difference in Activity (Treated-DMSO)					Difference in Vision (Treated-DMSO)				
	1hr	P15	Hab	S	E	R	G	B	FR	RGB
DMSO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nebivolol 5uM	20.3	8.4	-4.7	2.5	16.7	-2.0	-6.6	-0.1	-8.0	-4.9
Nebivolol 10uM	20.8	10.2	-5.0	-1.2	15.8	-1.1	-4.4	-4.8	-15.1	-7.7
Nebivolol 20uM	10.1	-2.7	-9.4	-2.1	11.7	-3.8	-8.8	-3.1	-10.8	-7.3

Table 9: Table containing the data analysis from the behavioral assay of all 3 concentration experiments combined.

Swim Pattern Analysis

The swim pattern analysis provided images of larval movements at 10 minute intervals for periods 2-6, then 2 minute, 5 minute and 10 minute intervals for periods 7-18, and finally 8 minute intervals for period 1. All experiments had 8 minute intervals for period 1 because there was a two minute interval in the beginning of every imaging session where there was slight movement from the plates. This could be caused from closing the cabinet door or bumping into the cabinet. This caused the camera to shake and take images that were slightly blurry. Those images would not run or work with the macro when placed in ImageJ. This was only an issue for period 1, and once the images that made up the first two minutes were removed, the macro ran smoothly.

Figure 2 represents an image of the 8 minute interval in period 1 from the third confirmation experiment. In this image there are four 96-well ProxiPlates, but the two plates on top are the only ones filled with zebrafish (Fig 2). The red color represents the zebrafish and their movements. When looking closely, the eyes and yolks of the zebrafish are red and the tails remain uncolored (Fig 2). The macro in ImageJ focused on the eyes and yolk when tracking larval movements. All the larval movements that occurred during the 8 minute time interval is shown in each well (Fig 2). Technically there is only one fish in each well, but this image shows the overall movements of that fish during the first period (Fig 2).

Figure 3 shows an example of 3 different time intervals of the same period. The images in Figure 3 are of period 15 from the third concentration experiment and contain both ProxiPlates filled with fish in each time interval. The top image represents a 2 minute interval of the period, so it only contains larval movements that occurred in the first two minutes of this period (Fig 3). The middle image contains larval movements that occurred in the first 5 minutes of period 15 (Fig 3). The bottom image shows the larval movements of the entire 10 minute interval that makes up period 15 (Fig 3).

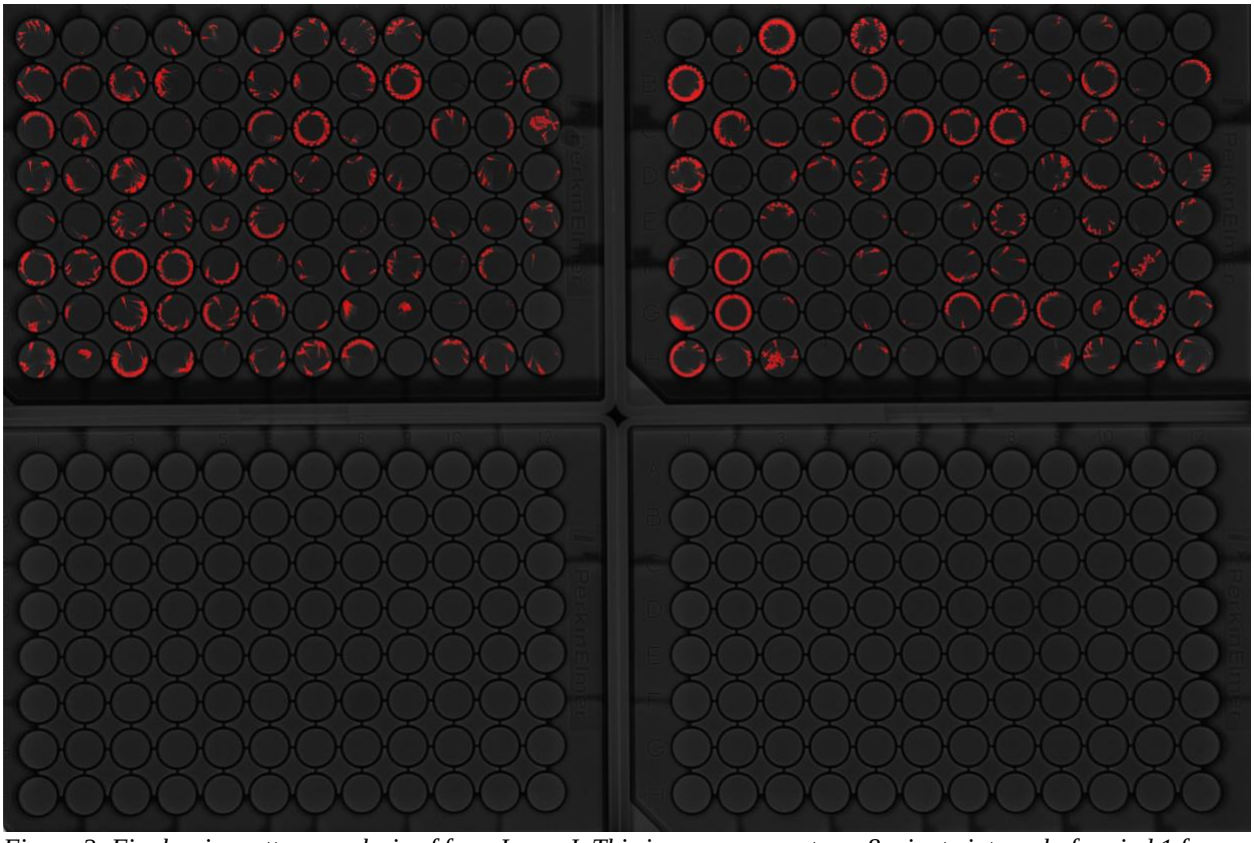

Figure 2: Final swim pattern analysis of from ImageJ. This image represents an 8 minute interval of period 1 from the third confirmation experiment.

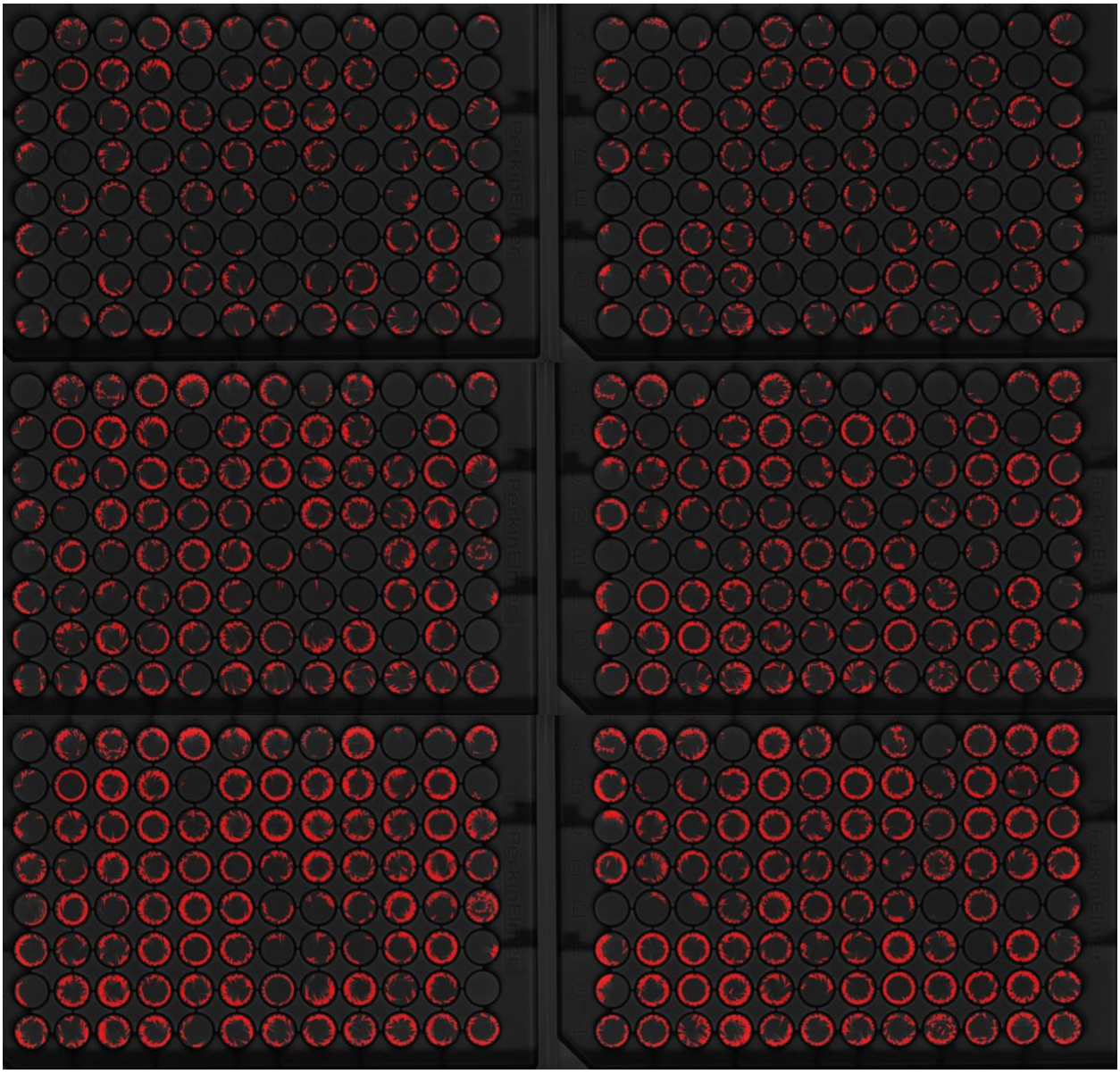

Figure 3: Final swim pattern analysis of period 15 from the third concentration experiment. Top Image: 2 minute interval of period 15. Middle Image: 5 minute interval of period 15. Bottom Image: 10 minute interval of period 15. There were no visual or acoustic stimuli present while these images were taken.

After having the larval movements at different time intervals for each experiment, the next step was to start labeling and categorizing the movements of the larvae. When trying to name the swim patterns that were seen, a study from *Kalueff et al,* who created a long catalog of zebrafish movements and behavior, was referenced[14]. After going through the catalog, a list of 5 behaviors that best relate to the larval movements seen in the experiments was created. The larval movements chosen were circling, thigmotaxis, dashing, jittery swimming, and freezing[14].

Circling is when the zebrafish swim in a repetitive, circular direction[14] (Fig 4). Thigmotaxis is when the zebrafish prefers to stay close to the edge and avoid the middle of the well[14] (Fig 4). Dashing is, "A series of directed (propulsive) darting movements; commonly seen as an escape response"[14](Fig 5). This involves the fish making repeated movements in multiple directions. Jittery swimming involves short, jerky movements, and there tends to be a lack of smoothness when it comes to how the fish are swimming[14] (Fig 6). Finally, freezing is when the fish completely stop moving, except for their gills and eyes[14](Fig 7).

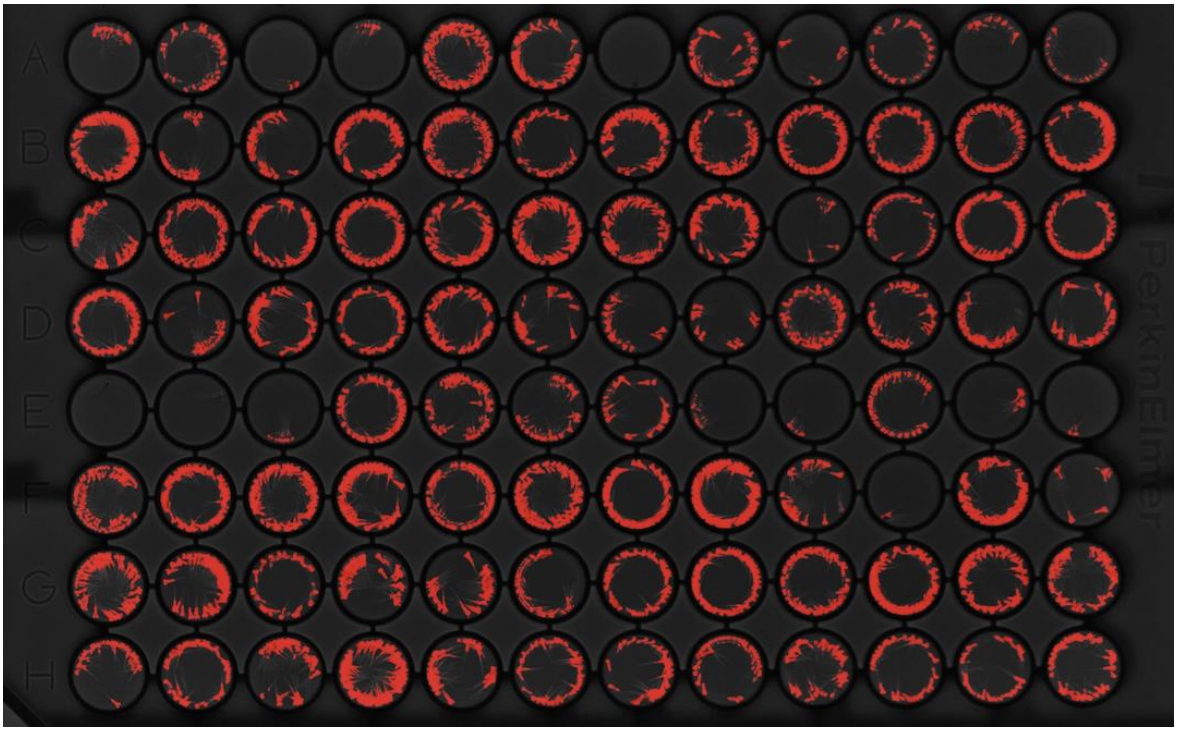

Figure 4: Swim pattern analysis image of a 10 minute interval of period 17 from the third concentration experiment. This image is an example of circling and thigmotaxis.

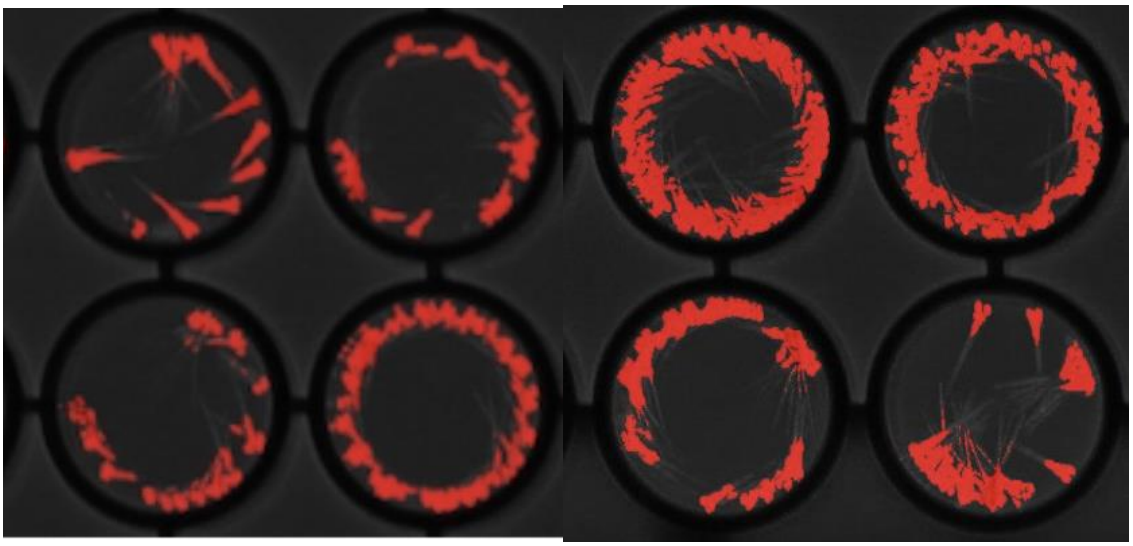

Figure 5: Left Image: Swim pattern analysis image of a 10 minute interval of period 16 from the third confirmation experiment. Top left well in the image is an example of dashing. Right Image: Swim pattern analysis image of a 10 minute interval of period 18 from the third confirmation experiment. Bottom right well is an example of dashing.

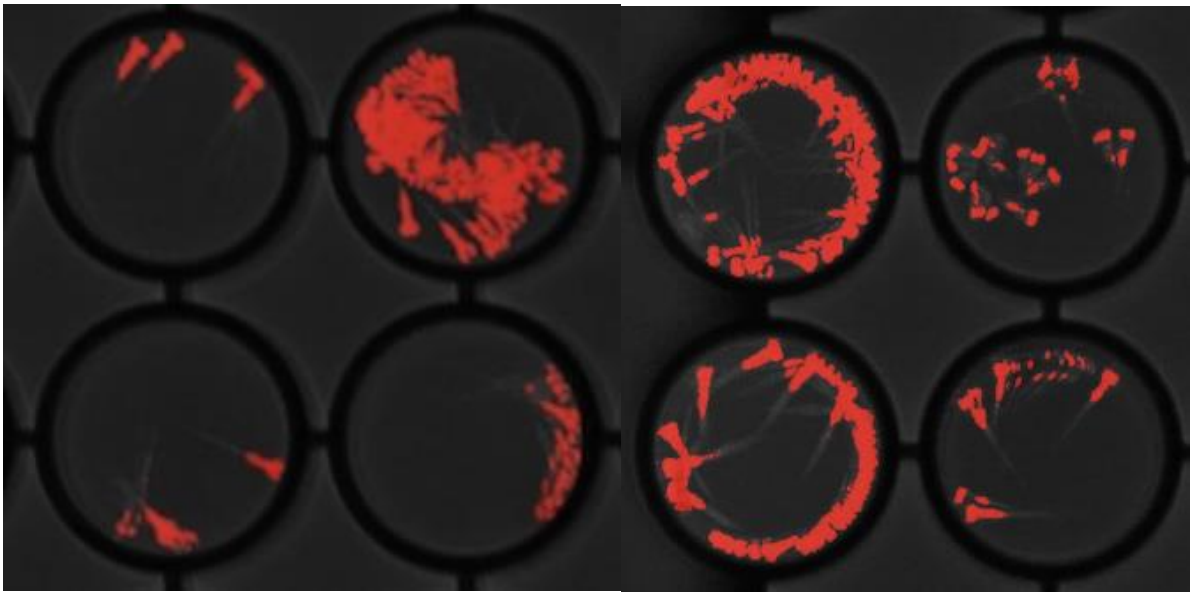

Figure 6: Left Image: Swim pattern analysis image of a 10 minute interval of period 5 from the third confirmation experiment. Top right well is an example of jittery swimming. Right Image: Swim pattern analysis image of a 10 minute interval of period 18 from the third confirmation experiment. Top right well is an example of jittery swimming.

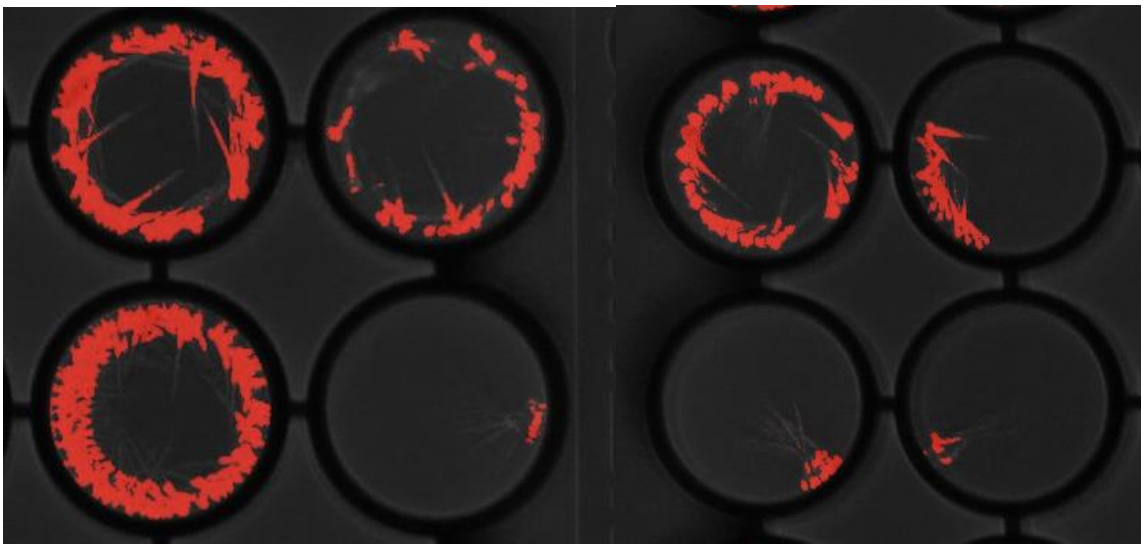

Figure 7: Left Image: Swim pattern analysis image of a 10 minute interval of period 17 from the third confirmation experiment. Bottom right well is an example of freezing. Right Image: Swim pattern analysis image of a 10 minute interval of period 17 from the third confirmation experiment. Bottom left and bottom right wells are examples of freezing.

From the swim pattern images, it is also possible to see the changes in movements during the transition from period to period. In this study, the focus was mainly on periods 1-6 and periods 15-17. This is because these periods relate the most to the categories that were focused on in the excel sheets. The goal is to see how the treatment affects the initial activity, seen in periods 1-6 and the habituation to acoustic stimuli, seen in periods 15-17. Vision was not as important for the swim pattern analysis because it does not provide as much information regarding early activity and habituation.

In the confirmation experiments from periods 1-6, there is increased activity in the zebrafish as the periods progress (Fig 8). As the periods transition, it is easier to see the increase in activity (Fig 8). Even though there are no visual or acoustic stimuli in periods 1-6, it is still possible to see the formation of patterned swimming during these periods (Fig 8). The most common patterns seen in this experiment during these periods were circling and thigmotaxis (Fig 8). Overall, there is a general increase in activity from the zebrafish in period 6 compared to the zebrafish in period 1 (Fig 8).

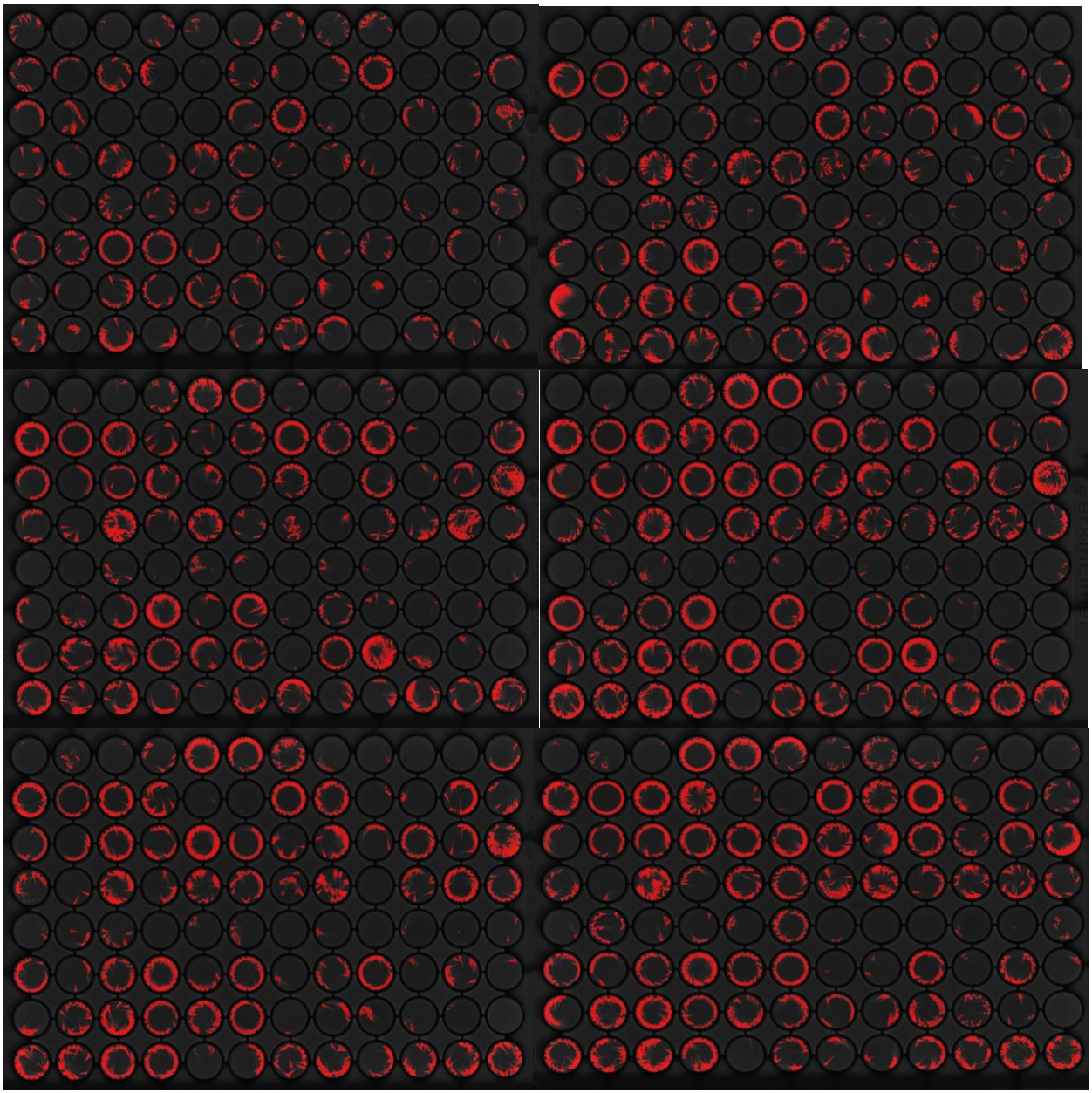

Figure 8: Swim pattern analysis of an 8 minute interval from period 1 and 10 minute intervals from periods 2-6 of the third concentration experiment. No visual or acoustic stimuli were present during these periods. Top Left Image: 8 minute interval of period 1. Top Right Image: 10 minute interval of period 2. Middle Left Image: 10 minute interval of period 3. Middle Right Image: 10 minute interval of period 4. Bottom Left Image: 10 minute interval of period 5. Bottom Right Image: 10 minute interval of period 6.

When looking at periods 15-17, it was interesting to see how the zebrafish activity levels, larval movements, and ability to habituate changed from period to period. Figure 9 shows the larval movements in 10 minute intervals of periods 15-17 from the third concentration experiments. The top left image shows the larval movements of the fish during period 15 when

there are no visual or acoustic stimuli present (Fig 9). The top right image shows the larval movements of period 16, which is the first introduction of acoustic stimuli that occurs in 20 second intervals (Fig 9). Looking back and forth between the top two images, it is possible to see the changes, which are sometimes very small, in larval movements as the assay transitions from period 15 to 16 (Fig 9). The fish do become slightly more active in the well in period 16 compared to period 15 (Fig 9). The bottom left image is the same 10 minute interval of period 16 and the bottom right image represents a 10 minute interval of period 17, which contains an acoustic stimulus that occurs in 1 second intervals (Fig 9). From these images it is possible to see if the fish are habituating to the acoustic stimuli. In the bottom right image, DMSO-treated fish are habituating in period 17 and nebivolol-treated fish are not habituating (Fig 9). The DMSO-tread fish are noticeably less active than the nebivolol fish, confirming that nebivolol prevents the zebrafish from being able to habituate to the sounds, just like cyclosporine (Fig 9). As a reminder, in the images in figure 9, the top row of the plate is DMSO fish, the second row is 5uM fish, the third row is 10uM fish, and the fourth row is 20uM fish (Fig 9). This pattern is repeated until the plate is filled.

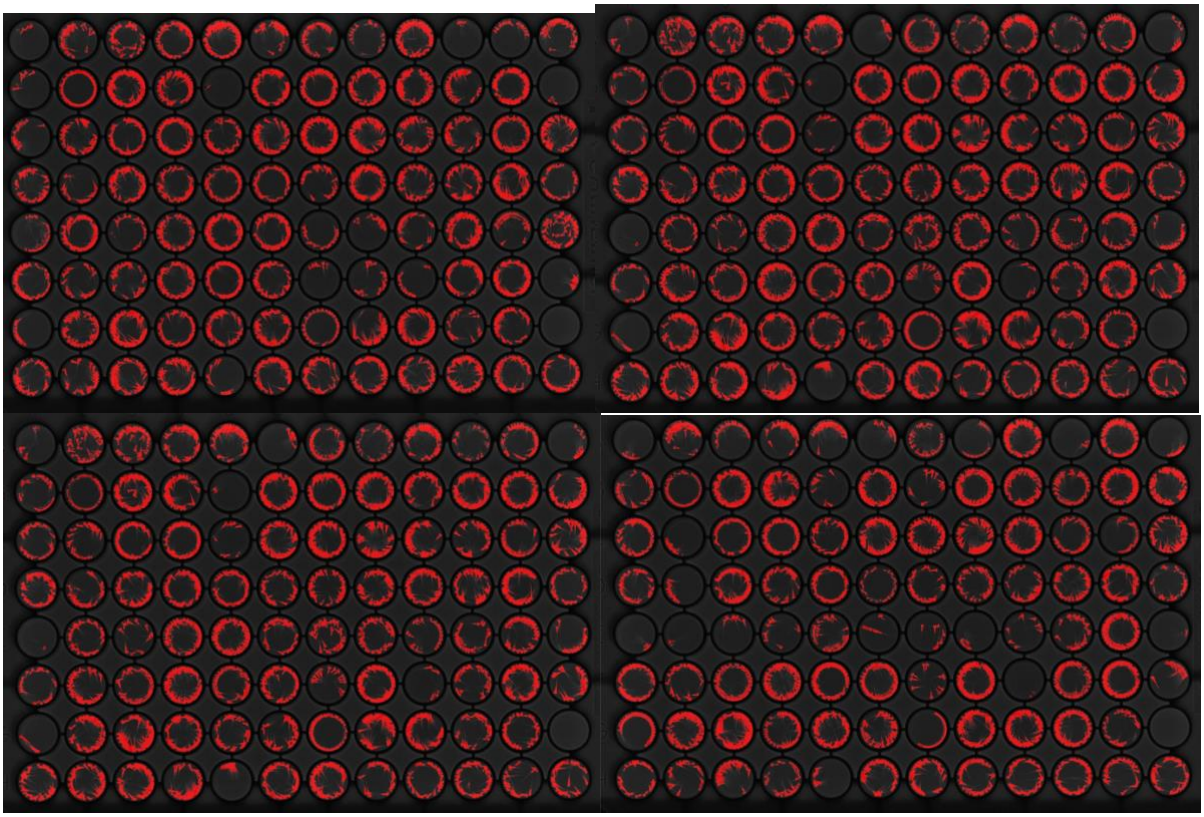

Figure 9: Swim pattern analysis of 10 minutes intervals from periods 15-17 of the third concentration experiment. Top Left Image: 10 minute interval of period 15. No visual or acoustic stimuli was present. Top Right Image: 10 minute interval of period 16. Acoustic stimuli occurred in 20 second intervals. Bottom Left Image: 10 minute interval of period 16. Acoustic stimuli occurred in 20 second intervals. Bottom Right Image: 10 minute interval of period 17. Acoustic stimuli occurred in 1 second intervals.

Discussion

In this study, I looked at how nebivolol could affect zebrafish behavior and swim patterns during a behavioral assay. From these results, I can conclude that nebivolol does affect zebrafish behavior similarly to cyclosporine, therefore it should be considered a CsA-like compound. During the confirmation experiments, nebivolol generally increased activity in the first hour and in period 15. It also increased excitability during period 17, decreased the optomotor responses in the fish, and caused a decrease in habituation. With that being said, there were some results from the confirmation experiments that did not align with cyclosporine or with data from previous nebivolol experiments conducted in the lab. This was mainly seen in the first confirmation experiment (Table 2). The P15 was decreased, excitability was decreased, habituation was increased, and the optomotor response for red lines was increased as well (Table 2). The data from this experiment was the opposite of what is expected from a CsA-like compound, but I later found that this was not because of nebivolol. After this experiment, I discovered that there was an issue with the imaging cabinet and the behavioral assay. During this experiment, the PowerPoint presentation froze for a bit in the beginning. This caused the camera to take too many images of the earlier periods and not enough of the later periods. When these images were analyzed in ImageJ, it gave results that were very different from previous cyclosporine experiments. I believe that without the technical issues, these results would not have occurred.

The next two confirmation experiments did not have the same technical issues as the first, so that data was more in line with cyclosporine and with previous nebivolol experiments. There were increases in activity and excitability and decreases in habituation and vision (Table 3-4). With the results from the last two experiments, I was able to confirm that the data from the first experiment was due to a technical error and was not an accurate representation of how

nebivolol affects zebrafish behavior. After the three experiments were combined, the data still showed that nebivolol was similar to cyclosporine. The only category that was slightly off was P15, and that was most likely due skewed data that occurred in the first experiment.

This experience with my confirmation experiments was one of the reasons why I wanted to carry out 3 trials for the confirmation and concentration experiments. Having multiple trials of the same experiment not only provides a lot of data to feel confident with the conclusions being made, but also allows us to control for any technical errors or disturbances that might have occurred. Even though I had different results for the first experiment that makes it appear as if nebivolol is not a CsA-like compound, my results from the other two confirmation experiments allow me to still confirm that nebivolol should be grouped with cyclosporine. I can also say that my results, and the results from previous nebivolol experiments, are repeatable. This provides confirmation that nebivolol should be studied further to see if it is a viable therapeutic option to prevent Alzheimer's.

After the confirmation experiments, I was able to see how different concentrations of nebivolol affect zebrafish behavior. Initially, I wanted to be able to determine the optimal dosage of nebivolol, or in other words, determine which concentration of nebivolol affected zebrafish behavior most similarly to cyclosporine. After looking at the data from all three concentration experiments, it is not possible to make a confident conclusion on the optimal concentration of nebivolol. This was due to the data in the first two concentration experiments giving results that showed that nebivolol differed from cyclosporine (Table 6-7). Even 10uM nebivolol, which already produced effects on zebrafish behavior that were like cyclosporine in the confirmation experiments, was not producing results that were expected of nebivolol during the concentration experiments. In the first concentration experiment, vision was increased for all concentrations

(Table 6). In the second concentration experiment, vision was still increased for all concentration but only in a few line colors and speeds, not all (Table 7). These results are completely unexpected, and I am not sure why they occurred. It could be caused by the zebrafish that were bred for this experiment not responding well to treatment. It also could be caused by technical errors like the PowerPoint presentation, how bright and sharp the lines are projected onto the fish, or if the camera is taking clear images. As of now, there is not an explanation for why these different results occurred.

The third concentration experiment was very different from the first two. In this experiment, the data that was produced was more along the lines of what is expected of nebivolol. Activity, habituation, and excitability were increased, and vision was decreased for all concentrations (Table 8). It is unclear why these results were so different from the first two experiments. The same protocol for breeding, treating, and imaging the fish were used every time. The fish were also imaged in the same imaging cabinet every time, so I am not sure why the data is so different from each other. When the data from all 3 experiments were combined into one excel sheet, the results were similar to the third concentration experiment (Table 9). Overall, the variability in my data from the three experiments makes me hesitant to make conclusions on which concentration is optimal. If I had to hypothesize on which concentrations were going to behave most similarly to cyclosporine, I would say 5uM and 10uM nebivolol. This hypothesis is formed because these two concentrations affected zebrafish behavior in the same way as cyclosporine more consistently during my experiments than 20uM nebivolol. 20uM nebivolol resulted in data that did not completely align with cyclosporine, until in the last experiment. This could be due to the errors that occurred in the first two experiments, or it could be a combination of that and that maybe 20uM is too much for the zebrafish. To confirm that

hypothesis and make confident conclusions about whether 20uM is too much nebivolol for the fish, there needs to be more concentration experiments. But for this study, these results still provide some insight into how the different concentrations of nebivolol affect the behavior of the zebrafish during the behavioral assay.

Finally, a macro was created that allowed for the analysis of larval movements in each period during both sets of experiments. Through this macro, I was able to visualize the movements of the zebrafish, and look at them in different time intervals, including 2, 5, 8 and 10 minute intervals, depending on the period. From there, I was also able to begin to characterize and label the swim patterns that I saw in both sets of experiments. With the information from *Kalueff et al* study, and my own images, I was able to name the swim patterns that I saw. My final list of swim patterns included circling, thigmotaxis, dashing, jittery swimming, and freezing. Although there were many different swim patterns seen in these wells, these five occurred the most during the behavioral assay.

I was also able to see the changes in excitability, overall activity, and swim patterns as the periods progressed. Periods 1-6 were interesting because they showed the formation of patterned swimming in the fish, even before any visual or acoustic stimuli was presented to the fish (Fig 8). With these images, it was possible to visually see the fish becoming more active as their time in the wells increased and as the periods progressed. It was also interesting to see the individuality in how the fish swim. Like humans, the fish appear to have different responses to being in a new environment and being given a treatment. Even though there were some common swim patterns, some fish were still swimming completely different from other fish given the same treatment and stimuli.

Images from periods 15-17, provided information on how acoustic stimuli affected zebrafish activity and their ability to habituate. In the transition from period 15 to 16 it was possible to see the general increase in activity and excitability in all the fish once they were introduced to the acoustic stimuli for the first time (Fig 9). When the loud pulses occurred in period 16, the fish visually became more active and that was seen in the image. It was also possible to see certain swim patterns becoming more common. Circling and thigmotaxis were a lot more prevalent in period 16 than in period 15 (Fig 9). Similar changes in excitability and activity were seen in the transition from period 16 to period 17. Habituation was also seen in the transition from period 16 to 17 (Fig 9). It is important to remember that DMSO-treated fish should show less excitability and increased habituation in period 17. Fish that were treated with cyclosporine show decreased habituation and remain very excited. These fish struggle to adjust to the acoustic stimuli during this period. Since nebivolol is a CsA-like compound, I would expect there to be decreased habituation in the transition from period 16 to 17 as well as increased excitability to the acoustic stimuli that occurs in 1 second intervals. DMSO-treated fish were less excited overall during period 17 compared to nebivolol-treated fish (Fig 9). The DMSO-treated fish calmed down in the transition from 20 second intervals to 1 second intervals of acoustic stimuli (Fig 9). Nebivolol-treated fish, on the other hand, remained excited during this transition (Fig 9). From these images, I was able to see how a CsA-like compound inhibits habituation and increases excitability in zebrafish.

I will acknowledge, it is a little more difficult to see the changes in swimming in the transition from periods 15 to 17 compared to the transition from period 1-6. It does require looking at the images very closely and picking up on the small changes that occur in individual wells within different periods. The changes that are seen are not very large, but they still are

enough to show that a larvae's swim movement is changing in that they went from swimming in a circling manner to swimming more in the middle. Or that they went from swimming slower with less movement, to swimming a lot quicker with a lot more movement. Future improvements on the macro could provide clearer images that could make it easier to see the changes in activity, habituation, and larval movements during the transition between periods and as the fish are exposed to different stimuli. Having the ability to look at larval movements over a period is great because it provides a visual representation of what is seen in the data in the excel sheets. These results from the swim pattern analysis can be a great supplementary tool to the numerical data that is given from ImageJ analysis.

As interesting as the information from this study is, it is only the tip of the iceberg. There is still a lot of information to learn about nebivolol and its effects on zebrafish behavior. The data from this study provides the background that allows for more research to be carried out in the future. Now having the confirmation that nebivolol is a CsA-like compound, more studies can be done to determine the best concentration of the compound along with if it should be used alone or in combination with other drugs. It would be interesting to see if nebivolol is strong enough on its own, or if it performs better with other CsA-like compounds. Future research could also focus on the swim pattern analysis and finding ways to connect zebrafish activity, behavior, stress, or anxiety, with how they are moving within the wells during the assay. It is also important to have studies that use other animal models. From there we would be able to see if nebivolol only has these effects on zebrafish, or if the data can be replicated in all animals used. Depending on the results, this data could eventually give the answer of whether nebivolol could be a potential therapeutic option for Alzheimer's.

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