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Impacts of Embryonic Exposure to Cannabidiol or Δ9-Tetrahydrocannabinol on Zebrafish (Danio rerio) Frailty in F0 and F1 Generations

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IMPACTS OF EMBRYONIC EXPOSURE TO CANNABIDIOL or Δ9-TETRAHYDROCANNABINOL ON ZEBRAFISH (*Danio rerio*) FRAILTY IN F0 AND F1 GENERATIONS

By

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ABSTRACT
ANIKA FARUQUE: IMPACTS OF EMBRYONIC EXPOSURE TO CANNABIDIOL or Δ9-TETRAHYDROCANNABINOL ON ZEBRAFISH (*Danio rerio*) FRAILTY IN F0 AND F1 GENERATIONS
(Under the direction of Dr. Nicole Ashpole)

Exposure to cannabinoids during critical development periods has increased with epileptic children being commonly prescribed CBD for seizures and pregnant women taking it recreationally. Many studies have been done on the possible benefits and drawbacks of cannabinoid exposure on the human brain, but not much is known about how it can affect the developing brain long-term. To see the potential adverse effects of cannabinoid exposure during critical stages of development and discover potential developmental origins of disease in consuming cannabinoids during embryogenesis, zebrafish embryos (6-96 hours post fertilization) were exposed to varying concentrations of CBD (0.02, 0.1, 0.5 µM), THC (0.08, 0.4, 2 µM) and a control (0.05% DMSO). Exposed parents (F0) were bred to produce an F1 generation to see if effects were cross-generational. Effects of aging were assessed 30 months after exposure. As zebrafish age, increased incidents of kyphosis are observed as well as decreased physical activity. The aged male fish exposed to 0.1 µM CBD during embryogenesis rotated significantly more times than the aged control male fish, demonstrating a significant deviation from the expected age-related change. These changes were not observed in the female fish or at any other experimental concentration of CBD or THC. Furthermore, the aged male fish treated with 0.4 µM THC swam with significantly more mobility than the aged control male fish, showing a significant deviation in mobility from the expected age-related

change in aged fish. This was not observed in the female fish or at any other experimental concentration of CBD or THC. Exposure to THC and CBD during embryogenesis did not significantly alter the expected increase in kyphosis in aged males or females, despite a visual trend of reduced curvature as concentrations of both cannabinoids decreased. These findings demonstrate that exposure to cannabinoids during critical development periods can cause significant effects on the long term health of zebrafish.

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I. Background

The *Cannabis sativa* plant has a long history of therapeutic use that traces back 5,000 years to China, when it was documented in the first Chinese pharmacopeia as having medicinal value for treating a variety of ailments including fatigue, rheumatism and malaria (Bonini 2018). Today, there is evidence supporting its pharmacological relevance to a number of conditions including Alzheimer's Disease, cancer, and glaucoma (Maurya 2018). Its extensive medical uses notwithstanding, the cultural and legal landscape around cannabis is continuously shifting. Currently, it is the most commonly used illicit drug in the United States and has been legalized for recreational use in eleven states and Washington D.C. (Kuhathasan 2019). It was first legalized for medical use by California in 1996 for the treatment of issues like nausea and pain, and since then over thirty jurisdictions in the United States have followed suit. Some states allow any medical condition to be treated by cannabis with a doctor's approval, while others are stricter in what they allow to be treated by cannabis. States also differ on what kind of cannabis products consumers can use, with some only allowing cannabis that is low in Δ9-tetrahydrocannabinol (THC) and high in Cannabidiol (CBD) (Hall 2019). These laws vary, as cannabis contains hundreds of compounds whose effects are not completely understood yet (Maurya 2018). Two of the most well-known compounds associated with the cannabis plant are THC and CBD, which can make up as much as 40% of cannabis extract (Hausman-Kedem 2018). CBD has been shown to have a therapeutic potential for treating nausea, inflammation, and epilepsy (Maurya 2018).

THC is known for giving cannabis its psychoactive properties but has also been shown to have anti-oxidant properties, protecting neurons from oxidative stress caused by glutamate induced excitotocity (Maurya 2018).

Animal models have shown that CBD and THC both have the ability to reduce epileptic activity, with CBD demonstrating superior anticonvulsive properties compared to THC. A pediatric clinical study found that CBD-enriched extract showed great potential as an add-on anti-seizure treatment for pediatric patients with refractory epilepsy, but noted that more studies are necessary to see how it affects the developing brain long-term (Hausman-Kedem 2018). While showing promising therapeutic potential for the adult brain, the long term effects of THC on the embryonic brain are not yet well understood. In vivo studies with pregnant mice have shown that prenatal exposure to THC can have persistent detrimental effects on adult-brain function due to its ability to interfere with CB_1 signaling responsible in cortical neuron development (Salas-Quiroga 2015). Though research is still being done to delineate the effects these compounds can have on the developing brain, children are already being prescribed cannabinoids for their antiepileptic effects and treatment for autism spectrum disorder (Koren 2019). In 2018, the Food and Drug Administration approved EPIDIOLEX® oral solution, the first medication approved by the FDA that contains a purified drug substance derived from cannabis. Containing active ingredient CBD, this medication was shown to reduce seizures in two rare epilepsy syndromes: Lennox-Gastaut syndrome and Dravet syndrome. EPIDIOLEX® was approved for use in people two years of age or older (FDA).

Some unborn children are also subject to the effects of cannabinoids in the case of pregnant women taking these compounds recreationally. As a possible consequence of its expanding legalization status, Cannabis is being commonly viewed as an all-natural substance that is safe to use during pregnancy (Hasin 2018). Furthermore, there are studies being done on cannabinoids' effectiveness against the severe nausea associated with hyperemesis gravidarum (Koren 2019). The chances of cannabinoid exposure during developmental stages of life are increasing, yet little is known about the long term effects that these compounds can have on those still developing: embryos and children.

This study looked at the potential adverse effects that can occur later in life due to cannabinoid exposure in critical development periods. The Developmental Origins for Health and Disease (DOHaD) model shows a connection between exposure to environmental stressors during early development and effects on long-term health (Barker 2007). Exposure to drugs, air pollution, and a number of other environmental chemicals can lead to changes in gene expression, causing abnormalities in tissues and organ development that can make one more susceptible to disease (Heindel 2017). Thus, we were interested in learning about the potential Development Origins of Disease implicated in the embryonic exposure to two different types of phytocannabinoids: CBD and THC. Zebrafish were used as test subjects to see what the long-term behavioral and morphological effects of embryonic exposure to CBD and THC would be. Zebrafish were ideal models for these studies given their conserved endocannabinoid system with mammals, easily identifiable morphology and behavioral phenotypes, and high fertility (Pandelides et al., 2020).

As the proportion of elderly people in the population is predicted to double in the next 50 years, it is now more important than ever to study the physical and cognitive deterioration often associated with aging (Lutz et al 2008). In humans, aging is often met with degeneration of skeletal muscle and reduced locomotor activity (Marck 2017). Aged zebrafish exhibit many of the same hallmarks of aging as humans, including reduced locomotor function and the onset of musculoskeletal changes like kyphosis that predict frailty. Additionally, aged zebrafish show increased inflammation, senescence markers expression, and other molecular signs of aging (Gilbert 2014).

This study looked at the locomotion and kyphosis of male and female zebrafish after they were exposed to THC or CBD during embryogenesis. The developing fish were exposed to a solvent control (0.05% DMSO), specified concentrations of CBD ranging from 0.02-0.5 µM, or concentrations of THC ranging 0.08 - 2 µM, as reported by Carty *et al*. These concentrations were proportional to therapeutic doses for THC and CBD in humans, which can fluctuate greatly based on who the patient is and method of delivery but which are generally below 100 mg/kg per dose (Klotz et al., 2018, Mersiades et al., 2018). Exposure began at 6 hours post fertilization (hpf) and fish were removed from treatment at the beginning of larval stages at 96 hpf. Fish were entered into the study at 12 months old and locomotor changes were assessed at 30 months of age.

II. Methods

Zebrafish Care and Exposure

Zebrafish THC and CBD exposure was performed by Dennis Carty, Cammi Thornton, and other members of Dr. Kristine Willett's laboratory prior to the initiation of this thesis work. In brief, zebrafish were acquired from the Zebrafish International Resource Center (ZFIN, Eugene, Oregon). Once obtained, adult zebrafish were bred in tanks filled with water supplied from an Aquatic Habitats Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, Florida) with pH 7.0-7.5 at 25–28°C (Pandelides et al., 2020). The fertilized zebrafish eggs were then placed in vials containing water 60 parts per million (ppm) Instant Ocean, (Cincinnati, OH). At 6 hours post fertilization, the developing embryos were given new water containing sublethal concentrations of 0.08, 0.4, 2 μM (0.024, 0.12, 0.6 mg/L) THC, 0.02, 0.1, 0.5 μM (0.006, 0.03, 0.15 mg/L) CBD, or 0.05% DMSO control water in tightly controlled settings until 96 hours post fertilization, when they were removed from treatment exposure. At 6 months of age, the treated adult F0 zebrafish that were exposed during embryogenesis were bred to produce the F1 generation. Twelve months after developmental exposure, both the exposed parent fish and their offspring were entered into the study and remained until they were aged at 30 months. Then, aged fish were examined for age-linked behavioral and morphological changes. Fish were exposed to 14:10 light-dark cycle and fed two times a day with Gemma Micro 300 diet made by Skretting (Pandelides et al., 2020). Two groups of 7-

month old fish raised under identical conditions as the aged fish were used as a young control.

Open Field Behavior

For the behavioral study, the tanks carrying the fish were transported to a dark room that contained a camera to record the fishes' movements; a computer with Noldus Ethovision 14 software to process the movements; a light to illuminate the movements; and a bucket, or maze, filled with water to provide a controlled observational environment for the fish to swim in. The behavioral testing room had tightly controlled settings, including temperature, sound, and consistent lighting, so as to get an accurate reading of the fishes' locomotive behavior. During recordings, observers tried to keep sound and sudden movements to a minimum to prevent scaring the fish and altering results. Temperature was maintained around 27-28°C. An LED light with an intensity of 9 Lux was fastened above the bucket, which had a diameter of 23.495 cm and a depth of 24.765 cm (Pandelides et al., 2020) . An observer would take a single fish from its tank using a capture net and place it in the maze for five minutes to swim freely. Before placing the fish in the water, the observer would start recording on the computer program. The video camera above the bucket recorded the fish swimming and the Ethovision tracked the fish's movements. Ethovision then calculated the distance moved by the fish, velocity, activity, mobility, rotation frequency, and time spent in the central area (inner 50% of the arena) and periphery (outer 50% of the arena).

Phenotypic Assessment of Adults

Upon completing the behavioral assessment, the zebrafish were euthanized in 300 mg/L MS-222 and 600 mg/L sodium bicarbonate (Pandelides et al., 2020). The fish were then measured for length and wet weight by Dr. Zach Pandelides. They were laid flat on their side and photographed so that raters could later analyze their back curvature and look for kyphosis. Curvature was judged by three different double-blinded observers. Raters designated a score to each fish based on how much its back curved using a scale of 1-5, with 1 representing little to no curve, 3 representing some curving, and 5 representing extreme curving. Each rater was given identical reference photos of the different degrees of curvature. Scoring between observers showed more than 90% similarity and 76% identity. The scoring between observers was highly correlated with Pearson coefficient values of .638-.870. Using scores from all three raters, median scores were calculated for each fish and used to compare curvature between groups.

Statistical Analysis

To study the effects of different concentrations of CBD and THC on aging, a one-way ANOVA followed by Dunnett's post-hoc test was used to analyze open field behavior compared to the old controls ($p \leq 0.05$). Locomotive behavioral data was displayed using bar graphs. Chi squared ($p \leq 0.05$) was used to analyze kyphosis (Pandelides et al., 2020). Prism8 and Sigmaplot 14.0 software were used for statistical analysis and graphing (Pandelides et al., 2020).

III. Results

In terms of distance moved, the young fish moved significantly more than the aged control and aged treated fish (**Figure 1**). Exposure to different concentrations of CBD during embryogenesis did not alter the expected age-related change because, just as was seen with the aged control fish, the distance moved by the aged treated fish was significantly less than that moved by the young fish (**Figure 1A**). We noticed this in both males and females (**Figure 1A-C**). When exposed to varying levels of THC, distance moved by the aged treated fish was significantly less than the young fish and did not significantly alter from the expected age-related change (**Figure 1B-D**). This was seen in both males and females.

When examining the velocity of the fish, we noticed that the aged control swam at a significantly slower velocity than the young zebrafish (**Figure 2)**. Exposure to varying concentration of CBD during embryogenesis did not alter this expected age-related change. No alteration was observed in males or females (**Figure 2A-C)**. Treatment with different concentrations of THC during development also did not alter the expected age-related change for velocity in males or females (**Figure 2B-D**).

As far as rotation frequency, both male and female aged control fish rotated fewer times than the young fish (**Figure 3)**. The aged male fish exposed to 0.1 μM CBD during embryogenesis rotated significantly more times than the aged control male fish, demonstrating a significant deviation from the expected age-related change (**Figure 3A)**. This same effect by exposure to 0.1 μM CBD treatment was not seen in the aged female

fish (**Figure 3C)**. When exposed to 0.5 µM CBD during embryogenesis, aged male fish rotated significantly fewer times than the young male fish. Exposure at 0.02 µM CBD during developmental periods did not alter the expected age-related change for rotation frequency in aged male fish (**Figure 3A)**. Exposure to varying levels of CBD concentrations during developmental periods did not alter the expected age-related change for rotation frequency in aged female fish (**Figure 3C**). Treatment of different concentrations of THC during embryogenesis did not alter the expected age-related change for rotation frequency in males or females (**Figure 3B-D**). At concentrations of 0.08 and 2 µM THC, aged male fish rotated significantly fewer times than young male fish (**Figure 3B)**. In terms of the activity of the fish, exposure to different concentrations of CBD during embryogenesis did not alter the expected age-related change in male or female fish (**Figure 4)**. Similarly, exposure to varying concentrations of THC did not alter the expected age-related change for activity in either males or females (**Figure 4B-D)**.

When measuring mobility of the fish or how functionally they move, the aged control males had significantly less mobility than the young male fish (**Figure 5A**). Exposure to different concentrations of CBD did not alter the expected age-related change in mobility. This same observation was made with the aged female fish (**Figure 5A-C**). The aged control female fish had less mobility than the young females. When exposed to a concentration of 0.02 µM CBD during embryogenesis, the aged female fish swam with significantly less mobility than the young female fish (**Figure 5C)**. Exposure to 0.08 and 2 µM of THC during embryogenesis did not alter the expected age-related change in mobility for aged males, as their mobility did not significantly differ from the

aged control males. Treatment with 2 µM of THC during embryogenesis caused the aged males to have significantly less mobility than the young male fish. The aged male fish treated with 0.4 µM THC swam with significantly more mobility than the aged control male fish, showing a significant deviation in mobility from the expected age-related change in aged male fish (**Figure 5B)**. This same effect by 0.4 µM THC treatment was not observed in aged female fish (**Figure 5B-D**). Exposure to different concentrations of THC during embryogenesis did not alter the expected age-related change in mobility for female fish (**Figure 5D**).

In terms of how long the fish spent in the periphery of the bucket, the aged control fish spent less time in the outer edges than the young fish (**Figure 6)**. When exposed to different concentrations of CBD during critical development periods, the expected age-related change was demonstrated in both male and female fish (**Figure 6 A-C**). At a concentration of 0.02 µM CBD, the aged treated male zebrafish spent significantly less time in the periphery of the maze than the young male fish (**Figure 6A**). The controlled aged female fish swam for significantly less time in the periphery than the young female zebrafish (**Figure 6C**). Exposure to different levels of THC did not alter the expected age-related change in males or females (**Figure 6B-D**). Aged male fish treated with 0.08 µM THC spent significantly less time in the periphery of the maze than the young male fish (**Figure 6B**). Aged female fish treated with 2 µM THC spent significantly less time in the outside edges of the maze than young female fish (**Figure 6D**). In terms of how much time was spent in the central location of the maze, the aged male fish spent more time in the center than the young male fish and the aged control female fish spent significantly more time in the center than the young female fish (**Figure 7**). The aged fish

treated with different concentrations of CBD demonstrated the expected age-related change and did not significantly differ from the aged control in either males or females (**Figure 7A-C**). Treated at a concentration of 0.02 µM CBD, these aged male fish spent significantly more time in the center of the maze than the young male fish (**Figure 7A**). Exposure to varying levels of THC did not alter the expected age-related change in males or females (**Figure 7B-D)**. When exposed to a concentration of 0.08 µM THC during embryogenesis, aged treated male fish spend a significantly greater amount of time in the center of the maze than young male fish (**Figure 7B**). Aged female fish treated with 2 µM THC during embryogenesis spend significantly more time in the central area of the maze than the young female fish (**Figure 7D)**.

Phenotypic Changes in Aged Fish

Locomotor changes can often be attributed to changes in musculoskeletal structure. As seen in other vertebrates, zebrafish are known to demonstrate increasing incidence of kyphosis as they age (Gerhard 2002). We also observed significant increases in the incidence of kyphosis in both the aged male and female fish (**Figure 8**). Exposure to CBD in early life did not significantly alter incident of kyphosis in aged males despite a visual trend of reduced curvature. Likewise, CBD exposure did not decrease incidence of kyphosis in females either (**Figure 8A-B**). Exposure to THC during embryogenesis did not significantly alter the expected increase in kyphosis in aged males or females, despite an apparent reduction in curvature as concentrations of THC decreased.

Figure 1

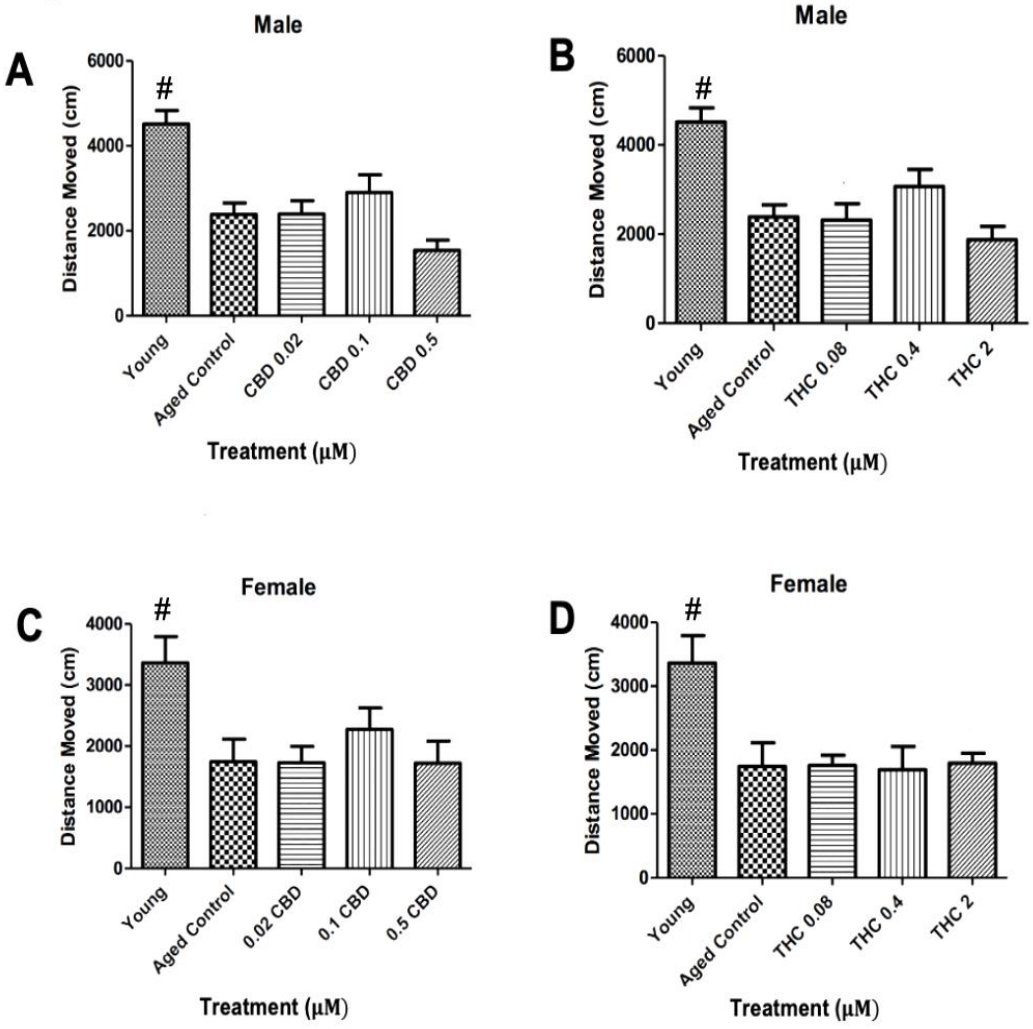

Figure 1: Distance moved by aged fish after exposure to varying concentrations of CBD and THC 6-96 hours post fertilization, n=8-10, #$p<0.05$, One-Way ANOVA post-hoc Dunnett's test vs Aged Controls. Exposure to cannabinoids did not alter the expected age related change.

Figure 2

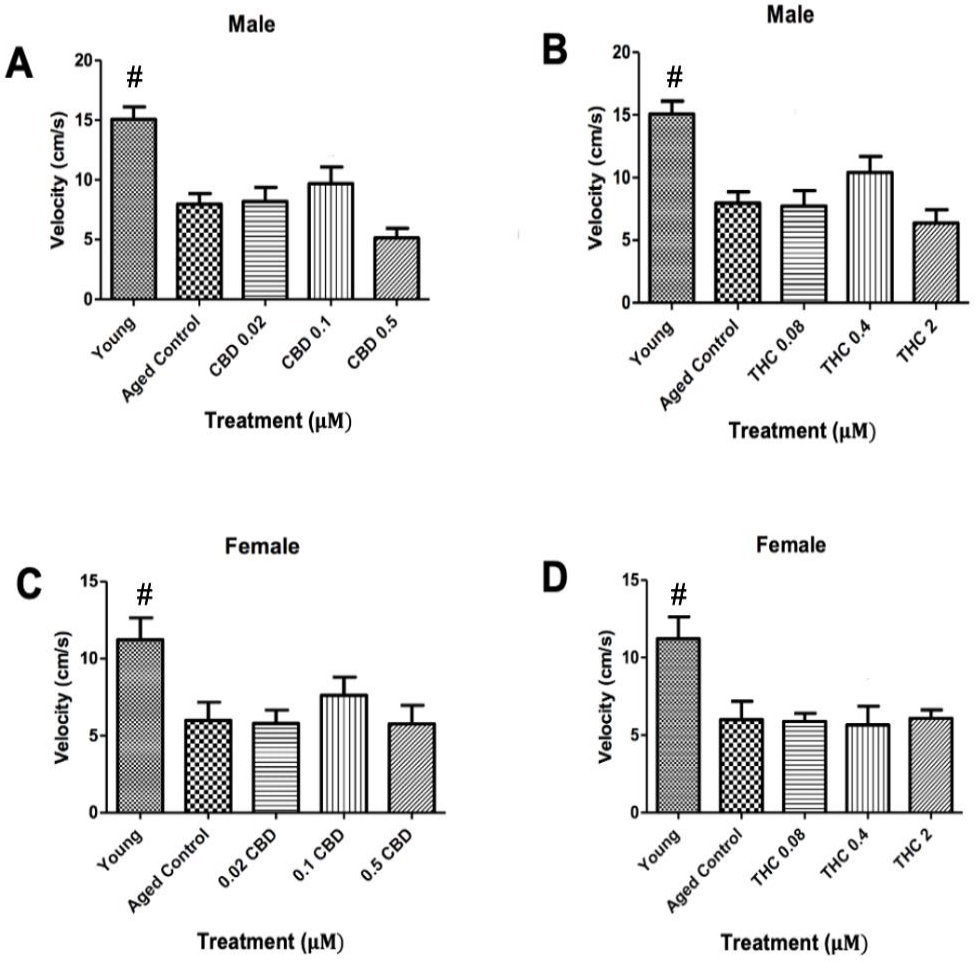

Figure 2: Velocity of aged fish after exposure to varying concentrations of CBD and THC 6-96 hours post fertilization, n=8-10, #p<0.05, One-Way ANOVA post-hoc Dunnett's test vs Aged Controls.

Figure 3

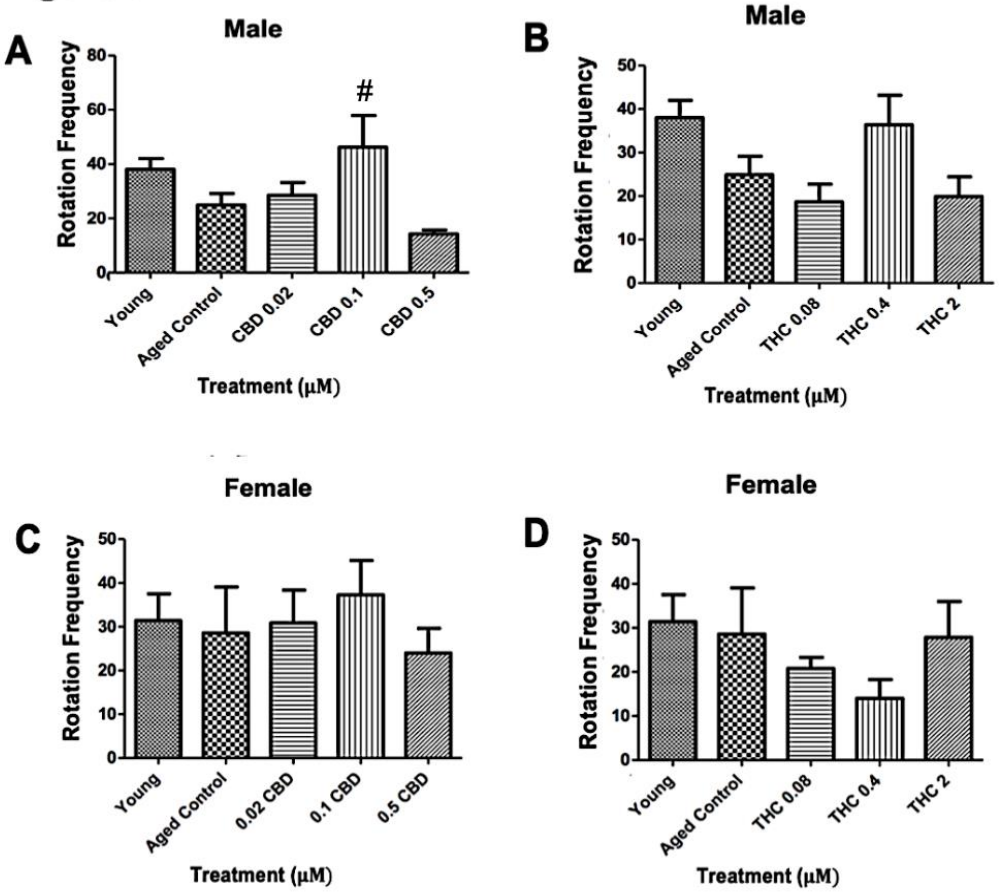

Figure 3: Rotation Frequency of aged fish after exposure to varying concentrations of CBD and THC 6-96 hours post fertilization, n=8-10, #$p<0.05$, One-Way ANOVA post-hoc Dunnett's test vs Aged Controls.

Figure 4

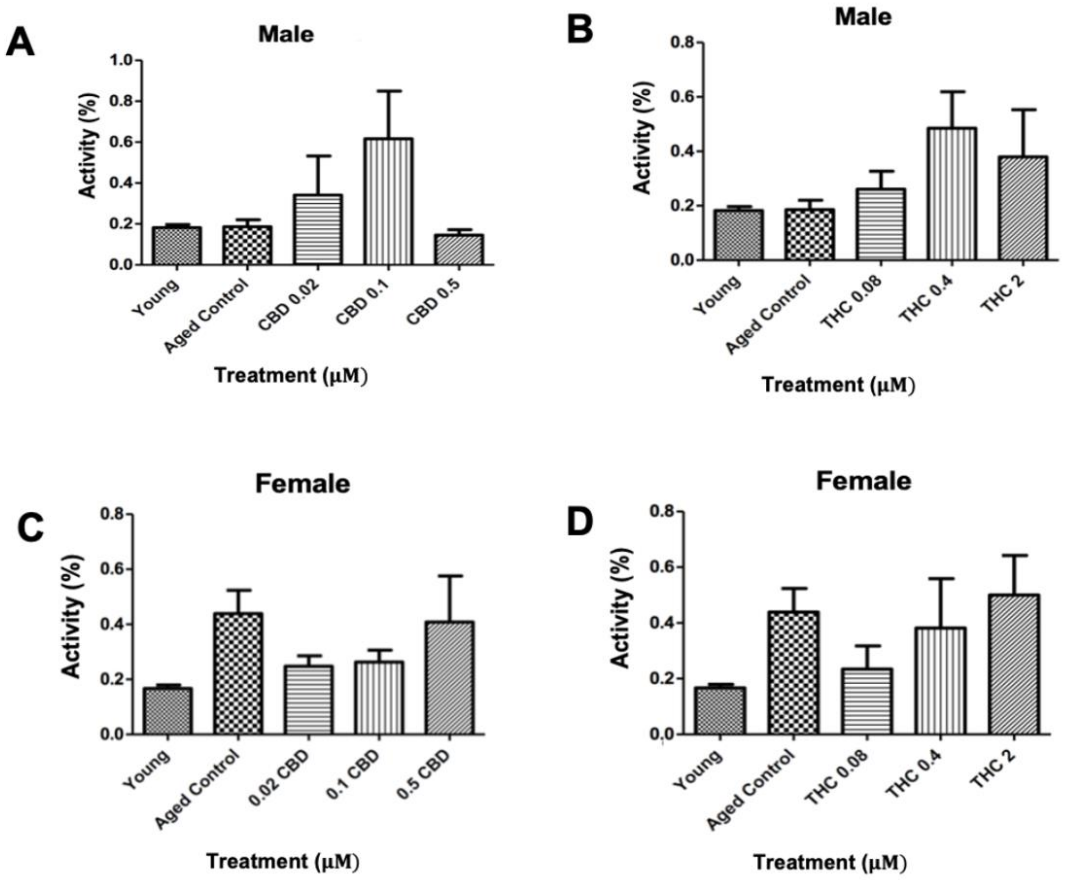

Figure 4: Activity of aged fish after exposure to varying concentrations of CBD and THC 6-96 hours post fertilization, n=8-10, #$p<0.05$, One-Way ANOVA post-hoc Dunnett's test vs Aged Controls.

Figure 5

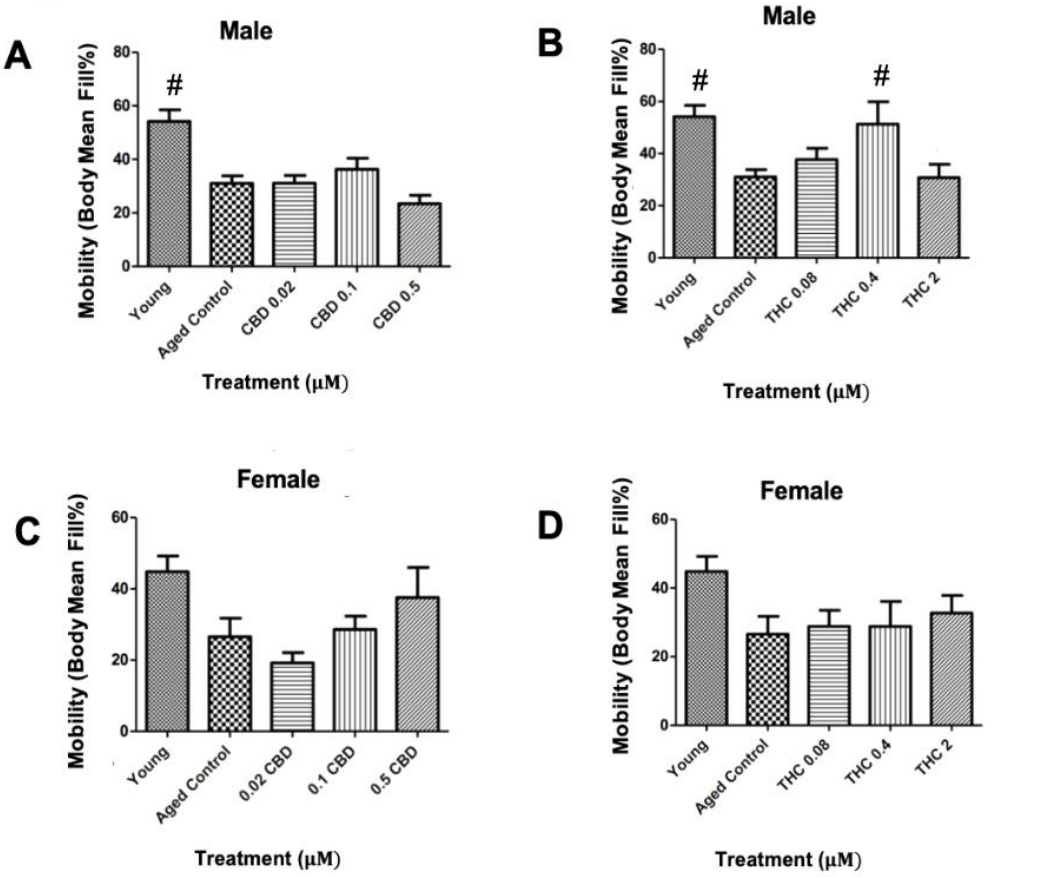

Figure 5: Mobility of aged fish after exposure to varying concentrations of CBD and THC 6-96 hours post fertilization, n=8-10, #p<0.05, One-Way ANOVA post-hoc Dunnett's test vs Aged Controls.

Figure 6

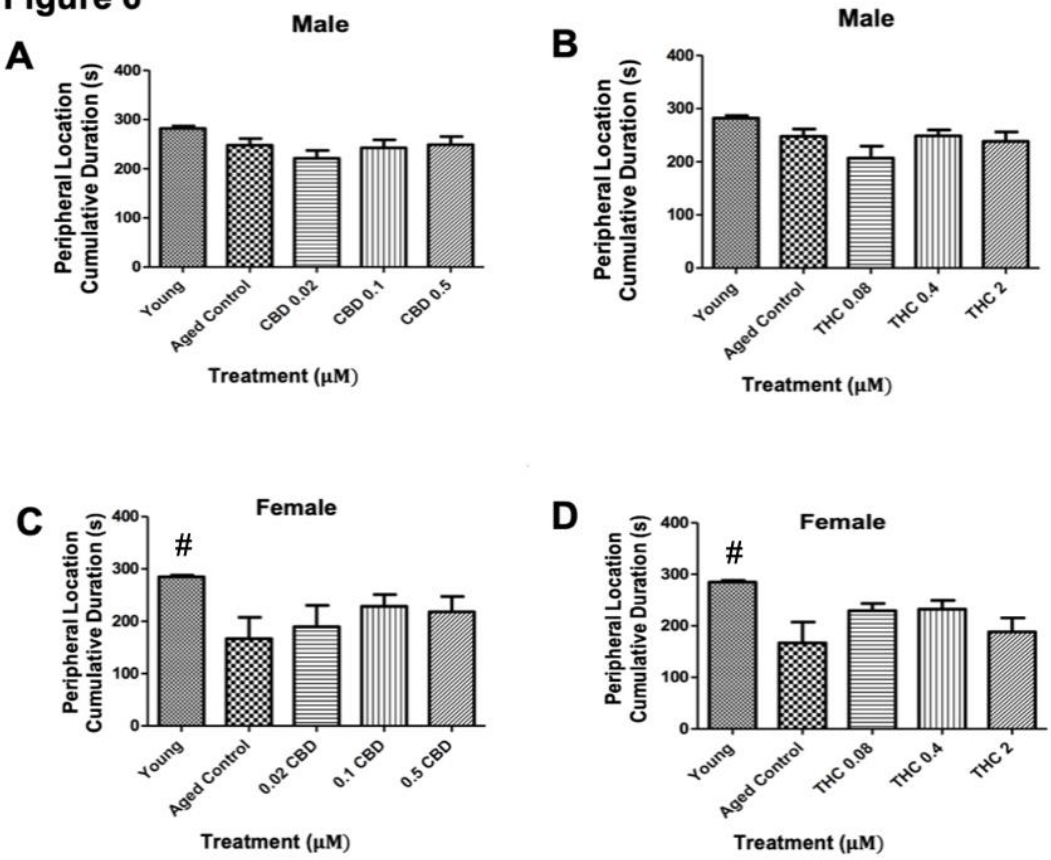

Figure 6: Total time spent by aged fish in peripheral location of maze after exposure to varying concentrations of CBD and THC 6-96 hours post fertilization, n=8-10, #$p<0.05$, One-Way ANOVA post-hoc Dunnett's test vs Aged Controls.

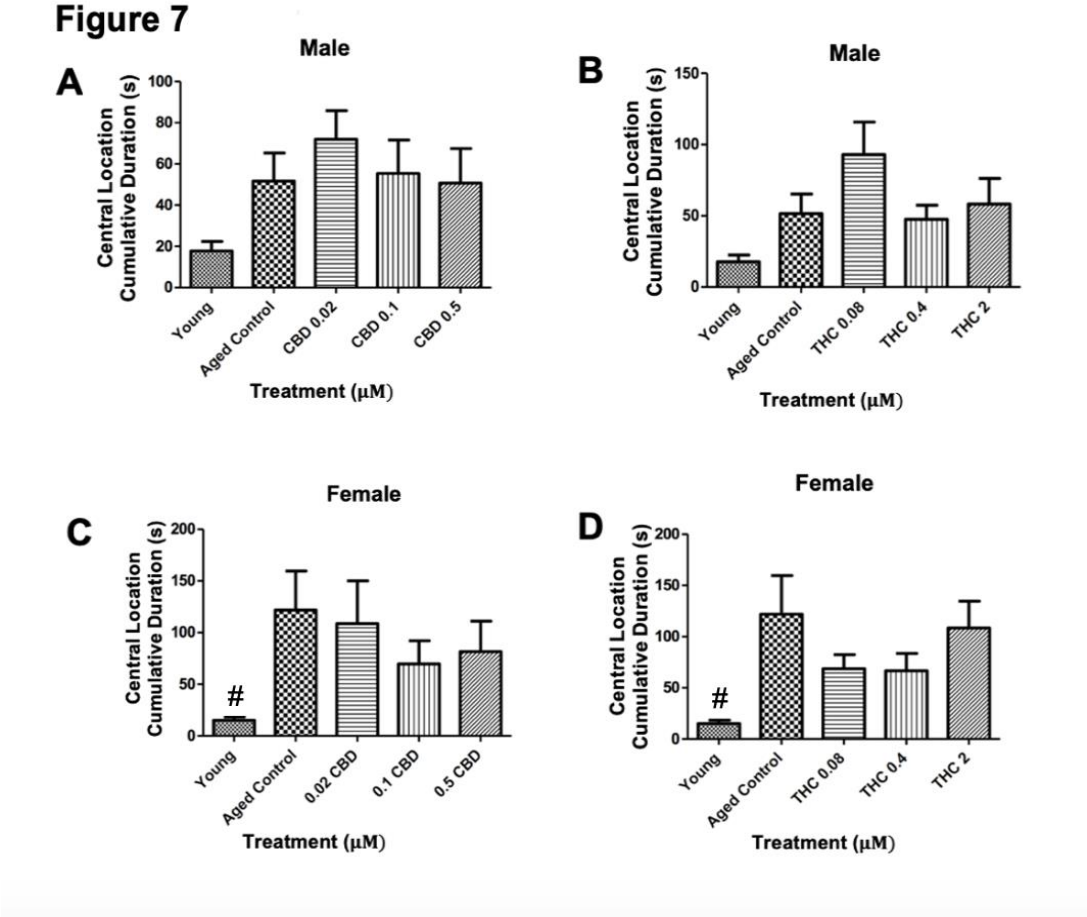

Figure 7: Total time spent by aged fish in central location of maze after exposure to varying concentrations of CBD and THC 6-96 hours post fertilization, n=8-10, #$p<0.05$, One-Way ANOVA post-hoc Dunnett's test vs Aged Controls.

Figure 8

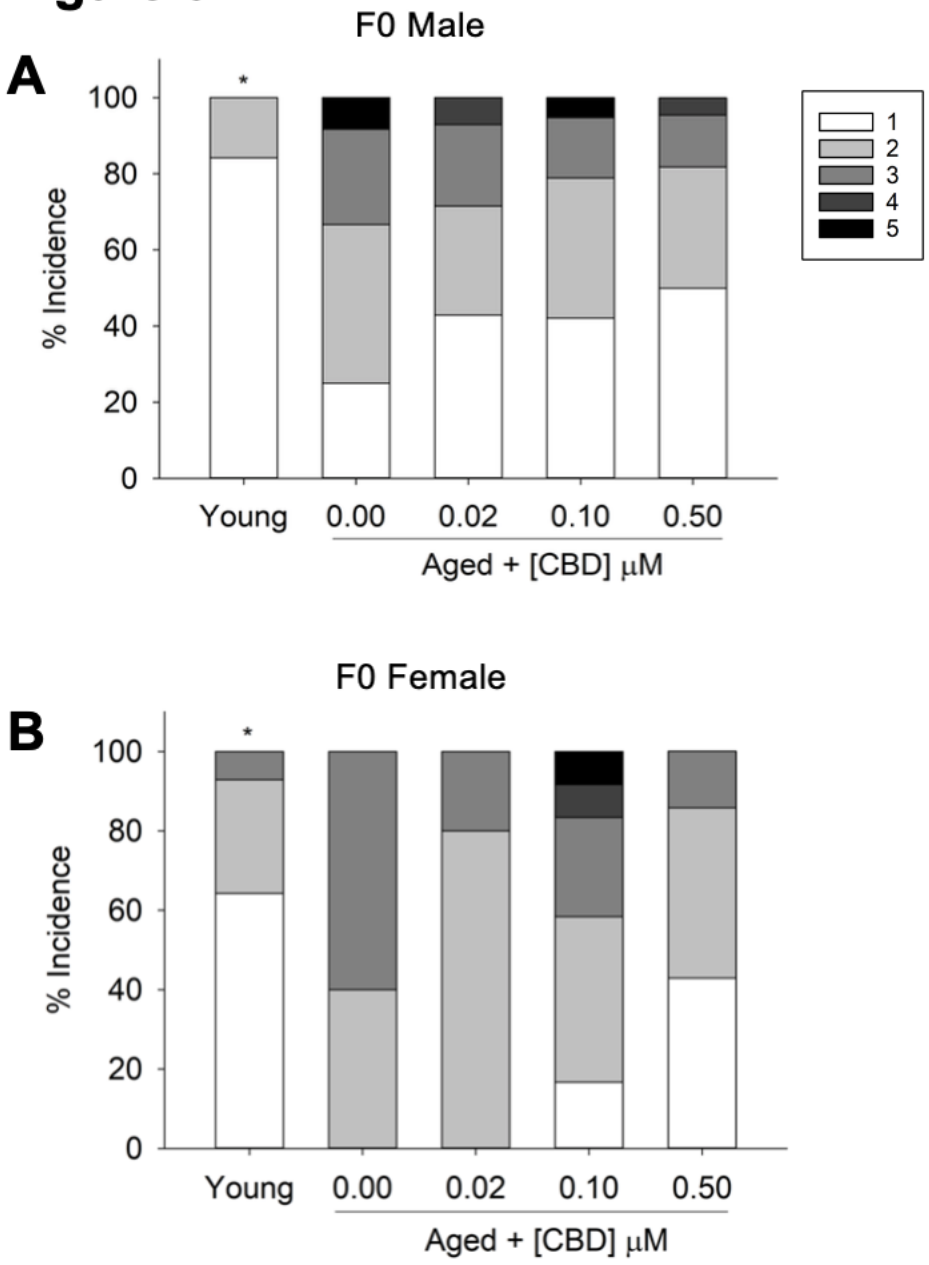

Figure 8: Incidence of spinal curvature (kyphosis) of adult F0 (young = 7 months old, aged = 30 months old) male (n=8-22) & female (n=5-12) zebrafish exposed to varying concentrations of CBD 6-96 hours post fertilization. Kyphosis was ranked on a scale from 1-5, 1indicating no curvature and 5 showing severe curvature. (#$p<0.05$, One-Way ANOVA post-hoc Dunnett's test vs Aged Controls)

IV. Discussion

Aged zebrafish showed higher indication of kyphosis compared to young fish. The male aged fish demonstrated significantly less mobility than young fish. Distance moved and velocity both decreased as fish got older. Turning frequency was reduced in aged males. We observed several deviations from the expected age related change when developing fish were exposed to cannabinoids. When exposed to concentrations of 0.02 and 0.1 micromolar CBD, the aged fish demonstrated less activity than the aged control. This data shows the possible neurodegenerative properties CBD can have later in life due to exposure during critical development periods. Previous studies have shown that CBD and THC exposure during gastrulation can cause disruptions in motor neuron development in zebrafish. Fish that were exposed to cannabinoids for five hours during critical development periods showed alterations in motor neuronal morphology (Ahmed 2014). More studies should be done to see how taking CBD during childhood can affect neurodevelopment into adulthood.

Similar to humans, zebrafish experience a reduction in overall movement and routine activity as they age (Gilbert 2014). As zebrafish get older, they become more trepidatious and prefer to remain in the periphery of their environment, a condition known as thigmotaxis that can be used as a measurement of anxiety (Schnörr 2012). Conversely, young zebrafish are more curious and are more likely to explore throughout their environment. In this experiment, the young control were expected to spend more time in the central area of the maze than the aged control. It was expected that the aged

control fish would spend relatively more time in the outer edges of the maze than the young fish. At 0.02 µM CBD, the aged male fish demonstrated decreased thigmotaxis. Aged male fish treated with 0.08 µM THC also showed decreased thigmotaxis. The increased amount of time in the center of the arena indicate that cannabinoids could have a beneficial impact on reducing the age-related anxiety observed in zebrafish.

At 0.4 µM THC, aged male fish demonstrated significantly more mobility than the aged control. At 2 µM THC, this observation did not hold true and mobility was decreased. Many studies suggest that cannabinoids exhibit a hormetic effect where small concentrations have beneficial effects and higher doses prove to be harmful (Hodges 2019). However, our findings showed that aged female fish exposed to CBD during development showed a dose-dependent increase in mobility as concentration rose. These findings suggest the need for more testing on the impact of cannabinoid developmental exposure at different concentrations. At 0.1 µM CBD, rotation frequency was significantly higher in the aged male fish than the aged control. The work of Gilbert et al. showed that zebrafish turning frequency decreased with age as did physical ability (Gilbert 2014). Reduced turning frequency was also demonstrated in the aged control in this study. The increase of rotation frequency in aged fish treated with 0.1 µM CBD indicated the significant impact developmental cannabinoid exposure can have on the healthspan of zebrafish. This same age-related change was not seen in aged fish that were exposed to 0.5 µM CBD. These results further indicated the need for more research on dosing of cannabinoids and their long term impact.

Results from animal model studies suggest that low doses of THC may prevent certain characteristics of cognitive decline associated with aging (Sarne et al., 2018). Likewise, this study showed that developmental exposure to 0.4 μM THC significantly increased mobility in aged male zebrafish compared to the aged control. Previous work by Carty et al. showed that THC and CBD caused a dose-dependent reduction in locomotor behavior in zebrafish exposed to cannabinoids during critical development periods, with the highest concentrations leading to the most significant reduction in locomotor activity (Carty et al., 2018). These findings underline the need to do more dose-response studies on the effects of THC and CBD on development and aging. With the increase of cannabinoid containing anti-epileptic medications being approved for child consumption, it is more important than ever to understand the mechanisms of cannabinoids on the brain and their long term impact on human health.

Future Directions

These studies were only performed in zebrafish. It would be useful to conduct them again in mice or other mammals to see how they would affect an animal model closer to humans. A mammalian model could give insight into the possible adverse effects these compounds could have on the rest of the human body. A mammalian model would also allow researchers to study the maternal-embryo interaction during gestation, which the zebrafish model does not allow.

V. Additional Assays

Background

In the Neuropharmacology Core, I performed many in vitro studies. The Core Lab received samples from scientists throughout the Thad Cochran Research Center and the remainder of the University. I tested for therapeutic potential of novel natural products with a particular emphasis on Cannabinoid and Opioid receptors. I worked at the front end of drug discovery. I tested the sample's binding affinity to various receptors in the brain: cannabinoid receptors like Cannabinoid Receptor Type 1 (CB1) and Cannabinoid Receptor Type 2 (CB2) and Opioid receptors including the mu opioid receptor (MOR), kappa opioid receptor (KOR), and delta opioid receptor (DOR). I performed screening assays to see whether agonists could produce response in receptors. Binding affinity and selectivity assays demonstrated if the compound showed specificity for one target.

Membrane Isolation

Overview

I isolated the cell membrane containing the desired Cannabinoid and Opioid receptors. I ordered the whole cells and grew them up in flasks. I removed the cells from the flasks by scraping them from the plate into the buffer. To isolate the membrane receptors from the whole cell, I centrifuged the solution, resuspended it, and sonicated it three times. I then aliquoted it and stored it in the freezer at -80°C to use in assays later. Then, I determined the concentration of membrane receptor I needed to use for radioligand binding assays.

Methods

I waited for the cells to reach 90-100% confluency. I confirmed confluency by checking under a microscope. The centrifuge was precooled to 4°C. Then I collected all the items I needed for membrane isolation including PBS, 50 mM Tris-HCl buffer, and cell scrapers and placed them all in a large ice bucket. One scraper was used for every five flasks. One plate at a time, I removed the media and washed the plate with 10 mL chilled PBS. Then I removed the PBS and replaced it with 5 mL chilled Tris-HCl. The Tris-HCl membrane was then scraped off using a chilled cell scraper. I pipetted the Tris-HCl with cells into a 50 mL conical tube sitting in ice. To collect any remaining cells, I scraped the plate again after putting 3 mL of Tris-HCl in the plate. Again, I pipetted the cells and Tris-HCl into the 50 mL conical tube. I performed these steps for each plate and placed all the cells and buffer collectively into one 50 mL conical tube until it filled up and another one was needed. I centrifuged the Tris-membrane solution for 10 at 4°C and 3100 revolutions per minute (rpm). After centrifugation, I carefully poured out the supernatant without disturbing the pellet. I discarded the supernatant. I resuspended the pellet in 2-5 mL of Tris-HCl depending on the size of the pellet. If there was more than one pellet, I combined all into one solution in a single centrifuge tube. I sonicated the membrane until the solution was well mixed and then centrifuged again for 10 minutes at 4°C and 1000xg. After centrifugation, I decanted the supernatant again but this time, I saved it in a 50 mL tube. I resuspended the pellet in 10 mL Tris-HCl. I repeated this cycle of sonicating and centrifuging two more times, each time remembering to save the supernatant. After the third centrifugation, I resuspended the pellet in the saved supernatant. I centrifuged the final solution in a high-speed centrifuge tube for 40 minutes

at 4°C and 13650 rpm. I resuspended the pellet in Tris-HCl and sonicated for 30 seconds. I aliquoted the membrane by putting 1 mL of the membrane into each 2 mL tube and labeled correctly. Finally, I placed the tubes in the -80°C freezer.

BCA protein assay

Overview

To determine what concentration of membrane should go in each well, I performed the BCA protein assay.

Methods

I performed a BCA protein assay, where I plated BSA standards. I prepared the dilutions, A thru I. I plated the BSA standard serial dilutions. Then I added the working reagent to it which gives it the color gradient. I also plated the cannabinioid or opioid membrane receptor. The plate was kept in the incubator for 30 min at 37°C. The color gradient correlated with concentration. To get an accurate reading of the concentration, I read the absorbance on a Biotek Synergy Microplate Reader. Using that data, I made a BSA standard curve.

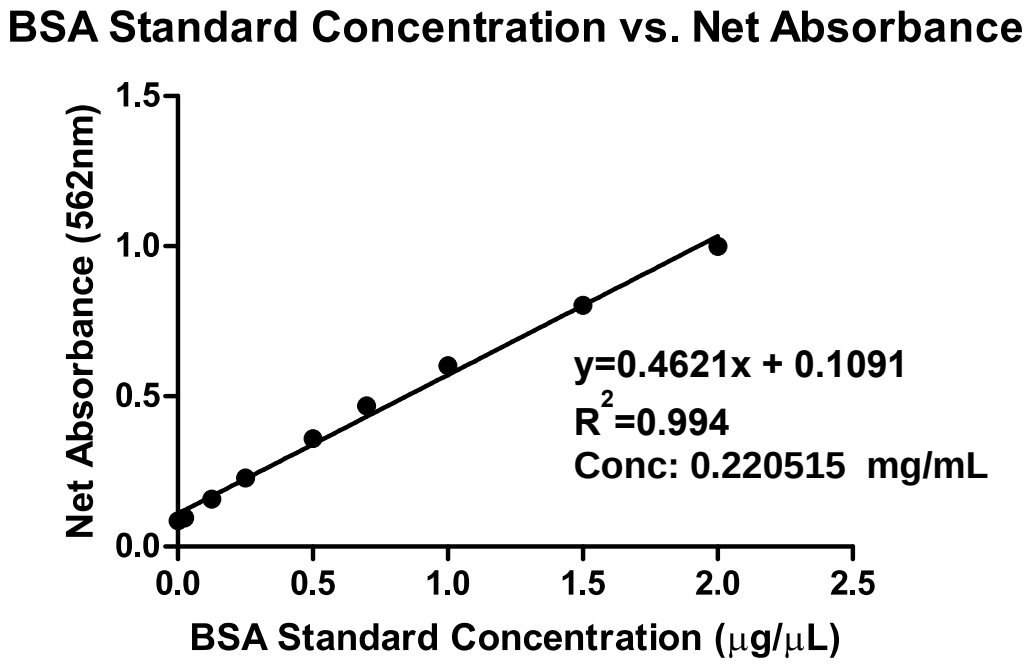

Figure 9: BSA calibration curve. This standard curve of BSA standard concentration vs net absorbance used to estimate protein concentrations from samples. R^2 =0.994; slope = 0.422, and intercept = 0.1091 was used to calculate protein concentration of 0.220515 mg/mL from absorbance readings of samples.

Ligand Binding Assay

Overview

I had to validate that the radioligand was working. There was a maximum concentration of radioligand I wanted in each well. I made serial dilutions of the radioligand, which was tritiated CP55940 or DAMGO, used for cannabinoid and opioid receptors, respectively. I plated them against controls and tested their binding to their corresponding receptor. The radioligand bound to its receptor and remained attached to it. When I washed the plate later in this assay, the bound radioligand stuck to the GF/C or GF/B filter plate. Everything that was not bound washed away. This told me how much radioligand bound to the receptors. There was a maximum amount of binding that I needed to get a signal for me to be able to determine binding affinity.

Methods

I prepared the membrane and radioligand tubes. Then I prepared the assay plate controls. Using a 96-well plate, I made dilutions of the test compounds. I transferred 50 microliters of control and test dilutions to the experiment plate. For opioid screening, I added an additional 100 microliters of Tris-HCl buffer (50mM Tris-HCl, 6.25 mM NaOH, 7.4 pH) to the "no membrane" wells. For cannabinoid screening, I used a Roth buffer (50mM Tris-HCl, 5mM $MgCl_2$, 15 uM BSA, 6.25mM NaOH). I added 50 microliters of the diluted radioligand to each well in the assay. [3H]-CP-55,940 was the radiochemical used in cannabinoid screening, while [3H]-DAMGO was used in opioid screening. I incubated the plate for 60 minutes at room temperature for opioid screening and 90 minutes at 37°C with agitation for cannabinoid screening. I washed the plate appropriately so that the bound radioligand would stick to the GF/B filter plate or the GF/C filter plate, used for

opioid and cannabinoid screening, respectively. I allowed the filter plate to dry at 50°C for at least 15 minutes. I added 50 microliters of MicroScint20 to the plate and sealed it. I read the binding on a PerkinElmer TopCount plate reader.

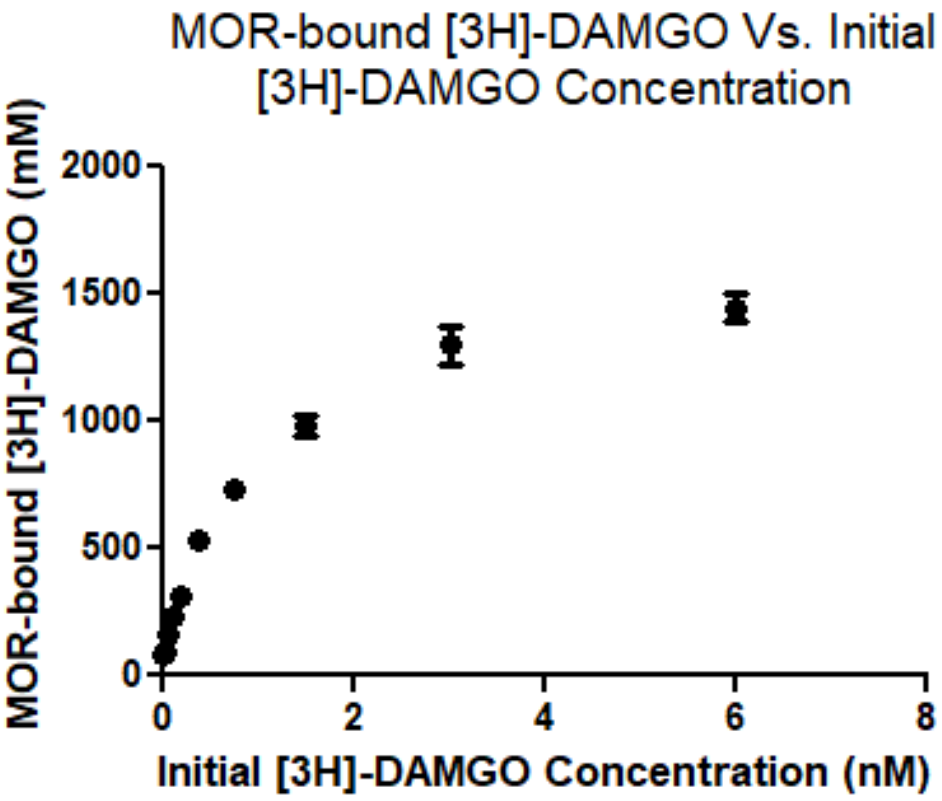

Figure 10: Saturation Binding Assay Curve. Binding curve of Initial [3H]-DAMGO Concentration vs [3H]-DAMGO bound to Mu Opioid Receptors (MOR).

Competitive Receptor Binding Assay

The overall goal was to compare samples to a standard and see how those binding affinities corresponded to each other. I plated test compounds against a variety of controls and then observed how those bindings compared to each other. Once I read the plate, I could tell if the sample had a higher binding affinity than the control. Then the compounds that had really high binding affinity, I could move into functional assays or into animals later.

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