Spring 5-4-2022

Characterizing the roles of cannabinoid receptor 1 & 2 in zebrafish behavior, metabolism, and seizure-induced activity

Kayci Kimmons
University of Mississippi

CHARACTERIZING THE ROLES OF CANNABINOID RECEPTOR 1 & 2 IN ZEBRAFISH BEHAVIOR, METABOLISM, AND SEIZURE-INDUCED ACTIVITY

By:
Kayci Kimmons

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements to the Sally McDonnell Barksdale Honors College

Oxford, MS
April 2022

Approved by:

Advisor: Dr. Kristine Willett

Reader: Dr. Davita Watkins

Reader: Dr. Noa Valcarcel

ACKNOWLEDGEMENTS

I will be forever grateful for this experience and the endless knowledge that it has brought me. I would like to thank Dr. Kristine Willett for always inviting me in and making me feel welcome. I have cherished the many years we have gotten to know each other and without your guidance, I would not have made it this far. The joy and kindness that you bring to the team has always given me motivation to continue on. I would also like to thank Cammi Thornton for being the best mentor and guiding light throughout this process. I have always admired your endless patience and dedication to your craft, and you have inspired me to continue pursuing my passions and striving to achieve my future goals. I will sincerely miss hearing your many stories and talking about life with you. Thank you to Dr. Zach Pandelides for being such a phenomenal master of software and technology and for always bringing a new perspective. You have always challenged me to learn and keep an open mind. And to the Sally McDonnell Barksdale Honors College, thank you always for believing in me and supporting me throughout my college journey. I have renewed faith and confidence in the person I am becoming because of the lasting impressions of the SMBHC staff and the numerous opportunities that I have been awarded. Lastly, thank you to my additional readers, Dr. Davita Watkins and Dr. Noa Valcarcel, for volunteering your time and assistance to my work.

ABSTRACT

Epileptic disorders like Dravet Syndrome require novel studies to determine the most ideal treatment. New research linking the endocannabinoid system (ECS) to epileptic disorders is arising, but there is still much to be discovered about the function and regulatory impact of the endocannabinoid system and its receptors in epilepsies like Dravet. In this study, knockout models of larval and adult zebrafish (*Danio rerio*) were used to investigate the roles of cannabinoid receptors 1 & 2 in behavior, brain mitochondrial metabolism, and seizure-induced activity following exposure to THC and CBD. Larval zebrafish which lacked cannabinoid receptor 1 exhibited increased locomotion compared to the wild-type (5D) line and cannabinoid receptor 2 null line. Conversely, adult zebrafish which lacked cannabinoid receptor 1 exhibited decreased locomotion, while adult zebrafish which lacked cannabinoid receptor 2 exhibited increased time spent in the periphery in the open field test compared to the wild-type line. Adult males significantly spent more time in the periphery than females and exhibited higher metabolic rates during specific stages of mitochondrial respiration regardless of line. Larval zebrafish that were exposed to THC and CBD following seizure induction by pentylenetetrazole (PTZ) were unaffected by the cannabinoid treatment, displaying no significant response. While we could not determine the exact mechanism behind the function of cannabinoid receptors in seizure-induced activity, this study found that cannabinoid receptors 1 & 2 do play a role in the behavior of zebrafish and are possibly involved in pathways that may link the ECS to epileptic behavior.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AED	Anti-epileptic drug
DS	Dravet Syndrome
Cnr1	Cannabinoid Receptor 1
Cnr2	Cannabinoid Receptor 2
CBD	Cannabidiol
THC	Δ^9-tetrahydrocannabinol
dpf	Days Post Fertilization
ECS	Endocannabinoid System
PTZ	pentylenetetrazole
OCR	oxygen consumption rate

INTRODUCTION

1.1 Epilepsy

Epilepsy is one of the most broadly defined and oldest neurological disorders in the world, affecting 65 million people worldwide (Epilepsy Foundation, 2019). About 3.4 million people in the U.S. have epilepsy, with about 470,000 of those being children (Centers for Disease Control and Prevention, 2021). It is a noncommunicable disease that is characterized by seizures, which are sudden, abnormal electrical disturbances that occur between brain cells. There are two major types of seizures that can occur. The first is a generalized seizure, meaning both sides of the brain are affected. The second is a focal seizure, which affects one area of the brain. For two out of every three people experiencing epileptic seizures, the cause of epilepsy is unknown and deemed idiopathic or cryptogenic (CDC, 2021). Many common causes of epilepsy include stroke, brain tumors, infections, trauma, anoxia, genetic disorders or neurological disorders.

Because epilepsy is so broadly defined, it can be difficult to find the appropriate treatment for a specific epilepsy disorder. Anti-epileptic drugs (AEDs) are the most common treatment (Boon et al., 2021).There are about 20 readily available AEDs, and they can be selected for a specific case depending on a person's size, age, comorbidities, sex, childbearing ability, and adverse effects. In most cases, AED monotherapy can control seizures (National Institute of Neurological Disorders and Stroke, 2020). Common side effects of AEDs include dizziness, drowsiness, mental slowing, fatigue and weight gain, but some are more severe, like depression, allergic reactions, and damage to the liver and bone marrow. Some AEDs can even cause other drugs to become less effective if taken simultaneously. However, the effectiveness of current AEDs is not universal because about 30-40% of patients are nonresponsive to these drugs

(Schmidt, 2011). Novel treatments are needed in order to target epilepsy disorders in a more effective and less detrimental way.

1.2 Dravet Syndrome

Dravet Syndrome (DS), or Severe Myoclonic Epilepsy in Infancy (SMEI), is a rare, genetic epileptic disease that is part of a group called developmental epileptic encephalopathies (DEEs). The devastating condition is often linked to a heterozygous loss-of-function mutation in the SCN1A (sodium voltage-gated channel alpha subunit 1) gene which causes sodium ion channels in the brain to malfunction (Strzelczyk & Schubert-Bast, 2020). Sodium channels are essential to brain cells, and give rise to the neuronal excitability needed for the body to function properly. This genetic mutation is found in approximately 85% of patients who are diagnosed with DS (Strzelczyk et al., 2019).

DS onset occurs in infancy, typically within the first year of life. Tonic-clonic or grand mal seizures tend to occur first along with fever (Epilepsy Foundation, 2019). Following the first seizure event, more types of seizures may recur, such as myoclonic seizures, atypical absence seizures, and more. These may be triggered by stress, photosensitivity, and infection. Child development is usually normal until after age 2, which is when children begin to develop motor problems, like a crouched gait or unsteady walking, and growth problems. Prognosis of DS is unfavorable, often resulting in severe neurophysiological impairment and death. DS is associated with a high risk of premature mortality and sudden unexpected death in epilepsy (SUDEP) (Strzelczyk & Schubert-Bast, 2020).

Adding to the devastating prognosis of DS is the fact that it is highly drug resistant. Current treatments, which can be costly, often involve a combination of modestly effective drugs. Two well-known primary treatments are valproic acid and clobazam (NORD, 2019).

However, most patients still do not reach a seizure-free point throughout their lifetime (Wirrell & Nabbout, 2019). Some developed anti-seizure medications that block sodium channels can even exacerbate DS symptoms. An ideal drug treatment has not been determined, but new studies that target altered metabolism in DS reveal new ways to identify anticonvulsants to treat catastrophic symptoms that may be related to bioenergetics.

1.3 Endocannabinoid System & Epilepsy

Endogenous cannabinoids or endocannabinoids (ECs) are naturally occurring cannabinoids that are present in the vertebrate central nervous system (CNS). The human body produces these endocannabinoids as a way to regulate synaptic transitions in the CNS (Lu & MacKie, 2016). The two most well-known endocannabinoids, *N*-arachidonoyl-ethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), are arachidonic acid derivatives. These endocannabinoids act as lipophilic ligands that directly affect neuronal excitability by interacting with cannabinoid receptors on the surface of cells throughout the body (Zou & Kumar, 2018). AEA and 2-AG are known to affect mood, appetite, pain sensation, inflammatory response, and memory (Chakravarti et al., 2014). Cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) are G-protein coupled receptors (GPCRs) that produce cellular responses when directed by certain signals like neurotransmitters (Rosenbaum et al., 2009). CB1 receptors are expressed throughout the CNS, while CB2 receptors predominantly play a role in the mediation of the immune system (Rowley et al., 2017). Endocannabinoids function like neurotransmitters, binding to these cannabinoid receptors and causing downstream modulations within the cell. This process is known as the endocannabinoid system (ECS), and it is an important regulator of neuronal activity. By understanding the ECS, potential therapeutics may be used to target this pathway and treat various neurological and physiological conditions.

Because neuronal hyperexcitability is prevalent in epilepsy, the ECS in epilepsy may be a potential target (Cheung et al., 2019). CB1 receptors have especially become prevalent in studies about the ECS due to its direct suppressive action on signaling in the CNS, but there is evidence that CB2 receptors may also be capable of regulating neuronal activity (Rowley et al., 2017). Cannabinoid receptor agonists for CB1 and CB2 may have the capability of reducing neuronal hyperexcitability. There are numerous studies utilizing knockout animal models, in which the gene coding for the CB1 receptor, cnr1, and the gene coding for the CB2 receptor, cnr2, are altered so that the receptors are not expressed or function correctly. There is significant statistical evidence that single cnr knockout models have increased susceptibility to seizures induced by pentylenetetrazole (PTZ), which is a known GABA receptor antagonist and convulsing agent (Shapiro et al., 2019). There is also evidence that suggests the ECS plays a role in animal models of DS, because knockout models are more susceptible to DS seizures and compounds that enhance endocannabinoid signaling may reverse the effects of DS (Anderson et al., 2022).

1.4 Cannabinoids as Therapeutics

Cannabinoids are naturally occurring chemical compounds that have been utilized for centuries for their medicinal/pharmaceutical efficacy and for recreational purposes (Andre et al., 2016). Besides endocannabinoids, there are two other types of cannabinoids: phytocannabinoids and synthetic cannabinoids. Phytocannabinoids are derived directly from the *Cannabis sativa* plant. Of the roughly 100 different compounds present in the plant, two of them are commonly known and used as therapeutics. The first is Δ^9-tetrahydrocannabinol (THC), which is known to exhibit anti-spasmodic, anti-inflammatory, anti-cancer, and analgesic effects (Andre et al., 2016). THC also has a psychoactive component. The second is cannabidiol (CBD), and it is a structural analog of THC. A major difference between THC and CBD is that CBD does not have a

psychoactive component. There is anecdotal evidence that CBD can be used as an analgesic, anxiolytic, appetite stimulant, and for the reduction of nausea and vomiting, but more concrete studies are necessary to investigate the efficacy of treatment. Additionally, THC acts as an agonist to CB1 and CB2, while CBD acts as an allosteric modulator to these two receptors (Ahmed et al., 2018).

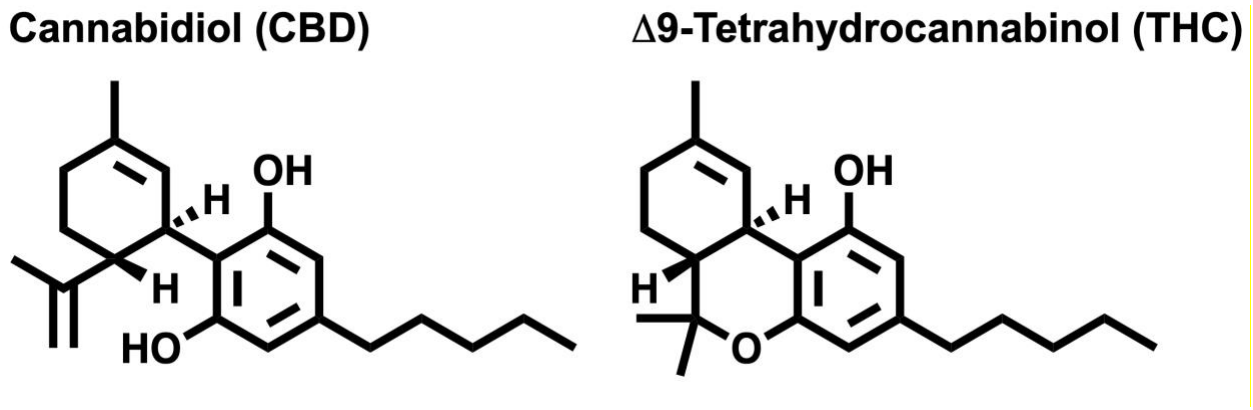


Figure 1: Structures of CBD and THC

Cannabinoids are currently being used to treat multiple diseases and conditions, such as glaucoma, cardiovascular disorders, obesity, inflammatory pain, epilepsy, and neurodegenerative diseases (Chakravarti et al., 2014). For example, THC has been considered for the treatment of epilepsy. However, experimental trials have produced mixed results. In some animal trials, THC has anticonvulsant properties, but in others, THC causes pro-convulsant effects (McCoy et al., 2018). CBD, in the Epidiolex formulation, is currently approved by the Food and Drug Administration as therapeutic for three types of epilepsy (DS and Lennox Gastaut syndrome and more recently tuberous sclerosis complex) (FDA, 2020). There is clinical evidence that CBD exhibits anticonvulsant properties in those affected by treatment-resistant epilepsy (Pamplona et al., 2018). Further research is ongoing to determine the efficacy, long-term safety and the molecular mechanisms of action of THC and CBD in the treatment of epilepsy disorders.

1.5 Glycolysis & Mitochondrial Oxidative Phosphorylation

Glycolysis is the first sequence of reactions that are part of a catabolic pathway present in many eukaryotic organisms. During glycolysis, one molecule of glucose is converted into two molecules of pyruvate, and this process generates two molecules of adenosine triphosphate (ATP) through the aid of reducing agents and enzymes (Teslaa & Teitell, 2014). Pyruvate is a 3-carbon metabolic intermediate that may go on to participate in various other energy-producing processes depending on the presence or absence of oxygen. If oxygen is not present in the environment, pyruvate is directly converted to lactate, a process that regenerates the production of nicotinamide adenine dinucleotide (NAD+) for use in glycolysis. If oxygen is present, pyruvate participates in the tricarboxylic acid (TCA) cycle. When pyruvate enters the TCA cycle within mitochondria, this cycle produces carbon dioxide (CO_2) and generates the reducing agents NADH and flavin adenine dinucleotide ($FADH_2$). Both of these agents then participate in the electron transport chain, which results in the oxidation of NADH and $FADH_2$ and reduction of molecular oxygen to water. The effective coupling of the spontaneous oxidation of NADH/$FADH_2$ to the nonspontaneous phosphorylation of ADP results in the net production of about 34 ATP per glucose molecule. This process is known as mitochondrial oxidative phosphorylation.

1.5.1 Roles of Metabolic Pathways in Epilepsy

Altered metabolism has been linked to seizure activity, resulting in a pattern of ictal hypermetabolism and increased glycolysis followed by interictal hypometabolism (Kumar et al., 2016). Diet control has been explored as a nonpharmacological treatment to combat epileptic seizures. The ketogenic diet, a low-carb, high-fat diet, is known to effectively treat some childhood epilepsies, but it is generally the final treatment option for epilepsies that do not

respond to antiepileptic drugs (Kossoff et al., 2009). Because of the efficacy of the ketogenic diet, it may be beneficial to explore the role of metabolism in epileptic disorders. The Seahorse Bioscience extracellular flux analyzer can be used to study metabolism in the laboratory. With this instrumentation, it is possible to conduct *in vitro* metabolic studies on glycolysis and oxidative phosphorylation. Rates of glycolytic flux are given in terms of the extracellular acidification rate (ECAR), which is determined by the excretion of lactic acid per unit time (Teslaa & Teitell, 2014). The oxygen consumption rate (OCR) also allows for the determination of the amount of mitochondrial oxidative phosphorylation happening within cells. When applied to biological systems, the use of the Seahorse analyzer can reveal changes in glycolytic and mitochondrial oxidative phosphorylation rates that may be linked to seizure disorders.

1.6 Zebrafish as an Epilepsy Model

Animal models for epilepsy are crucial for the understanding of epileptic mechanisms in specific disorders and how to treat them. For genetic epilepsies like DS, genetic mutations that cause disrupted gene function must be studied by generating these mutations in animal models. The development of new AEDs also relies on preclinical testing in animal models. Rodent models have been utilized for the study of DS because of genetic and physiologic similarities with humans and the ability to introduce the *Scn1a* mutation in their genome (Schutte et al., 2016). However, there are potential limitations to the rodent model, like high maintenance costs. Preclinical testing in rodent models also tends to elicit a broad-spectrum suppression against a range of different seizure types, which does not help to identify and develop more disease-specific AEDs (Griffin et al., 2016a).

The use of zebrafish as an alternative model for epilepsy studies has grown significantly over the past decade. Zebrafish models are capable of behavior-based high-throughput assays,

showing responses to established AEDs like valproate and diazepam (Schutte et al., 2016). Additionally, zebrafish are genetically similar to humans. The zebrafish genome can be altered for the study of certain behaviors or phenotypes (Griffin et al., 2016b). The scn1a mutant zebrafish model is an example of this. De novo mutations of the human *SCN1A* gene are causative in DS, and zebrafish possess two orthologues of this gene, *scn1laa* or *scn1lab*, that can be mutated to recapitulate the epileptic seizures seen in humans affected by DS (Sourbron et al., 2017).

The ECS is also highly conserved between mammals and zebrafish (Krug & Clark, 2015). Zebrafish express cnr1 and cnr2 (which encode for CB1 and CB2) in the brain and peripheral tissues, and these receptors function as homeostatic regulators of the neurological system. There is much to be discovered about the role of the ECS in epilepsies like DS, so the use of the zebrafish model may be beneficial for further studies. Manipulating the zebrafish genome to obtain cnr1 and cnr2 knockout models could help to determine the role of the ECS in epileptic activity and acute effects of cannabinoids like THC and CBD on seizure-induced behaviors (Bailone et al., 2022).

Zebrafish have many other advantages, including physiological homology to humans, high availability, and short generation time. These advantages give zebrafish the potential to be an efficient system for studying seizure-induced behavior, metabolic interventions and novel pharmaceuticals.

1.7 Study Goals & Hypotheses

The main goals of this study were to:

1. Evaluate the effects of non-functional cannabinoid receptors on larval and adult behavior.
2. Assess differences in basal mitochondrial bioenergetics among wild-type and cannabinoid receptor 1 and 2 null zebrafish lines.
3. Determine the roles of cannabinoid receptors 1 and 2 in PTZ-induced seizures.
4. Determine the efficacy of THC and CBD in reducing PTZ-induced seizures in cannabinoid receptor 1 and 2 null lines compared to wild-type.

Hypotheses:

1. *Cnr1*$^{-/-}$ larvae will experience higher locomotion and higher anxiety than WT (5D) in the larval strain. *Cnr2*$^{-/-}$ larvae will not experience changes in behavior. Adult mutant lines are expected to follow this pattern of behavior.
2. *Cnr1*$^{-/-}$ larvae will experience increased OCR compared to 5D. *Cnr2*$^{-/-}$ larvae will not experience significant changes in OCR.
3. In PTZ-treated *cnr1*$^{-/-}$ larvae, the addition of THC will result in no significant reduction of seizures and the addition of CBD will result in decreased seizure activity compared to WT fish.
4. In PTZ-treated *cnr2*$^{-/-}$ larvae, the addition of THC & CBD will result in no significant reduction of seizures compared to WT fish.

METHODS AND MATERIALS

2.1 Zebrafish husbandry

5D, *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$ fish were used in this study. 5D fish, which were used as a wild-type control, were obtained from Dr. Robyn Tanguay at Oregon State University. Cannabinoid receptor 1 and 2 mutant lines (*cnr1*$^{-/-}$ and *cnr2*$^{-/-}$) were obtained from Dr. Wolfram Goessling at

Harvard Medical School in the Genetics Division of Brigham and Women's Hospital. Adult mutant lines were genotyped prior to assessing behavior or conducting larval experiments. Fish were maintained in Aquatic Habitats Zebrafish Flow-through System under the following conditions: pH 7.5-8, dissolved oxygen 7.2-7.8 mg/L, conductivity 730-770 µS, temperature 26-29°C, and light:dark cycle of 14:10. All protocols were in accordance with the Institutional Animal Care and Use Committee guidelines.

Embryos were collected prior to larval exposures. Adult fish were placed in breeding tanks overnight, and in the morning the fish were exposed to light. During the light period, eggs were laid. To collect, eggs were filtered from the tanks and placed into petri dishes. Debris was removed from each dish and eggs were counted, raised and incubated in sterilized embryo water (pH 7.4-7.7, 60 ppm (parts per million) Instant Ocean, 0.05% methylene blue) at 28°C. Embryos were assessed daily for developmental defects.

2.2 Constitutive larval behavioral assay

At 5 days post fertilization (dpf), unexposed fish (n=46-48 per line from 5D, *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$ lines) were placed in a 96 well plate with 1 larva per well (300 µL zebrafish embryo water per well) and behavior in a larval photomotor response assay was measured by the Viewpoint Zebrabox. Movements were recorded in alternating periods of light and dark for 50 minutes (0-10 min acclimation, 12-20 min dark: 22-30 min light: 32-40 min dark: 42-50 min light). Once completed, the Viewpoint Zebrabox created an Excel spreadsheet which included total movement per fish within each of the alternating light cycles. Total distance was summed and divided by the minutes in each phase to show the differences in movement rates within the phases.

2.3 Adult behavioral assessment

Locomotor activity and anxiety-like behavior was studied in adult lines using the open field test. 5D**,** *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$ fish (n=12 fish per sex per line) were separated by sex and analyzed at age 15-18 months post fertilization. Fish were placed individually in a cylindrical arena (diameter = 30.5 cm) that was filled with 10 L of water and allowed to swim for 5 minutes at 9 Lux lighting (**Figure 2**). The Noldus EthoVision XT video tracking system was used to track movements. Differences in exploratory behavior were collected with the EthoVision software, which tracked velocity, time spent in periphery, and freezing duration.

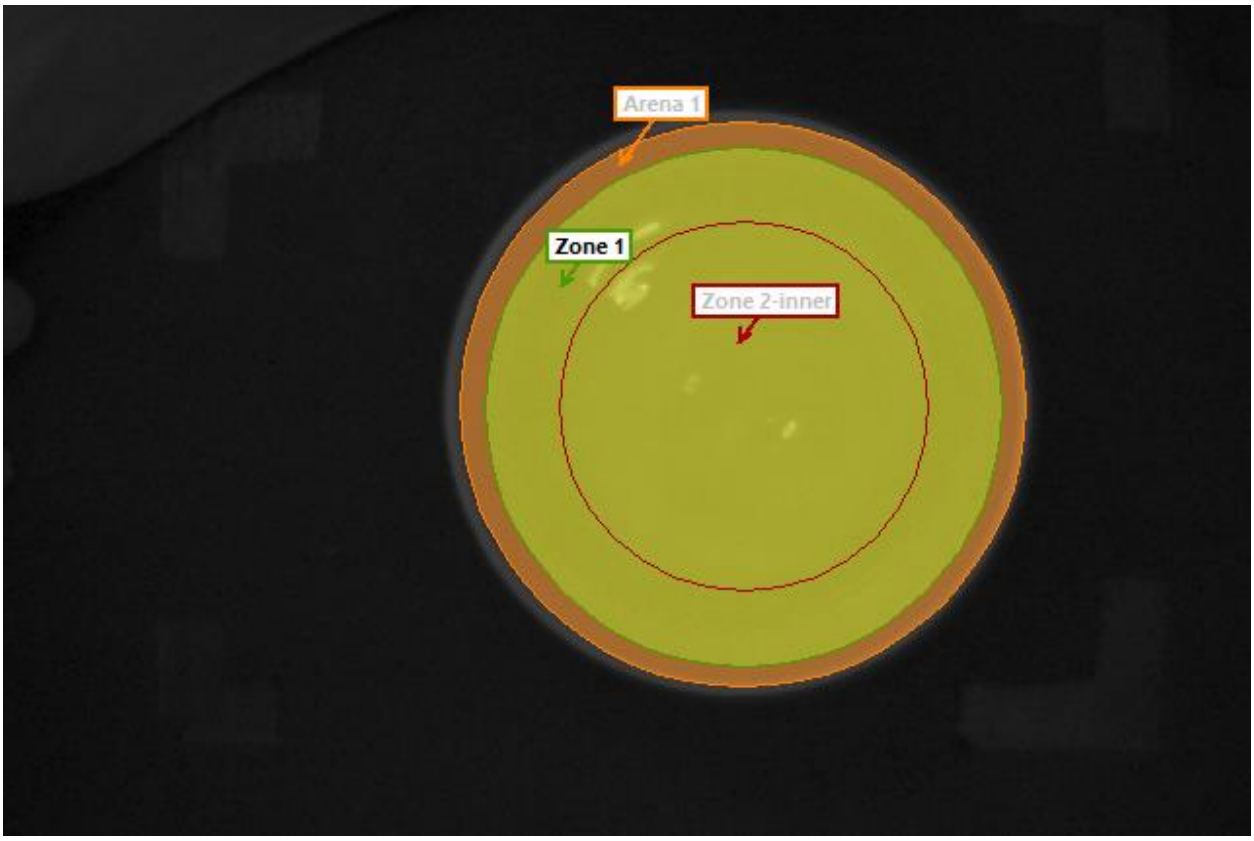


Figure 2: Cylindrical arena used in the open field test. There were two zones used for analysis: zone 1 (the outer zone (periphery)) and zone 2 (the inner zone). The total diameter was 30.5 cm.

2.4 Seahorse bioenergetics analysis

Adult brain mitochondria from each line was studied using the Cell Mito Stress Test (Agilent) conducted by the Seahorse XFe96 analyzer. 5D**,** *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$ adult brains were extracted from male and female fish and used to generate four biological replicates per sex consisting of 3-4 brains per biological replicate, and four technical replicates per biological replicate. Brain tissues were kept on ice and homogenized in Extraction Buffer with BSA (bovine serum albumin) and 1x protease inhibitors (1.6 mL per homogenizer tube). Protein samples were centrifuged twice at 700xg for 10 min at 4ºC and once at 10,000xg for 15 min at 4ºC. The resulting pellet was resuspended in Extraction Buffer without BSA or protease inhibitors (1 mL). Samples were centrifuged once again at 10,000xg for 15 min at 4ºC and resuspended in Extraction Buffer without BSA or protease inhibitors (50 µL). Mitochondrial protein was then quantified using the Nanodrop 2000 (ThermoFisher Scientific).

Following protein quantification, the oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone/antimycin A substrates for the assay were prepared and inserted into the XFe96 sensor cartridge plate for the calibration of the instrument. After calibration, the mitochondria (50 µg protein per replicate) was attached to the plate by centrifuging at 2000*xg* for 20 min at 4ºC, all wells had a final volume of 180 µL of DMEM complete (Dulbecco's Modified Eagle's Medium with 1 mM pyruvate, 10 mM glucose, and 2 mM glutamine), and the Cell Mito Stress Test was run following manufacturer's protocol (Agilent Seahorse XFe96 Cell Mito Stress Test). To assess mitochondrial function, oxygen consumption rates (OCR) were measured before the addition of metabolic activators/inhibitors (baseline respiration) and following the addition of oligomycin (1.5 µM final concentration; complex V (ATP synthase) inhibitor), FCCP (1 µM final concentration; mitochondrial

uncoupler), and rotenone/antimycin A (0.5 µM final concentration; complex I/III inhibitor) to the electron transport chain (**Figure 3**). For each parameter, OCR was measured three times over the course of an 18-minute cycle (3-minute mixing, 0-minute waiting, 3-minute measuring period).

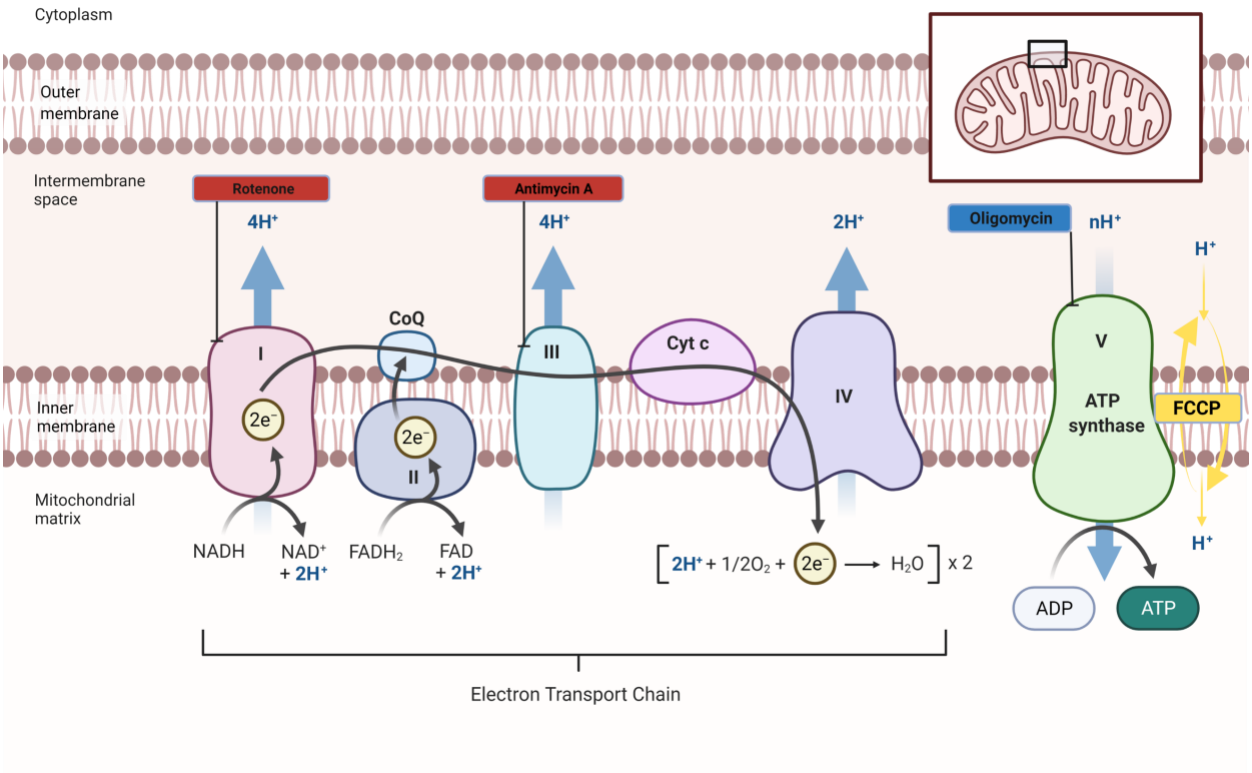


Figure 3: Metabolic modifiers added to the electron transport chain in mitochondria during the Cell Mito Stress Test. Oligomycin is added following the basal respiration stage, inhibiting complex V (ATP synthase). Next, FCCP is added, which uncouples electron transport to ATP production, destroying the proton gradient and allowing for maximal respiration through uninhibited electron flow. Lastly, rotenone/antimycin A, which inhibits complex III and I, prevent the electron transport chain operation, forcing the cell to move to non-mitochondrial respiration if possible. Created with BioRender.com.

2.5 CBD and THC larval exposures

At 5 dpf, 5D, *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$ larvae (n=24 larvae per treatment) were placed individually in wells of a 96-well plate. Fish were dosed with 150 µL dosing water: control (0.05% dimethyl

sulfoxide, DMSO), diazepam (DZP; 25 µM), CBD (0.6 and 1 µM), or THC (1 and 4 µM). These CBD and THC concentrations were chosen because they have previously reduced PTZ-induced total distance moved (Thornton et al., 2020). Plates were covered with aluminum foil and placed in a 28^{o}C incubator for 24 hours. After the 24-hour exposure, larvae were examined for deformities and mortality. Larvae with deformities were excluded from behavioral analysis.

2.5.1 Seizure induction

After 24 hours of exposure, seizures were induced by the addition of pentylenetetrazole (PTZ; 5 mM final concentration) with the exception of the control group. Total movement was recorded by the Viewpoint Zebrabox for 15 minutes in 100% light. Videos were scored for latency to seize where time to first seizure was recorded per fish.

2.6 Statistics

Data was tested for normality using the Shapiro-Wilk test and for equal variances using the Brown-Forsythe test. All graphing and statistical analysis were conducted using Sigmaplot 14.0 software.

Analysis on larval behavior was conducted on the total distance traveled during an acclimation phase and alternating phases of dark and light. Differences in behavior within each line were assessed using the ANOVA on Ranks, Dunn's posthoc, ($p \leq 0.05$) because data was non-parametric. Analysis on adult behavior was conducted on the total distance traveled, velocity, time spent in periphery, and freezing duration. Statistical analysis for mitochondrial bioenergetics were conducted on oxygen consumption rates for each stage. A two-way ANOVA was performed with sex and line as factors (Student-Newman-Keuls posthoc test, $p \leq 0.05$) when appropriate for adult behavior and mitochondrial bioenergetics. Since the data was parametric, significant differences among the three lines were assessed within each sex using a one-way

ANOVA (Tukey's posthoc test, $p \leq 0.05$). For the seizure-induced larval exposures, seizure latency and total distance were analyzed. Differences in treatment within each line were assessed using the ANOVA on Ranks, Dunn's posthoc, ($p \leq 0.05$) for seizure latency data because data was non-parametric. For total distance, a two-way ANOVA (Student-Newman-Keuls posthoc test, $p \leq 0.05$) was performed to assess treatment and line differences.

RESULTS

3.1 Roles of cnr1 and cnr2 in larval behavior

In the constitutive larval behavioral assay, unexposed larval fish from each line were placed in a 96-well plate at 5 dpf. Each plate experienced an acclimation period from 0-10 minutes in 100% light, two dark periods (from 12-20 and 32-40 minutes) and two light periods (from 22-30 and 42-50 minutes). The larval photomotor response and average total distance moved per period by each line is shown in **Figure 4**. Generally, locomotion increased during the dark periods, and locomotion decreased during the light periods (Figure 4A). Total distance during the acclimation phase (Figure 4B), dark phase (Figure 4C) and light phase (Figure 4D) was significantly increased in *cnr1*$^{-/-}$ larvae compared to 5D and *cnr2*$^{-/-}$ larvae.

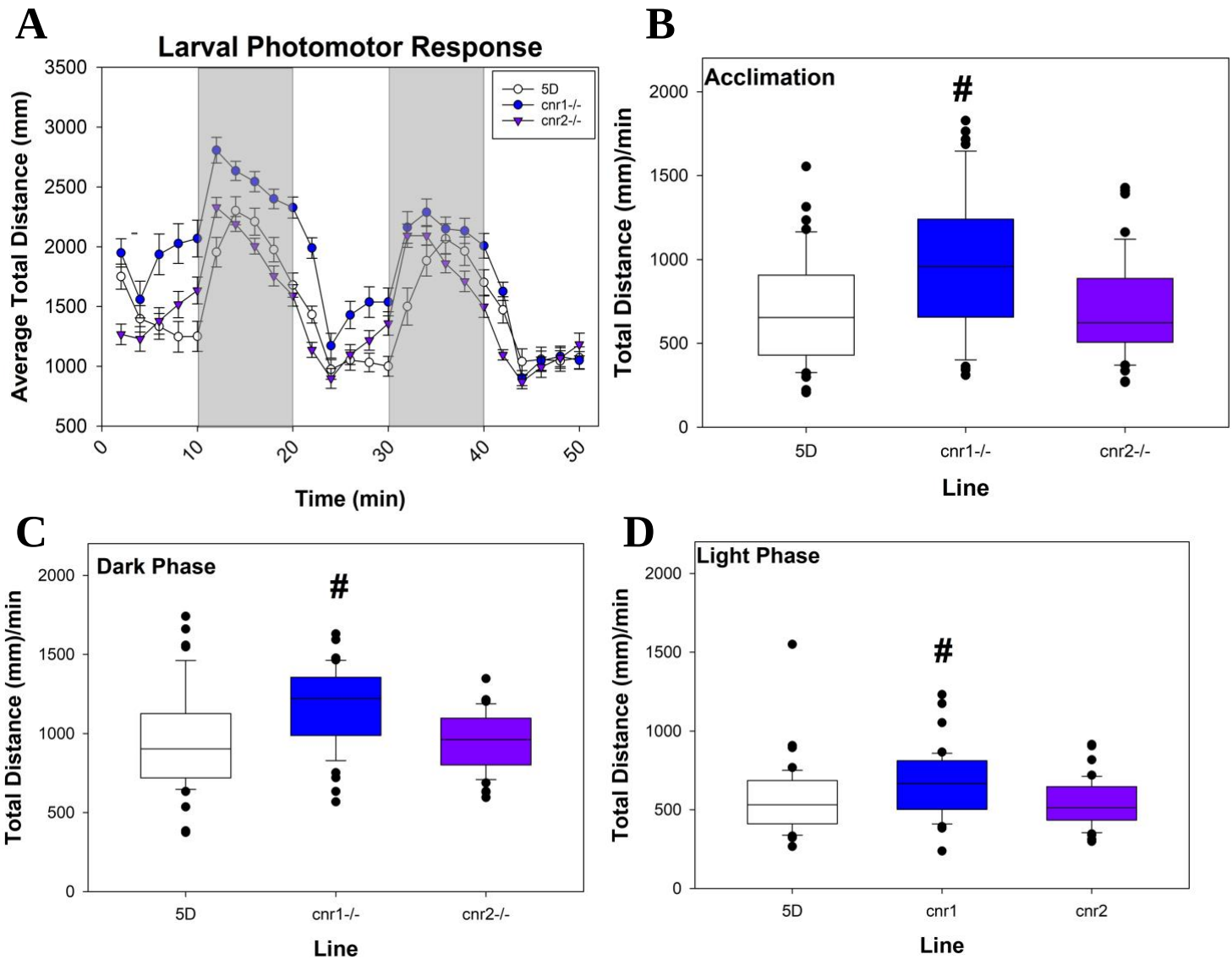


Figure 4: A Time course of larval photomotor response of 5D, *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$ larvae at 5 dpf. The total distance traveled (mm) per minute in the **B** Acclimation phase, **C** dark phase, and **D** light phase data was plotted. Box and whisker plots were used to plot phase data where the box represents the interquartile range (IQR) from 25-75%, the line inside the box is the median, the whiskers represent the top and bottom 25th percentiles, and outliers are represented with dots. For each phase, an ANOVA on Ranks followed by Dunn's posthoc test ($p \leq 0.05$, n = 46-48 per line) was used for analysis, and (#) indicates significant differences between lines.

3.2 Roles of cnr1 and cnr2 in adult behavior

In the adult behavioral assessment, the open field test was conducted using fish from each line. The total distance traveled, velocity, time in periphery, and freezing duration was analyzed as shown in **Figure 5**. There were no significant differences between total distance (Figure 5A) or velocity (Figure 5B) among female lines. However, total distance and velocity were significantly reduced in *cnr1*$^{-/-}$ males compared to 5D and *cnr2*$^{-/-}$ males. Time spent in the periphery was significantly different between males and females (Figure 5C). *Cnr2*$^{-/-}$ adults (male and female) significantly spent more time in the periphery than 5D and *cnr1*$^{-/-}$ adults. (Figure 5C). All *cnr1*$^{-/-}$ adults had significantly different freezing durations than 5D or *cnr2*$^{-/-}$ adults (Figure 5D).

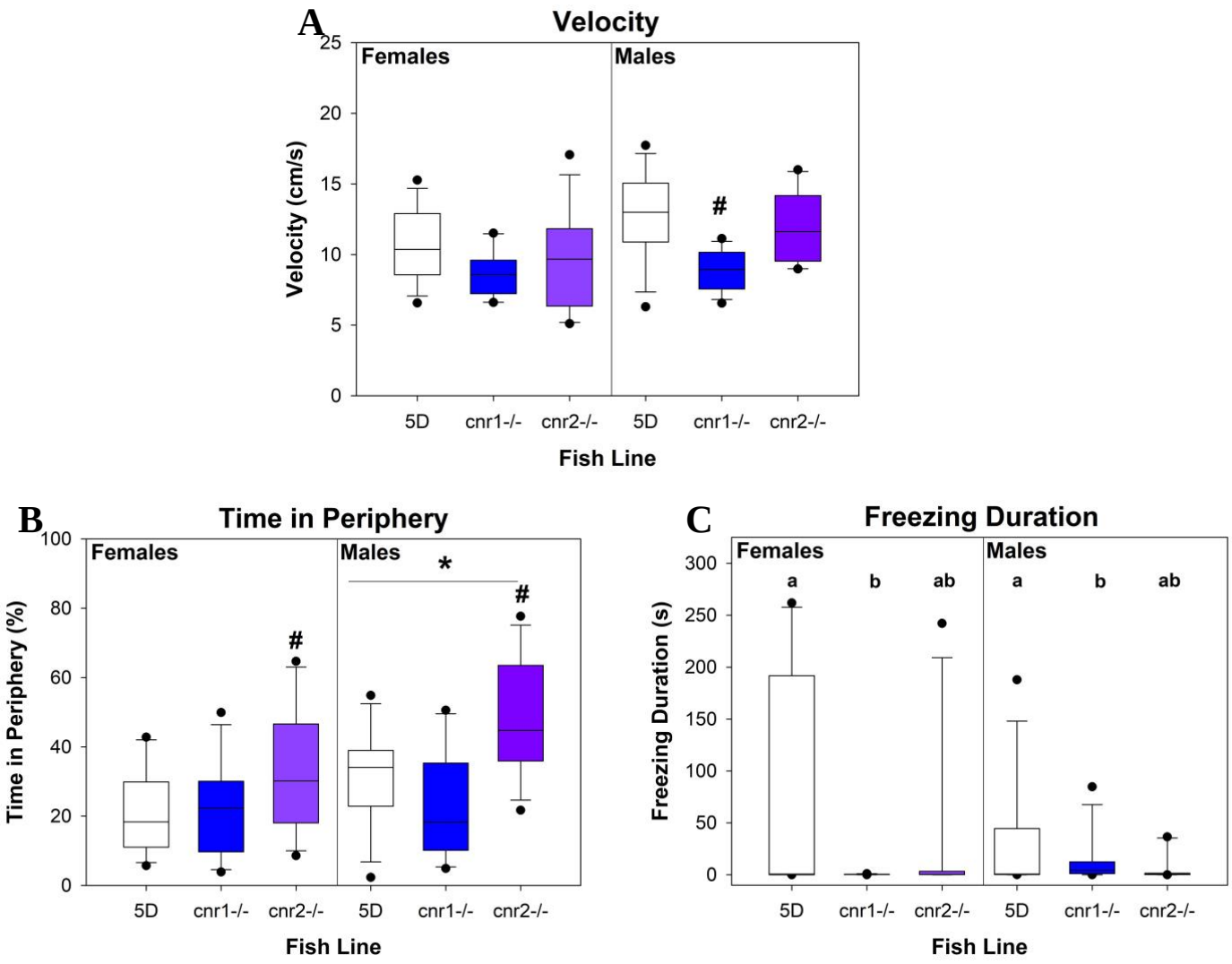
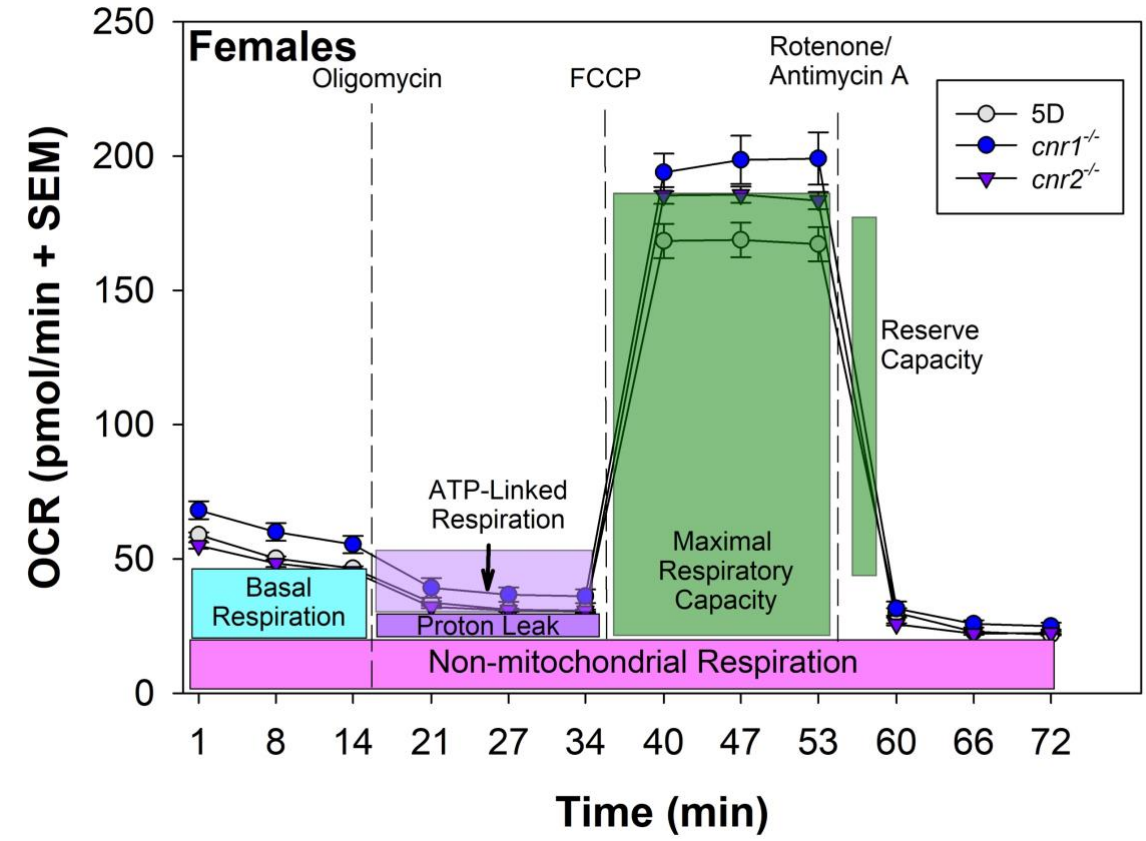


Figure 5: **A** velocity (cm/s), **B** time spent in periphery (%), and **C** freezing duration (s) of adult 5D, *cnr1*$^{-/-}$ and *cnr2*$^{-/-}$ zebrafish during the open field test conducted using the Noldus EthoVision XT video tracker. Box and whisker plots were used to plot data where the box represents the interquartile range (IQR) from 25-75%, the line inside the box is the median, the whiskers represent the top and bottom 25th percentiles, and outliers are represented with dots. For velocity analysis, differences between lines were compared using one-way ANOVA followed by Tukey's posthoc test ((#) indicates significant difference compared to 5D and *cnr2*$^{-/-}$ ($p \leq 0.05$, n = 12 adults per line per sex)). For time in periphery and freezing duration analysis, differences between sexes and lines were compared using two-way ANOVA followed by Student-Newman-Keuls posthoc test ((#) indicates significant difference compared to 5D and *cnr1*$^{-/-}$, different letters indicate significant differences compared to 5D; (*) indicates a significant difference between sexes ($p \leq 0.05$, n = 12 adults per line per sex)).

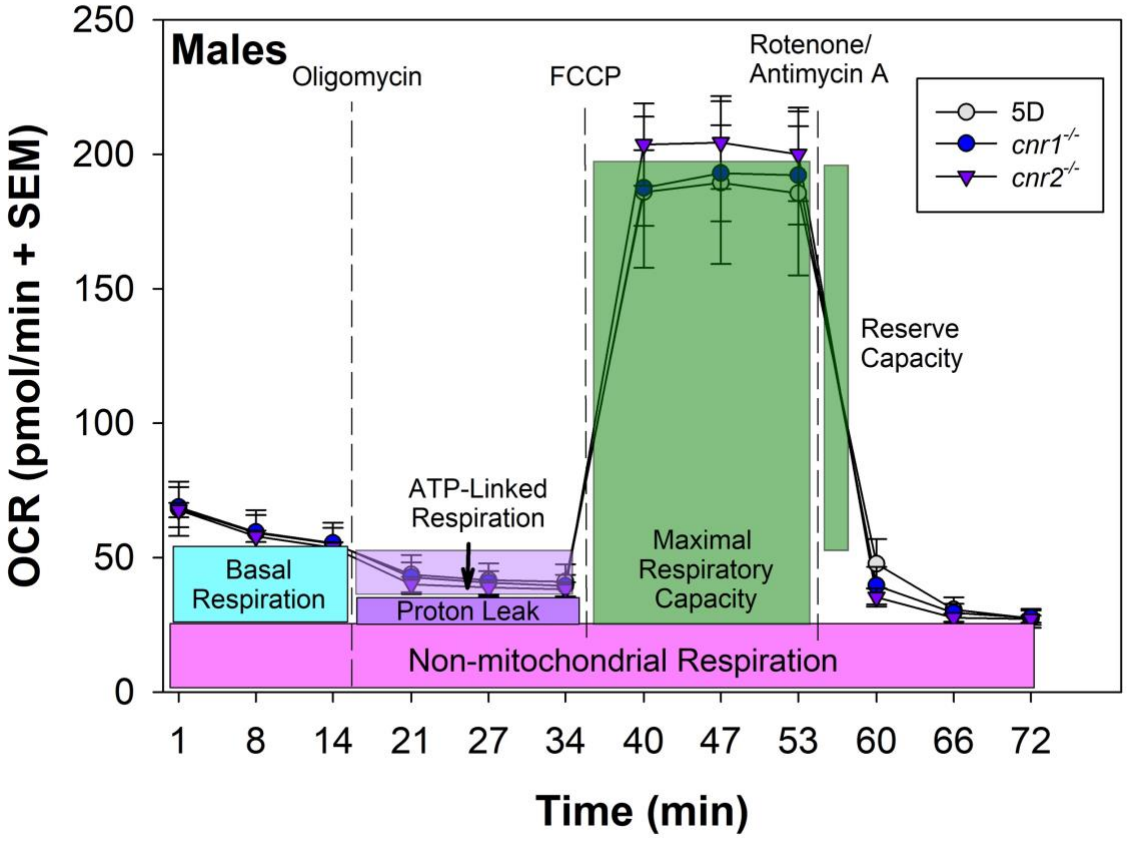
3.3 Roles of cnr1 and cnr2 in basal mitochondrial bioenergetics

The XF Cell Mito Stress test was used to assess mitochondrial bioenergetics by using adult brain mitochondria from each line. Female OCR (Figure 6A) and male OCR (Figure 6B) data was obtained from the Seahorse XFe96 analyzer. There were no significant OCR differences among the lines in any stage of the Cell Mito Stress Test, however sex differences were observed. There were no significant OCR differences between males and females during the basal respiration stage (Figure 6C) or after the addition of FCCP during the maximal respiratory capacity stage (Figure 6E). However, males had significantly higher OCR following the addition of 1) oligomycin during the ATP-linked respiration stage (Figure 6D), and 2) rotenone/antimycin A during the reserve capacity stage (Figure 6F).

A


Females
Oligomycin
FCCP
Rotenone/
Antimycin A
5D
cnr1-/-
cnr2-/-
Reserve
Capacity
ATP-Linked
Respiration
Maximal
Respiratory
Capacity
Basal
Respiration
Proton Leak
Non-mitochondrial Respiration
OCR (pmol/min + SEM)
Time (min)

B


Males
Oligomycin
FCCP
Rotenone/
Antimycin A
5D
cnr1-/-
cnr2-/-
Reserve
Capacity
ATP-Linked
Respiration
Maximal
Respiratory
Capacity
Basal
Respiration
Proton Leak
Non-mitochondrial Respiration
OCR (pmol/min + SEM)
Time (min)

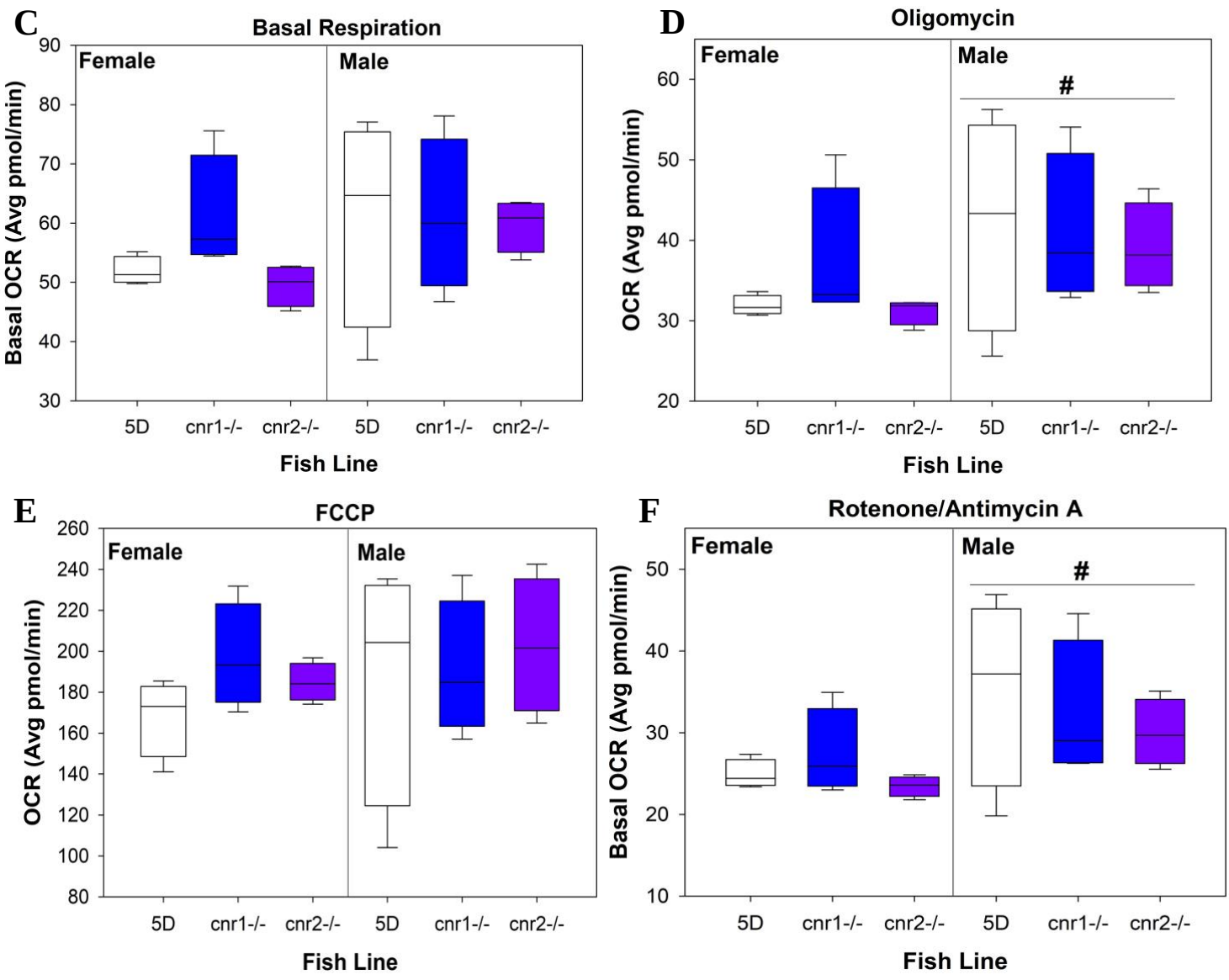


Figure 6: Oxygen consumption rates (pmol/min) in adult brains of 5D, *cnr1*$^{-/-}$ and *cnr2*$^{-/-}$ zebrafish following the Cell Mito Stress Test conducted with the Seahorse XFe96 analyzer. **A**, **B** show the rates as a function of time during the stages in which metabolic activators/inhibitors were added across the respiration period in females and males, respectively. **C**-**F** show the oxygen consumption rates prior to the addition of metabolic activators/inhibitors (baseline, C), following the addition of oligomycin (D), FCCP (E), and rotenone/antimycin A (F) plotted using box and whisker plots, where the box represents the interquartile range (IQR) from 25-75%, the line inside the box is the median, the whiskers represent the top and bottom 25th percentiles, and outliers are represented with dots. Line and sex differences were assessed with a two-way ANOVA followed by the Student-Newman-Keuls posthoc test (#) indicates a significant difference between sexes ($p \leq 0.05$, n = 3-4 brains per sex per line).

3.4 Roles of cnr1, cnr2, THC and CBD in PTZ-induced seizures

Larval fish behavior was evaluated following a 24-hour exposure to diazepam, THC, and CBD and subsequent seizure induction using PTZ. The time of the first seizure was recorded from each fish to determine seizure latency, which is shown in **Figure 7A**. Diazepam, the positive control, did significantly increase latency to seize in all three lines compared to PTZ. THC and CBD exposure did not significantly increase latency to seize in any of the lines compared to PTZ.

Total distance was also recorded following these exposures (**Figure 7B)**. 5D larvae had significantly different total distances compared to *cnr1*$^{-/-}$ and *cnr2*$^{-/-}$ larvae. THC and CBD exposure did not reduce total distance in any of the lines, but diazepam reduced distance in all lines compared to PTZ, mimicking the control group.

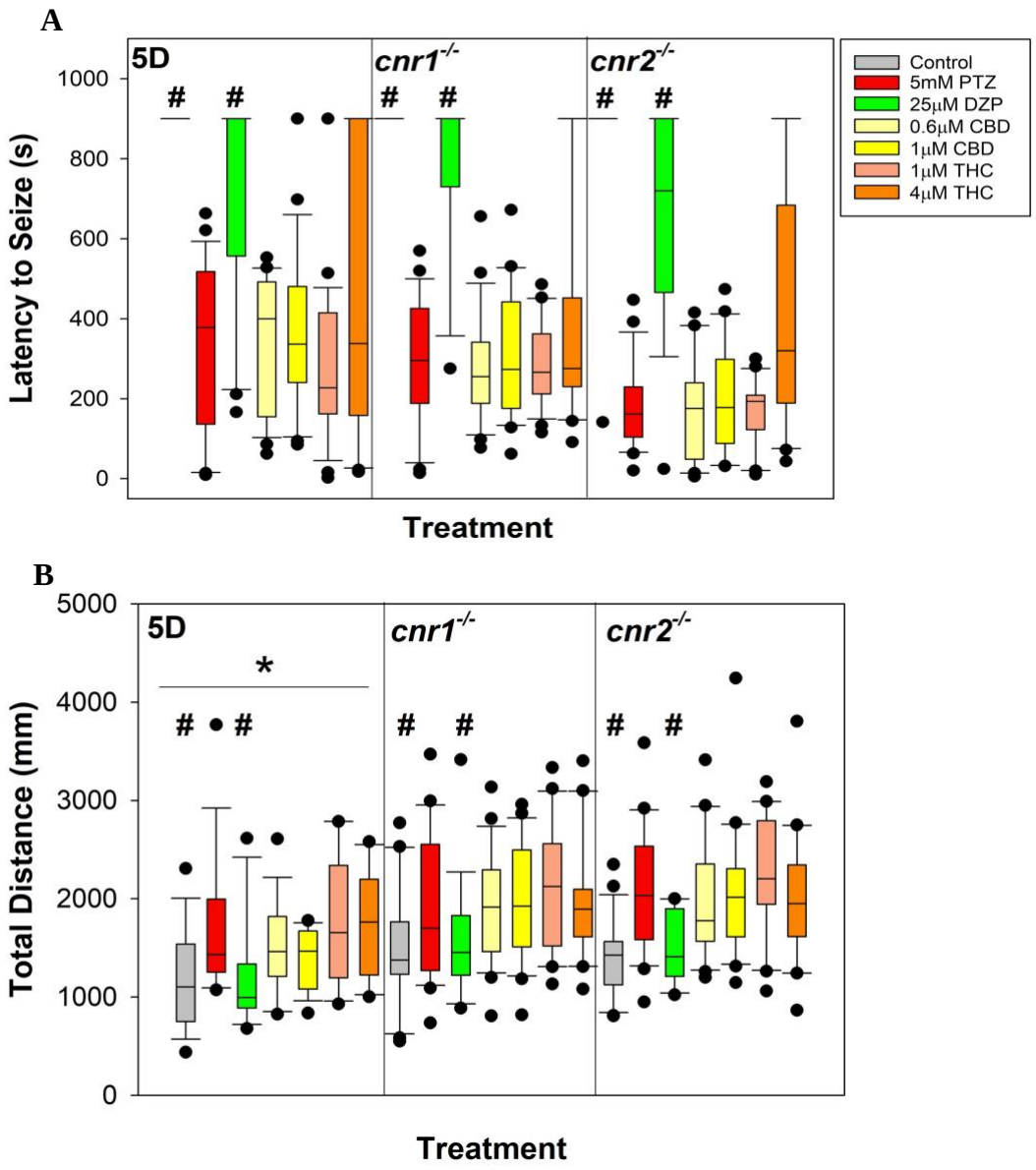


Figure 7: A Latency to seize (s) and **B** total distance (mm) of PTZ-induced seizures in 5D, *cnr1*$^{-/-}$ and *cnr2*$^{-/-}$ zebrafish following a 24-exposure from 5 to 6 dpf to 0.05% DMSO solvent control, DZP, CBD and THC. Latency to seize data was collected by scoring 15 min videos captured using the ViewPoint Zebrabox for time to first seizure. Total distance was measured from these videos. Box and whisker plots were used to plot data where the box represents the interquartile range (IQR) from 25-75%, the line inside the box is the median, the whiskers represent the top and bottom 25th percentiles, and outliers are represented with dots. Treatment groups were compared to 5 mM PTZ group using ANOVA on Ranks, followed by Dunn's posthoc test ((#) indicates significant difference compared to 5 mM PTZ group ($p \leq 0.05$, n = 24 larvae per treatment)) for latency to seize data, and differences in total distance traveled between treatment and line were assessed compared to the 5 mM PTZ group using a two-way ANOVA followed by Student-Newman-Keuls posthoc test ((#) indicates significant difference compared to 5 mM PTZ group; (*) indicates significant difference compared to *cnr1*$^{-/-}$ and *cnr2*$^{-/-}$ ($p \leq 0.05$, n = 24 larvae per treatment)).

DISCUSSION

4.1 Adult behavioral & mitochondrial assessment

The open field test was used to determine the effect of non-functional cannabinoid receptors on behavior in adult zebrafish. This test, when applied to animal models, allows for observation and study of exploratory behavior and interaction with a novel environment (Rosemberg et al., 2011). Anxious behaviors are assessed through the measurement of thigmotaxis, which refers to the tendency of animals to stay close to walls when exploring an open space. By applying this test to our fish, we could examine behavioral differences between adult lines and sexes.

According to our results, non-functional cannabinoid receptors do play a role in adult zebrafish behavior. *Cnr2*$^{-/-}$ adults spent more time in the periphery than the other two lines, which suggests more anxious behavior. This is consistent with studies in which cnr2 knockouts developed anxiety-like behavior under specific stressors, indicating that cnr2 expression is necessary to reduce anxious behaviors (Ishiguro et al., 2018). The results also indicated that among the males only, *cnr1*$^{-/-}$ adults had decreased total distances and velocities. These results suggest significant differences between sexes that may be linked to behavioral changes.

Seizure activity has been linked to altered metabolism in a previous study using DS animal models, but there is much to be discovered regarding metabolic changes due to loss of functional cannabinoid receptors (Kumar et al., 2016). One relevant study demonstrated the effects of the inhibition of cannabinoid receptor activity on liver metabolism and found that adult mutants experience abnormal liver development and metabolism following this inhibition (Liu et al., 2016). Another study suggested that metabolism may be indirectly altered by cannabinoid receptor binding of THC and CBD, which may contribute to behavioral and developmental

abnormalities (Pandelides et al., 2021). To assess constitutive changes in mitochondrial function among 5D and mutant lines without functional cannabinoid receptor 1 or 2, the Cell Mito Stress Test was used to create a metabolic profile of each line and each sex which shows the key stages of mitochondrial function (Gu et al., 2021). Oxygen consumption rates (OCR) were measured along each stage to indicate normal cellular function.

Our results indicated that there were no significant differences in OCR among lines, but there were sex differences in mitochondrial respiration across certain complexes of the electron transport chain. Males experienced higher OCR rates during the ATP-linked respiration stage (after inhibiting complex V), which may be indicative of higher ATP demand, and higher OCR after inhibiting complexes I and III, which is indicative of increased non-mitochondrial respiration (Wang et al., 2013). These elevated rates in the adult males may contribute to the more anxious behavior observed in the *cnr2*$^{-/-}$ adult males, but because there were no significant differences among lines, we cannot conclude this from the mitochondrial bioenergetics assessment. The metabolic assessment also does not explain why the adult behavioral assessment resulted in a significant decrease in total distance and velocity in the *cnr1*$^{-/-}$ adult male fish. However, epilepsy prevalence tends to be higher in males when compared to females (Banerjee et al., 2009). If higher metabolic rates are indicative of higher epileptic activity and abnormal behavior, our results are consistent with this data. Further studies are needed to investigate behavioral and metabolic differences among sexes that could characterize the role of the ECS in epileptic disorders.

4.2 Larval behavior and PTZ-induced activity

The larval photomotor response assay was used to assess constitutive changes in zebrafish swimming behavior in the 5D, *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$ lines. Following swim bladder development (4 dpf), zebrafish larvae show a specific pattern of movement when exposed to cycles of light and dark conditions (Burgess & Granato, 2007). The light-dark transition increases locomotion due to an increased stress response caused by scotophobia, while the dark-light transition decreases locomotion. Deviations from this pattern are typically indicative of neural abnormalities because locomotion is dependent on brain function, nervous system development, and visual pathways (Ali et al., 2012).

Locomotion was significantly increased across the acclimation phase, dark phase, and light phase of the larval photomotor response assay in *cnr1*$^{-/-}$ larvae compared to 5D and *cnr2*$^{-/-}$ larvae. One previous study found that *cnr1*$^{-/-}$ larvae experienced increased locomotion that was observed during the dark phase, suggesting that this could be because of developmental changes due to the cnr1 deficiency (Luchtenburg et al., 2019). CB1 signaling is known to mediate neuronal and endocrine stress responses, so the absence of the cnr1 gene may contribute to an increased stress response and result in hyperactivity (Beins et al., 2021). C*nr2*$^{-/-}$ larvae behaved similarly to the 5D wild-type line, indicating that the lack of cnr2 has no significant effects on locomotion in the larval photomotor response assay.

THC and CBD exposures were proposed due to their previous reduction of PTZ-induced seizure activity in zebrafish larvae in a previous study (Thornton et al., 2020), in which total distance was significantly reduced by THC (1 and 4 μM) and CBD (0.6 and 1 μM) in wild-type models and DS fish (*scn1Lab*$^{-/-}$). In the present study, neither total distance traveled nor latency to seize were significantly altered by cannabinoid treatments compared to PTZ. However, total distance traveled was significantly reduced in the 5D wild-type model compared to the other

lines. The knockout models were more hyperactive overall, which matched the increased locomotion observed in *cnr1*$^{-/-}$ larvae during the constitutive photomotor response assay. Following exposure, *cnr2*$^{-/-}$ larvae were also more hyperactive across all treatment groups compared to the 5D, deviating from the behavior observed in the constitutive assay.

THC is a known agonist of CB1 and CB2, with THC having a higher binding affinity for the CB1 receptor. Our results indicated that THC, compared to the PTZ group, had no significant effects on latency to seize or total distance in any of the lines. In a previous study, wild-type larvae that were pre-exposed to THC and CBD and induced using 2.5 and 5 mM PTZ experienced a reduction in total activity (Samarut et al., 2019). If THC is a CB1 agonist, these results suggest that the effects of THC are cnr1- and cnr2-mediated. However, although total distance was reduced in the 5D larvae compared to the knockout lines, we could not conclude whether the cnr1 and cnr2 genes were necessary for THC to be able to elicit a response due to the lack of significant differences between treatments in each line.

A similar conclusion for CBD, an allosteric modulator of the cannabinoid receptors, was reached. Our results indicated that CBD had no significant effects on latency to seize or total distance compared to the PTZ group in any of the lines. Although CBD has been proposed as a viable anticonvulsant therapy, perhaps more research is needed to determine the role of CBD in the ECS, especially because there are studies which indicate that CBD may have different modes of action that are independent from cnr1 and cnr2 (Pertwee, 2008).

4.3 Conclusion

In order to characterize the effects of non-functional cannabinoid receptors in larval and adult zebrafish models, we investigated whether the absence of cannabinoid receptor 1 or 2 would result in changes in behavior, metabolism, and/or seizure-induced activity. In summary,

we identified significant differences in behavior and metabolism in cnr1 and cnr2 knockout adult fish models that were consistent with previous studies. This study also found significant differences among sexes that may play a role in behavior and metabolism of adult fish. However, we were not able to determine the specific roles that cannabinoid receptor 1 and 2 may play in seizure-induced activity. Our data demonstrated that exposure to THC and CBD had no significant effects in any of the lines, which may suggest that these cannabinoids may have different modes of action. In this study, THC and CBD were not effective in reducing PTZ seizure-induced activity.

We hope that this research will inform further studies on the roles of cannabinoid receptors in other epilepsy models in order to determine the best antiepileptic drug treatments. An important research question raised is why are the adverse behavioral effects observed in cnr-null animals as adults but not observed in the larval stages? Delving into further research on larval behavior and metabolism in fish models should provide clarity and lead to a better understanding of the role of the ECS in epilepsy.

REFERENCES

Ahmed, K. T., Amin, M. R., Shah, P., & Ali, D. W. (2018). Motor neuron development in zebrafish is altered by brief (5-hr) exposures to THC (Δ9-tetrahydrocannabinol) or CBD (cannabidiol) during gastrulation. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-28689-z

Ali, S., Champagne, D. L., & Richardson, M. K. (2012). Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds. *Behavioural Brain Research*, *228*(2), 272–283. https://doi.org/10.1016/j.bbr.2011.11.020

Anderson, L. L., Doohan, P. T., Hawkins, N. A., Bahceci, D., Garai, S., Thakur, G. A., Kearney, J. A., & Arnold, J. C. (2022). The endocannabinoid system impacts seizures in a mouse model of Dravet syndrome. *Neuropharmacology*, *205*. https://doi.org/10.1016/j.neuropharm.2021.108897

Andre, C. M., Hausman, J. F., & Guerriero, G. (2016). Cannabis sativa: The plant of the thousand and one molecules. *Frontiers in Plant Science*, *7*(FEB2016). https://doi.org/10.3389/fpls.2016.00019

Bailone, R. L., Fukushima, H. C. S., de Aguiar, L. K., & Borra, R. C. (2022). The endocannabinoid system in zebrafish and its potential to study the effects of Cannabis in humans. *Laboratory Animal Research*, *38*(1), 5. https://doi.org/10.1186/s42826-022-00116-5

Banerjee, P. N., Filippi, D., & Allen Hauser, W. (2009). The descriptive epidemiology of epilepsy—A review. *Epilepsy Research*, *85*(1), 31–45. https://doi.org/10.1016/j.eplepsyres.2009.03.003

Beins, E. C., Beiert, T., Jenniches, I., Hansen, J. N., Leidmaa, E., Schrickel, J. W., & Zimmer, A. (2021). Cannabinoid receptor 1 signalling modulates stress susceptibility and microglial responses to chronic social defeat stress. *Translational Psychiatry*, *11*(1), 164. https://doi.org/10.1038/s41398-021-01283-0

Boon, P., Ferrao Santos, S., Jansen, A. C., Lagae, L., Legros, B., & Weckhuysen, S. (2021). Recommendations for the treatment of epilepsy in adult and pediatric patients in Belgium: 2020 update. *Acta Neurologica Belgica*, *121*(1), 241–257. https://doi.org/10.1007/s13760-020-01488-y

Burgess, H. A., & Granato, M. (2007). Modulation of locomotor activity in larval zebrafish during light adaptation. *Journal of Experimental Biology*, *210*(14), 2526–2539. https://doi.org/10.1242/jeb.003939

Centers for Disease Control and Prevention. (2021). *Epilepsy Fast Facts*. U.S. Department of Health and Human Services . https://www.cdc.gov/epilepsy/about/fast-facts.htm

Chakravarti, B., Ravi, J., & Ganju, R. K. (2014). *Cannabinoids as therapeutic agents in cancer: current status and future implications* (Vol. 5, Issue 15). www.impactjournals.com/oncotarget

Cheung, K. A. K., Peiris, H., Wallace, G., Holland, O. J., & Mitchell, M. D. (2019). The interplay between the endocannabinoid system, epilepsy and cannabinoids. In *International Journal of Molecular Sciences* (Vol. 20, Issue 23). MDPI AG. https://doi.org/10.3390/ijms20236079

Epilepsy Foundation. (2019). *What is Epilepsy*. https://www.epilepsy.com/learn/about-epilepsy-basics/what-epilepsy

FDA NEWS RELEASE FDA Approves New Indication for Drug Containing an Active Ingredient Derived from Cannabis to Treat Seizures in Rare Genetic Disease. (2020). https://ghr.nlm.nih.gov/condition/tuberous-sclerosis-complex#statistics

Griffin, A., Krasniak, C., & Baraban, S. C. (2016a). Advancing epilepsy treatment through personalized genetic zebrafish models. In *Progress in Brain Research* (Vol. 226, pp. 195–207). Elsevier B.V. https://doi.org/10.1016/bs.pbr.2016.03.012

Griffin, A., Krasniak, C., & Baraban, S. C. (2016b). Advancing epilepsy treatment through personalized genetic zebrafish models. In *Progress in Brain Research* (Vol. 226, pp. 195–207). Elsevier B.V. https://doi.org/10.1016/bs.pbr.2016.03.012

Gu, X., Ma, Y., Liu, Y., & Wan, Q. (2021). Measurement of mitochondrial respiration in adherent cells by Seahorse XF96 Cell Mito Stress Test. *STAR Protocols*, *2*(1), 100245. https://doi.org/10.1016/j.xpro.2020.100245

Ishiguro, H., Horiuchi, Y., Tabata, K., Liu, Q. R., Arinami, T., & Onaivi, E. S. (2018). Cannabinoid CB2 receptor gene and environmental interaction in the development of psychiatric disorders. *Molecules*, *23*(8). https://doi.org/10.3390/molecules23081836

Kossoff, E. H., Zupec-Kania, B. A., Amark, P. E., Ballaban-Gil, K. R., Christina Bergqvist, A. G., Blackford, R., Buchhalter, J. R., Caraballo, R. H., Helen Cross, J., Dahlin, M. G., Donner, E. J., Klepper, J., Jehle, R. S., Kim, H. D., Christiana Liu, Y. M., Nation, J., Nordli, D. R., Pfeifer, H. H., Rho, J. M., … Yim, G. (2009). Optimal clinical management of children receiving the ketogenic diet: Recommendations of the International Ketogenic Diet Study Group. In *Epilepsia* (Vol. 50, Issue 2, pp. 304–317). https://doi.org/10.1111/j.1528-1167.2008.01765.x

Krug, R. G., & Clark, K. J. (2015). Elucidating cannabinoid biology in zebrafish (Danio rerio). *Gene*, *570*(2), 168–179. https://doi.org/10.1016/j.gene.2015.07.036

Kumar, M. G., Rowley, S., Fulton, R., Dinday, M. T., Baraban, S. C., & Patel, M. (2016). Altered Glycolysis and Mitochondrial Respiration in a Zebrafish Model of Dravet Syndrome. *Eneuro*, *3*(2), ENEURO.0008-16.2016. https://doi.org/10.1523/ENEURO.0008-16.2016

Liu, L. Y., Alexa, K., Cortes, M., Schatzman-Bone, S., Kim, A. J., Mukhopadhyay, B., Cinar, R., Kunos, G., North, T. E., & Goessling, W. (2016). Cannabinoid receptor signaling regulates liver development and metabolism. *Development (Cambridge)*, *143*(4), 609–622. https://doi.org/10.1242/dev.121731

Lu, H. C., & MacKie, K. (2016). An introduction to the endogenous cannabinoid system. In *Biological Psychiatry* (Vol. 79, Issue 7, pp. 516–525). Elsevier USA. https://doi.org/10.1016/j.biopsych.2015.07.028

Luchtenburg, F. J., Schaaf, M. J. M., & Richardson, M. K. (2019). Functional characterization of the cannabinoid receptors 1 and 2 in zebrafish larvae using behavioral analysis. *Psychopharmacology*, *236*(7), 2049–2058. https://doi.org/10.1007/s00213-019-05193-4

McCoy, B., Wang, L., Zak, M., Al-Mehmadi, S., Kabir, N., Alhadid, K., McDonald, K., Zhang, G., Sharma, R., Whitney, R., Sinopoli, K., & Snead, O. C. (2018). A prospective open-label trial of a CBD/THC cannabis oil in dravet syndrome. *Annals of Clinical and Translational Neurology*, *5*(9), 1077–1088. https://doi.org/10.1002/acn3.621

National Institute of Neurological Disorders and Stroke. (2020). *The Epilepsies and Seizures: Hope Through Research* . https://www.ninds.nih.gov/Disorders/Patient-Caregiver-Education/Hope-Through-Research/Epilepsies-and-Seizures-Hope-Through#3109_4

NORD. (2019). *Dravet Syndrome*. https://rarediseases.org/rare-diseases/dravet-syndrome-spectrum/

Pamplona, F. A., da Silva, L. R., & Coan, A. C. (2018). Potential clinical benefits of CBD-Rich cannabis extracts over purified CBD in treatment-resistant epilepsy: Observational data meta-analysis. In *Frontiers in Neurology* (Vol. 9, Issue SEP). Frontiers Media S.A. https://doi.org/10.3389/fneur.2018.00759

Pandelides, Z., Aluru, N., Thornton, C., Watts, H. E., & Willett, K. L. (2021). Transcriptomic Changes and the Roles of Cannabinoid Receptors and PPARγin Developmental Toxicities following Exposure to Δ9-Tetrahydrocannabinol and Cannabidiol. *Toxicological Sciences*, *182*(1), 44–59. https://doi.org/10.1093/toxsci/kfab046

Pertwee, R. G. (2008). The diverse CB 1 and CB 2 receptor pharmacology of three plant cannabinoids: Δ 9-tetrahydrocannabinol, cannabidiol and Δ 9-tetrahydrocannabivarin. *British Journal of Pharmacology*, *153*(2), 199–215. https://doi.org/10.1038/sj.bjp.0707442

Rosemberg, D. B., Rico, E. P., Mussulini, B. H. M., Piato, Â. L., Calcagnotto, M. E., Bonan, C. D., Dias, R. D., Blaser, R. E., Souza, D. O., & de Oliveira, D. L. (2011). Differences in Spatio-Temporal Behavior of Zebrafish in the Open Tank Paradigm after a Short-Period Confinement into Dark and Bright Environments. *PLoS ONE*, *6*(5), e19397. https://doi.org/10.1371/journal.pone.0019397

Rosenbaum, D. M., Rasmussen, S. G. F., & Kobilka, B. K. (2009). The structure and function of G-protein-coupled receptors. In *Nature* (Vol. 459, Issue 7245, pp. 356–363). https://doi.org/10.1038/nature08144

Rowley, S., Sun, X., Lima, I. v., Tavenier, A., de Oliveira, A. C. P., Dey, S. K., & Danzer, S. C. (2017). Cannabinoid receptor 1/2 double-knockout mice develop epilepsy. *Epilepsia*, *58*(12), e162–e166. https://doi.org/10.1111/epi.13930

Samarut, É., Nixon, J., Kundap, U. P., Drapeau, P., & Ellis, L. D. (2019). Single and Synergistic Effects of Cannabidiol and Δ-9-Tetrahydrocannabinol on Zebrafish Models of Neuro-Hyperactivity. *Frontiers in Pharmacology*, *10*. https://doi.org/10.3389/fphar.2019.00226

Schmidt, D. (2011). Efficacy of New Antiepileptic Drugs. *Epilepsy Currents*, *11*(1). https://doi.org/10.5698/1535-7511-11.1.9

Schutte, S. S., Schutte, R. J., Barragan, E. v., & O'Dowd, D. K. (2016). Model systems for studying cellular mechanisms of *SCN1A* -related epilepsy. *Journal of Neurophysiology*, *115*(4), 1755–1766. https://doi.org/10.1152/jn.00824.2015

Shapiro, L., Wong, J. C., & Escayg, A. (2019). Reduced cannabinoid 2 receptor activity increases susceptibility to induced seizures in mice. *Epilepsia*, *60*(12), 2359–2369. https://doi.org/10.1111/epi.16388

Sourbron, J., Smolders, I., de Witte, P., & Lagae, L. (2017). Pharmacological analysis of the anti-epileptic mechanisms of fenfluramine in scn1a mutant zebrafish. *Frontiers in Pharmacology*, *8*(APR), 191. https://doi.org/10.3389/fphar.2017.00191

Strzelczyk, A., & Schubert-Bast, S. (2020). Therapeutic advances in Dravet syndrome: a targeted literature review. *Expert Review of Neurotherapeutics*, *20*(10). https://doi.org/10.1080/14737175.2020.1801423

Strzelczyk, A., Schubert-Bast, S., Bast, T., Bettendorf, U., Fiedler, B., Hamer, H. M., Herting, A., Kalski, M., Kay, L., Kieslich, M., Klein, K. M., Kluger, G., Kurlemann, G., Mayer, T., Neubauer, B. A., Polster, T., von Spiczak, S., Stephani, U., Trollmann, R., … Rosenow, F. (2019). A multicenter, matched case-control analysis comparing burden-of-illness in Dravet

syndrome to refractory epilepsy and seizure remission in patients and caregivers in Germany. *Epilepsia*, *60*(8). https://doi.org/10.1111/epi.16099

Teslaa, T., & Teitell, M. A. (2014). Techniques to monitor glycolysis. In *Methods in Enzymology* (Vol. 542, pp. 91–114). Academic Press Inc. https://doi.org/10.1016/B978-0-12-416618-9.00005-4

Thornton, C., Dickson, K. E., Carty, D. R., Ashpole, N. M., & Willett, K. L. (2020). Cannabis constituents reduce seizure behavior in chemically-induced and scn1a-mutant zebrafish. *Epilepsy and Behavior*, *110*, 107152. https://doi.org/10.1016/j.yebeh.2020.107152

Wang, D., Green, M. F., McDonnell, E., & Hirschey, M. D. (2013). *Oxygen Flux Analysis to Understand the Biological Function of Sirtuins* (pp. 241–258). https://doi.org/10.1007/978-1-62703-637-5_16

Wirrell, E. C., & Nabbout, R. (2019). Recent Advances in the Drug Treatment of Dravet Syndrome. In *CNS Drugs* (Vol. 33, Issue 9, pp. 867–881). Springer International Publishing. https://doi.org/10.1007/s40263-019-00666-8

Zou, S., & Kumar, U. (2018). Cannabinoid receptors and the endocannabinoid system: Signaling and function in the central nervous system. In *International Journal of Molecular Sciences* (Vol. 19, Issue 3). MDPI AG. https://doi.org/10.3390/ijms19030833