Generation of *cnr1* knockout zebrafish line and assessment of their breeding competency

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Abstract

The endocannabinoid system is a signaling system found in the vertebrate species and consists of cannabinoid receptors, endogenous ligands, and metabolic enzymes. It is suggested that the endocannabinoid system has a role in maintaining various functions, such as reproduction. Interestingly, impairing the system has deleterious effects in fertility, but the mechanisms underlying these effects are unknown. The objective of our study was to develop a cannabinoid receptor 1 (CNR1) knockout line of zebrafish (*Danio rerio*) and investigate the function of CNR1 in breeding competency. It was hypothesized that there will be a decrease in CNR1 mutant fertility and fecundity. We determined the mRNA expression of *cnr1* and *cnr2* in wild-type male and female brain, and gonads, in which there was no significant interaction observed between receptor and organ. We also generated a *cnr1* knockout line via CRISPR-Cas9 and assessed the breeding competency of $cnr1^{+/-}$ females. Furthermore, we examined the phenotypic characteristics of the $cnr1^{+/-}$ mutants. We found no statistical difference in breeding competency, gonadosomatic index and condition factor between $cnr1^{+/-}$ and the wild-type females. Moving forward, we will assess the breeding competency of $cnr1^{-/-}$, as our results were inconclusive in determining the functional role of CNR1 in fertility. Overall, we were able to successfully generate a *cnr1* knockout line using CRISPR-Cas9. To my knowledge, there is little to no data available on the specific role of the endocannabinoid system in zebrafish reproduction. I anticipate that future research will provide a novel dataset to understand the involvement of this system in zebrafish reproduction.

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List of abbreviations

2-AG = 2-arachidonoyl glycerol (protein)

abhd12 = alpha/beta-hydrolase domain containing 12 (mRNA transcript)

abhd6a = alpha/beta-hydrolase domain containing 6 (mRNA transcript)

AEA = anandamide

ANOVA = analysis of variance

cAMP = cyclic AMP

CBD = cannabidiol

CBN = cannabinol

cDNA = complementary DNA

CNR = cannabinoid receptor

CNR1 = cannabinoid receptor 1 (protein)

CNR2 = cannabinoid receptor 2 (protein)

cnrip1 = cannabinoid receptor interacting protein 1 (mRNA transcript)

CNS = central nervous system

CRISPR-Cas9 = clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9

crRNA = CRISPR RNA

dagla = diacylglycerol lipase alpha (mRNA transcript)

daglb = diacylglycerol lipase beta (mRNA transcript)

DAG-lipase = diacylglycerol lipase (protein)

ddPCR = droplet digital PCR

DSB = double-stranded breaks

eCB = endocannabinoid

ECS = endocannabinoid system

FAAH = fatty acid amide hydrolase (protein)

faah = fatty acid amide hydrolase (mRNA transcript)

gde1 = glycerophosphodiester phosphodiesterase 1 (mRNA transcript)

GnRH = Gonadotropin releasing hormone

GPCR = G-protein coupled receptor

GSI = gonadosomatic index

HIV/AIDS = human immunodeficiency virus/acquired immune deficiency syndrome

hpf = hours post-fertilization

HPO = hypothalamic-pituitary-ovarian axis

indel = insertion/deletion

Kn = condition factor

KO = knockout

MAPK = mitogen-activated protein kinases

mgll = monoacylglycerol lipase (mRNA transcript)

mRNA = messenger RNA

NAPE = N-acyl phosphatidylethanolamine-specific phospholipase D (protein)

NAPE-PLD = N-acyl phosphatidylethanolamine specific phospholipase D (protein)

naple-pld = N-acyl phosphatidylethanolamine phospholipase D (mRNA transcript)

NHEJ = non-homologous end joining

NHEJ = non-homologous end-joining

PAM = protospacer adjacent motif

PKA = protein kinase A

PNS = parasympathetic nervous system

qPCR = quantitative PCR

rpl13a = ribosomal protein L13a (mRNA transcript)

rps18 = ribosomal protein S18 (mRNA transcript)

RT-PCR = real-time polymerase chain reaction

sgRNA = single guide RNA

TALEN = Transcription activator-like effector nucleases

THC = tetrahydrocannabinol

tracrRNA = trans-activating crRNA

WT = wild type

ZFN = zinc finger nucleases

Chapter I:

General Introduction

1.1 History of cannabinoid research

The marijuana plant (genus *Cannabis*) is one of the earliest domesticated plants, first documented over 12,000 years ago in Eastern Asia (Ren et al., 2021). Throughout time, it has had both recreational and medicinal purpose used by people all over the globe. Individuals often use marijuana because symptoms of euphoria and relaxation are associated with its use. On the other hand, it has also been used medicinally to treat symptoms of nausea associated with cancer therapy and to improve eating in human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) patients (Borgelt et al., 2013). Today, this plant continues to be one of the most popular drugs to use, comparable to alcohol and tobacco. A brief historical account along with the pharmacology of the plant will be discussed. Moreover, the discovery of the endocannabinoid system will be elucidated, which is the endogenous system that the active ingredients of the *Cannabis* plant interact with to generate effects within the organism.

Cannabis research initially began in the 1840s—in which psychiatrist Jacques-Joseph Moreau prescribed hashish to his patients and recorded their experiences (Moreau, 1845). Dr. Moreau noted that his patients experienced “delirium” and later expanded his research into the effects of marijuana in the central nervous system. Between the 1940s-1950s, more pharmacological research with marijuana derivatives tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN) was conducted. For example, it was determined that cannabis increased sleep duration in mice (Loewe, 1945) Furthermore, in 1946, Loewe had determined that THC rather than CBD caused catalepsy, which is a condition in which the body undergoes seizures

followed by a loss of sensation (Loewe, 1946). Results such as these gave hints to the psychotropic ability of the plant-derived cannabinoids.

However, its interest peaked during the 1960s Psychedelic Era, in which scientists began to investigate marijuana and its neurological effects. Particularly, in 1963, utilizing nuclear magnetic resonance spectroscopy (NMR) and column chromatography, young scientist Raphael Mechoulam successfully identified the structure of CBD (Mechoulam and Shvo 1963). A year later, the structure of tetrahydrocannabinol (THC) was determined. Moreover, it was hypothesized that the psychotropic effects of marijuana were due to THC (Paton & Pertwee, 1973). Such effects involved heightened anxiety, panic, perception, and memory. These essential discoveries prompted further inquiry into what is now known as the endocannabinoid system (ECS). Importantly, Dr. Mechoulam has been a prominent figure in cannabinoid research, having been responsible for identifying chemical structures and receptors essential in the endocannabinoid system.

1.2 The endocannabinoid system: its discovery and background

While the effects of cannabinoids were being studied, it was unclear as to how the cannabinoids worked within the body. It was postulated that these plant derivatives worked through nonspecific targeting, by disrupting the cell membrane (Pertwee, 2006). However, the next key discoveries clarified the existence of specific cannabinoid receptors. In the 1980s, Dr. Allyn Howlett and colleagues localized cannabinoid receptor 1 (CNR1, protein abbreviation) to the rat brain (Devane et al., 1988). In particular, synthetic radiolabelled ligands CP 55,940 (bearing a high affinity to G-protein receptors) were utilized to label the receptor sites. A different cannabinoid receptor was later identified as cannabinoid receptor 2 (CNR2, protein abbreviation); however, it was found in the rat spleen, not the brain (Munro et al., 1993).

At this point, the chemical structures of THC and CBD had been characterized, as well as the cannabinoid receptors. The discovery of the receptors was useful, as it allowed researchers to better understand the mode of action for these plant derivatives. However, researchers were still unaware as to whether these receptors were specific to the active compounds of marijuana, or if there are ligands naturally produced in the body. A key question lingered—is there an endogenous ligand? Such questions were addressed by Mechoulam and Bill Devane (Devane et al., 1992). More specifically, Devane and colleagues were able to demonstrate that the endogenous ligand now identified as anandamide (AEA; isolated from the pork brain) was able to displace radio-labelled synthetic cannabinoid from the rat brain membrane (i.e. receptor) in a competitive manner (Devane et al., 1992). The next endogenous cannabinoid to be discovered was 2-arachidonoyl glycerol (2-AG). Raphael Mechoulam isolated 2-AG from the canine spleen and its structure was determined via mass spectrometry (Mechoulam et al. 1995). AEA and 2-AG are the most studied endocannabinoids. For this reason, only they will be discussed.

Catabolic and anabolic enzymes are essential for synthesizing and degrading these ligands, and it is widely believed that both AEA and 2-AG are produced on demand, rather than stored in reserves (Basavarajappa and Hungund 1999; Hungund and Basavarajappa 2000). The pathways are complex, as there are multiple enzymes implicated in eCB synthesis and degradation. To start, it was determined that AEA was formed by endocannabinoid precursor and hormone N-acyl-phosphatidylethanolamine by N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD; protein abbreviation; enzyme that prepares the release of N-acyl-ethanolamine from N-acyl-phosphatidylethanolamine) (Okamoto et al., 2004). This discovery was determined via studying AEA formation in rat brain cultures and the dog brain (Di Marzo et al., 1994; Schmid et al., 1983). Alternatively, studying rat liver plasma membranes led to the discovery that AEA

degradation to arachidonic acid is catalyzed by an enzyme identified as fatty acid amide hydrolase (FAAH; protein abbreviation) (Cravatt et al., 1996; Deutsch & Chin, 1993). Next, it was determined that 2-AG is synthesized from diacylglycerol (DAG) by DAG-lipase alpha or beta (Bisogno et al., 2003). 2-AG is subsequently degraded by monoacylglycerol lipase (MGL; protein abbreviation) into glycerol and arachidonic acid (Bisogno et al., 2003). Further, Dinh and colleagues determined that increasing MGL expression by inserting MGL cDNA into rat neurons significantly reduced 2-AG production, suggesting the lipase's role in degrading the 2-AG (Dinh et al., 2002). Overall, different enzymes from alternate pathways aid in AEA and 2-AG synthesis/degradation, but the mentioned pathways remain among the most-studied (Fig 1.1.).

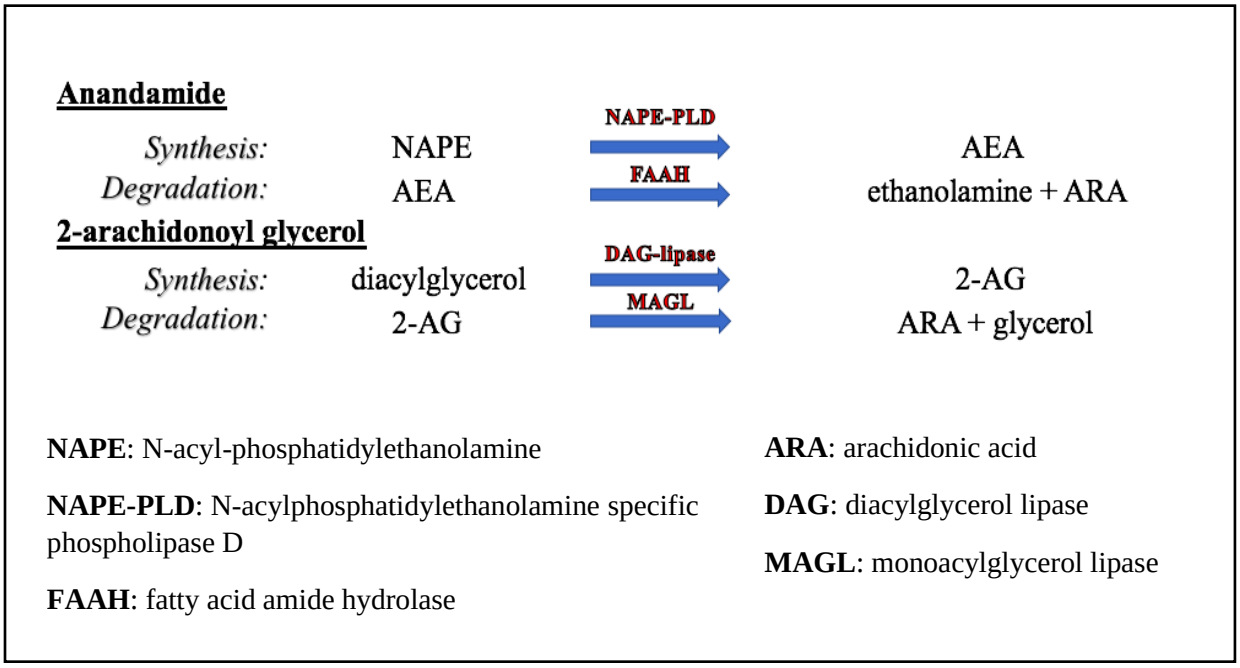

Figure 1.1. Simplified diagram of common anandamide and 2-arachidonoyl glycerol anabolic and catabolic pathway. N-acyl phosphatidylethanolamine specific phospholipase D (NAPE-PLD) catalyzes the formation of anandamide (AEA) from hormone precursor N-acyl phosphatidylethanolamine (NAPE). The enzyme fatty-acid amide hydrolase (FAAH) catabolizes AEA into arachidonic acid and ethanolamine. Moreover, the enzyme diacylglycerol lipase (DAG-lipase) catalyzes the synthesis of 2-AG from DAG. 2-AG is further degraded by the enzyme monoacylglycerol lipase (MAGL) into arachidonic acid and glycerol.

Moreover, endocannabinoid levels such as AEA and 2-AG varies between the organs and this phenomenon is referred to as the "Endocannabinoid Tone." (Silver, 2019). There are two categories of eCB signalling: *basal* signalling which influences the endocannabinoid tone and *phasic* signalling which refers to the change in eCB levels. Furthermore, it is mostly argued that the eCBs are retrograde synaptic messengers in the CNS (Castillo et al., 2012). This mechanism involves the eCB being carried from the post-synaptic neurons. The eCBs will then bind to the CNR1 located on the presynaptic neuron. Finally, the eCB will be transported back to the post-synaptic membrane, ultimately being degraded by enzymes FAAH and MAGL (Castillo et al., 2012; Silver, 2019).

Putting this information together, our understanding of the endocannabinoid system (ECS) is comprised of the following: (1) the endogenous cannabinoids (eCBs), (2) the cannabinoid receptors 1 and 2 that the eCBs bind to, and the (3) catabolic and anabolic enzymes for eCBs. To date, the ECS has been discovered in a variety of organ systems, namely the reproductive system. As a result, the focus of my master's has been on the role of the ECS on vertebrate reproductive health.

1.3 The major cannabinoid receptors

The major cannabinoid receptors, cannabinoid receptor 1 and cannabinoid receptor 2 (CNR1 and CNR2) are G-protein coupled receptors (GPCR) which facilitate the effects of endocannabinoids, synthetic cannabinoids, and phytocannabinoids. GPCR receptors are part of the most diverse receptors in the eukaryote species (Katritch et al., 2012). GPCR receptors (CNR1 and CNR2 included) have cell membrane proteins that pass through the membrane seven times and have two main signal transduction pathways. These pathways include the cAMP signal pathway and the phosphatidylinositol signal pathway (Di Marzo et al., 2004). Once a ligand binds to the

cannabinoid receptor, the receptor will undergo a conformational change to G_i (inhibitory protein) which then inhibits adenylyl cyclase, further preventing formation of cAMP. Overall, the conformational change of the G protein will send a signal to other proteins which ultimately lead to cellular responses, summarized in Figure 1.2. (Di Marzo et al., 2004). Moreover, the protein sequence for CNR1 and CNR2 share 44% similarity (Zou & Kumar, 2018). They are structurally similar, however the receptor distribution varies on the organ (Silver, 2019). For example, CNR1 is highly expressed in the central nervous system (CNS), whereas CNR2 is localized to immune system organs such as the spleen and kidney. Moreover, these cannabinoids can bind reversibly to either receptor and the receptors are also stereoselective (Devane et al., 1988).

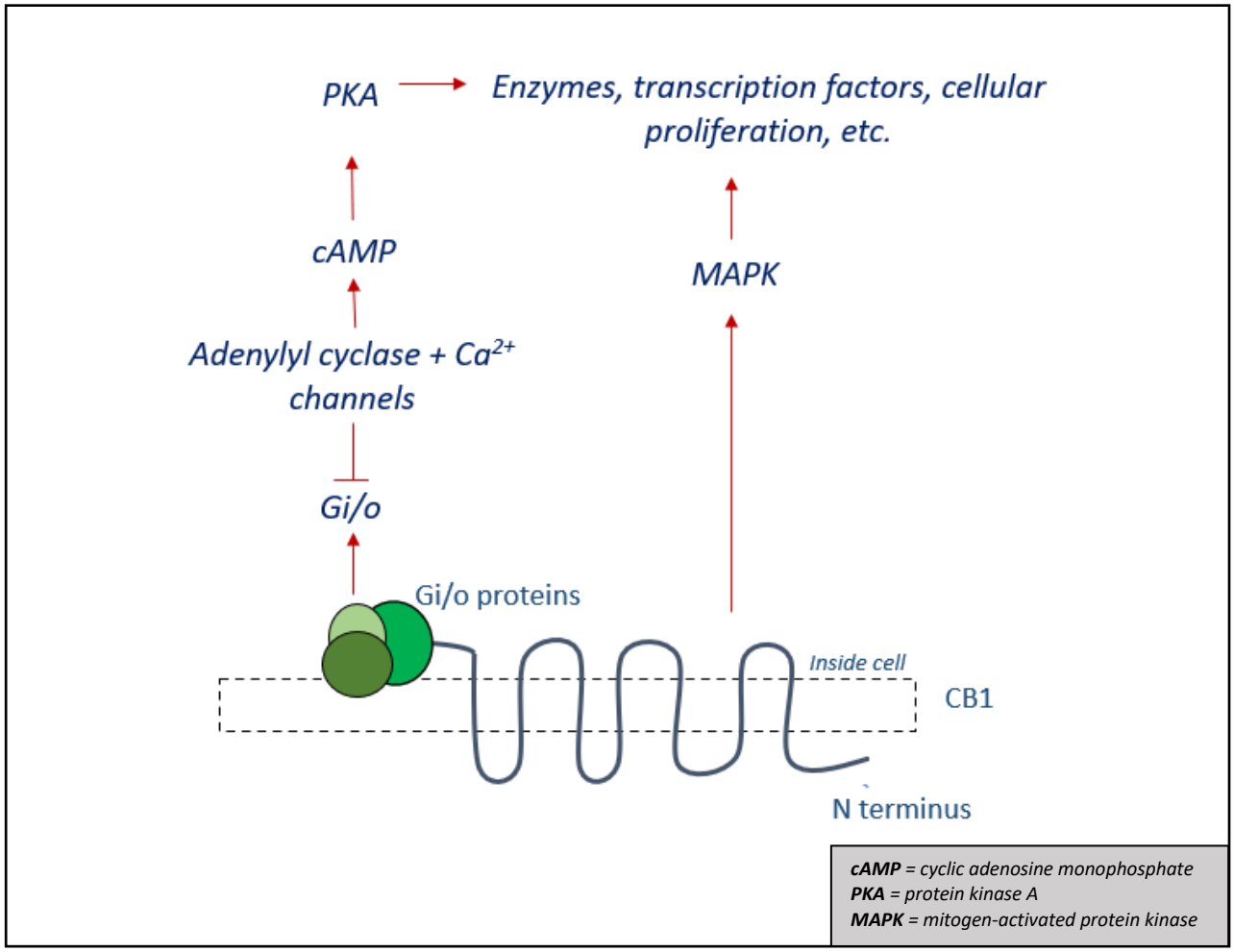

Figure 1.2. Major signalling pathways involved with cannabinoid receptor. Once a ligand binds to the cannabinoid receptor, the receptor will undergo a conformational change of the heterotrimeric $G_{i/o}$ protein which then inhibits adenylyl cyclase, further preventing formation of cAMP. Overall, the conformational change of the G protein will send a signal to other proteins which ultimately lead to cellular responses. Modified from Di Marzo, Bifulco, and De Petrocellis 2004.

1.4 CNR1 in the vertebrate organism

The endocannabinoid system has been detected throughout multiple organ systems, but first discovered in the central nervous system (CNS). To be specific, autoradiographic studies localized CNR1 in tissue sections of the rat pituitary (Jansen et al. 1992). Furthermore, there was also higher expression of the receptor in the basal ganglia, olfactory bulb, and hippocampal regions. There was some receptor binding in the amygdala and corpus collosum, less localization of the receptor in the forebrain, and markedly less in the brain stem and spinal cord (Jansen et al. 1992). More autoradiographic studies determined the receptor binding in the neocortex, and similarly extremely low levels of receptors in the spinal cord (Glass et al., 1997). Different analyses involving immunocytochemistry and fluorescence microscopy further proved the presence of CNR1 in the rat and mouse amygdala (Katona et al., 2001; McDonald & Mascagni, 2001). Receptors have also been characterized in the peripheral nervous system, specifically the parasympathetic nervous system (PNS). For example, there were several studies supporting the fact that the ECS modulated nociception (Bénard et al., 2012). Nociceptive processing refers to the nervous system's ability to respond to painful stimuli. Interestingly, one study determined that CBD exposure could modulate pain sensitivity. It was determined that exposure to synthetic CBD agonist WIN 55,212-2 reduced the activity of nociceptive neurons in the thalamus. Furthermore, THC and its synthetic analog similarly caused antinociception (suppression of nociception) in another brain region, the periaque ductal gray region (Lichtman et al., 1996). As well, it was determined that a synthetic compound acting as a FAAH prevented AEA degradation, thereby increasing AEA levels outside of the CNS (Clapper et al., 2012). This novel effect implicated the use of FAAH inhibitors to modulate pain. Other associated functions of CNR1 in the CNS include

but are not limited to anxiety, depression, modulating appetite, reward and addiction, sleep, memory, and cognition (Zou & Kumar, 2018).

Furthermore, other studies localized CNR1 in peripheral tissues. Immunohistochemistry and *in situ* hybridization demonstrated the distribution of CNR1 in the terminal ganglion, which is a parasympathetic ganglion (Price et al., 2003). Further proof of the ECS in the PNS is the detection of the major receptor in the gastrointestinal (GI) tract, specifically the enteric nervous system (Duncan et al., 2005). Moreover, THC exposure inhibited contractions in the urinary bladder, providing more evidence that the CNR1 acted on peripheral nerves (Pertwee & Fernando, 1996). Although this is a summary of the ECS involvement in the vertebrate organism, eCB signalling has been detected but not limited to in the cardiovascular system, GI tract, liver, immune system, muscles, bones, and skin (Mechoulam 2014). The ECS has also been implicated in the reproductive system, which is of particular concern to my thesis.

The function of the ECS in these multiple organ systems has also been widely studied. For instance, there are several hypotheses regarding the physiological role of the ECS in the central nervous system (CNS). To start, the endocannabinoid system has been implicated in brain development, as there was localization of the CNR1 in the brain at different neurodevelopmental stages (Begbie et al., 2004). By immunostaining cellular proliferation, there was evidence that the neural progenitor cells express CNR1 and produce FAAH (Aguado et al., 2005). The ECS has also been detected in areas of the brain such as the hippocampi that are associated with learning and memory (Skaper & Di Marzo, 2012). For example, it was found that rats exposed to THC exhibited memory impairment (Molina-Holgado et al., 1995). Further, it has also been suggested that fear responses can be influenced by endocannabinoid signalling. For example, utilizing CNR1 antagonist AM251 induced a fear response in rats (Kamprath et al., 2011). These are just a few

examples of the studies that have been conducted to clarify the mechanisms of the ECS in the central nervous system. In summary, the mentioned studies exemplify the ubiquitous nature of the ECS. It is not only implicated in the nervous system, but other peripheral tissues. As a result, the ECS plays multiple essential physiological roles in the organism.

1.5 The endocannabinoid system and reproduction in mammals

It is crucial to note that the reproductive system is also regulated by neuroendocrine factors (Bovolin et al., 2014). For instance, the gonadotropin releasing hormone (GnRH) is an important hormone in reproductive function (Bovolin et al., 2014). The GnRH neurons release the GnRH, which acts on the anterior pituitary to regulate the secretion of gonadotropins, the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). FSH and LH act on the gonadal organs (e.g., the ovaries) to regulate hormones such as estrogen and progesterone to aid in oocyte and follicular development (Meccariello et al., 2014; Sánchez & Smitz, 2012; Walker et al., 2019). Together, this system is commonly denoted as the hypothalamic-pituitary-ovarian (HPO) axis.

Moreover, the ECS has been suggested to affect the endocrine system, particularly by operating on the HPO axis. Evidence of its role in the reproductive system was the discovery of CNR1 and CNR2 in the human ovaries (El-talatini et al., 2009). In wild-type (WT) mice, expression of *cnr1* and *cnr2* messenger RNA (mRNA) was found to be differentially regulated during the preimplantation stage in mice (Paria et al., 1995). The implantation periods refer to the developmental stages the embryo undergoes before and after being implanted into the uterus. Moreover, enzymes FAAH and NAPE-PLD were also found in different follicular stages, suggesting they may play a role in folliculogenesis, the process by which an ovarian follicle matures (El-talatini et al., 2009). CNR1 and CNR2 were also localized to the mouse hypothalamus, which is a brain region containing GnRH neurons (Gammon et al., 2005). Furthermore, several

studies have demonstrated that ECS disruption negatively affects the HPO axis. For example, marijuana exposure to mammals inhibits GnRH, which later prevents estrogen and progesterone production (Gammon et al., 2005). In turn, this causes women to menstruate without ovulation, and in some cases, the follicular stages take longer to develop (Brents, 2016; Jukic et al., 2007). Together, these studies suggested that the ECS receptors may play a role in fertility.

The endocannabinoid AEA also been suggested to influence pregnancy outcome. Maccarronne and others examined FAAH levels in the lymphocyte homogenate of women during their pregnancy, in which women who suffered a miscarriage had lower FAAH enzyme levels (Mauro Maccarrone et al., 2000). It was also determined that NAPE-PLD levels in the mouse uterus influenced AEA production, and AEA levels resultantly fluctuated throughout the pregnancy stages (Guo et al., 2005). For example, when the blastocyst, the stage at which fertilization reaches five or six days, was implanted into the uterus, NAPE-PLD levels were lower, leading to reduced AEA levels (Guo et al., 2005). Interestingly, AEA levels and its related enzymes (i.e., NAPE-PLD and FAAH) could be utilized as a biomarker to predict fertility outcome. Women who experienced miscarriages had three-times higher levels of AEA in plasma versus women who delivered (Habayeb et al., 2021). Overall, these studies seem to suggest some involvement of the eCB-related enzymes in fertility processes.

Genetic knockout (KO) studies have also been useful in ascertaining the functional role of the ECS in mammalian reproduction (Das et al., 1995). When the ECS was disrupted, it interfered with the embryo development during the preimplantation and implantation period (Das et al., 1995). More importantly, it has been suggested that the dysregulated ECS has a negative impact on reproductive health. For instance, *cnr1*$^{-/-}$ and *cnr2*$^{-/-}$ mice had delayed blastocyst growth. Less *cnr1*$^{-/-}$/*cnr2*$^{-/-}$ KO mice had embryos reach the blastocyst at day 4, whereas nearly all wildtype mice

had embryos reach the blastocyst stage at this timepoint (Paria et al., 2002). Ectopic pregnancy was also observed in *cnr1*$^{-/-}$ mice (Wang et al., 2004). Together, these studies were suggestive that the ECS receptors may play an important role in reproductive success. Regardless, the physiological role of various ECS-regulatory genes, and the mechanisms underlying the role of the ECS in reproductive function, are not completely clear.

1.6 Involvement of the endocannabinoid system between sexes

Sexual dimorphism refers to the differences between the male and female sex. Such characteristics can be morphological, behavioural, genetic, etc. Interestingly, males and females exhibit differences in the endocannabinoid system. For instance, males have higher CNR1 levels than females in brain regions such as the hippocampus, hypothalamus, and cortex (Reich et al., 2009). Despite this, it is suggested that females are more sensitive to exogenous cannabinoids. For instance, CNR1 function was examined in adult rats after being exposed to THC (Rubino et al., 2008). Specifically, male rats had less changes in receptor binding and lower G-protein activation compared to females. Autoradiographic studies also determined that females had higher CNR1 G-protein activation, despite having less CNR1 density (Mateos et al., 2011). These studies suggest that females have a potentially more sensitive ECS compared to males.

Other differences between the sexes in the ECS involve cell proliferation (Krebs-Kraft et al., 2010). Female rats have higher rates of cell proliferation in the amygdala compared to males. Interestingly, treating young female rats with cannabinoid agonist WIN 55,212-2 reduced cell proliferation in the amygdala, reaching levels comparable to males. Females also had higher FAAH and MAG-L levels than males, and resultantly higher 2-AG and AEA levels. According to the same study, early exposure of the cannabinoid agonist also resulted in young females to exhibit

masculine play behaviour (Krebs-Kraft et al., 2010). Together, these studies indicate the dimorphic characteristics of the ECS.

While the mechanisms underlying the dimorphic characteristics of the ECS are not completely clear, there is some speculation that the hormonal system is at play. Specifically, the presence of estrogen in either the male or female may have a modulating effect. For instance, motor behaviour in rats exposed to THC was examined (Craft & Leitl, 2008). It was determined that gonadally intact and sexually mature females experiencing their estrous cycle had stronger behavioral effects to THC exposure. Furthermore, it was found that estradiol and CNR1/CNR2 agonists can reduce the cannabinoid effects on appetite and body temperature (Kellert et al., 2009). Interestingly, estrogen and THC also has effects on learning and memory (Daniel et al., 2002). Additional estrogen treatment in ovariectomized rats also blocked THC's ability to reduce response accuracy (i.e., learning and memory). Further, the fluctuating levels of estrogen during the female's development may influences eCBs and mRNA expression. It was found that 2-AG and AEA levels fluctuated the most at the time of ovulation (Bradshaw et al., 2006). Furthermore, *cnr1* mRNA transcripts also fluctuated during the estrous cycle in female rats. Female rats also had higher AEA levels than males (González et al., 2000). Overall, there is a need for clarifying the sex differences of the ECS in vertebrates. Such information would shed light on the mechanisms of endocannabinoid regulation in multiple organ systems.

1.7 Zebrafish reproduction

There are many factors that influence successful breeding and spawning in zebrafish. For instance, zebrafish are early morning and group breeders, typically breeding within the first few hours of daylight. Interestingly, one female can release 200-300 eggs in a single spawning event (Darrow & Harris, 2004; Hoo et al., 2016). Additionally, several courtship behaviours in males

and females are influential in effective reproduction. Males must chase, touch the female with their tail or nose, encircle, zig-zag, and rapidly quiver their tails against the female (Hoo et al., 2016; Spence et al., 2008). On the other hand, females must approach, escort, hide or present themselves, lead males, and egg-lay (Hoo et al., 2016; Spence et al., 2008). Furthermore, environmental factors such as photo-period, water temperature and pH, tank volume, and density of fish can impact the breeding performance of fish (Hoo et al., 2016; Lawrence, 2007). There is typically a photoperiod of 14 hours of light, and 10 hours of dark (Matthews et al., 2002). The water temperature should range between 24°C-30°C and a pH of 7-8 (Hoo et al., 2016). Physical features of either the male or female also considerably affect the reproductive capabilities of zebrafish. Larger females have more offspring and therefore higher fecundity than smaller females, and females also prefer to mate with males with a larger body size in comparison to smaller males (Hoo et al., 2016; Uusi-Heikkilä et al., 2012). Finally, for effective breeding, a ratio of 2 males to 1 female is recommended (Spence & Smith, 2005). Overall, these qualities determine the outcome of successful zebrafish breeding.

1.8 Why use zebrafish in endocannabinoid research?

Zebrafish are a great animal model in scientific research for a multitude of reasons. To start, zebrafish are cost-effective compared to rodent models such as mice and rats (Segner, 2009). Moreover, zebrafish have a short developmental time. Major organs develop after a full day (24 hours) and they also reach sexual maturity within 3-6 months. Females can also produce more offspring per week (200-300) compared to rodents who produce less offspring in that timespan (Hoo et al., 2016). These qualities make facilitating experiments easier, compared to mammals which have longer developmental times and process less offspring. Zebrafish also have similarities in anatomy and physiology to humans. Both humans and zebrafish contain bilateral ovaries (Li

and Ge 2020). The ovaries are the organs where female gametes or oocytes develop. Moreover, the full zebrafish genome was sequenced, and it was found that approximately 70% of human genes were discovered in zebrafish (Howe et al., 2013). Embryos are also transparent, making it possible to view their organs during the early stages of development. For instance, the circulatory system is visible, as it is possible to image blood vessels. Other organs such as the heart, liver, stomach, fins, and skin are visible as early as 2 days post fertilization (Teame et al., 2019). These characteristics make utilizing the zebrafish an ideal model in biological research.

To date, mammals such as rodents are the most popular scientific model for endocannabinoid research. More recently, zebrafish are being used and prove to be useful in both endocrinological and endocannabinoid research. The CNR1 protein is orthologous between mammals and zebrafish, sharing a 70% sequence identity to both humans and mammals (Oltrabella et al., 2017). CNR2 proteins are also homologous between zebrafish and humans, sharing up to 46% protein identity (Demin et al., 2018). CNR1 has also been identified via *in situ* hybridization and real-time PCR in the ovaries, particularly in stage III and IV oocytes (Migliarini & Carnevali, 2008). Western blot analysis determined higher levels of the CNR1 protein in stage III compared to IV, which had low levels. CNR1 protein was also detected in the embryonic stages, at the 3-somite stage and at 15 days post-fertilization (Migliarini & Carnevali, 2009a). The receptor has also been detected in the brain. For example, via *in situ* hybridization, *cnr1* mRNA expression was detected in both the larval and adult zebrafish brain (Lam et al., 2006). Even further, whole-mount *in situ* hybridization determined mRNA expression of genes related to the metabolism of 2-AG (*dagla, daglb*) in zebrafish at 2 and 4 days post fertilization (Watson et al., 2008). CNR1, which is the main receptor for 2-AG, was also expressed in the brain (Lam et al., 2006; Nishio et al., 2012; Watson et al., 2008). Cannabinoid receptor 2 (CNR2) has been less studied in

comparison to CNR1, but still detected in the brain, intestine, gills, cardiac system, and spleen (Rodriguez-Martin et al., 2007). A detailed description of the genes of interest is summarized in Table 1.1. Other eCB-related mRNA transcripts which have been examined included cannabinoid receptor interacting protein 1 (*cnrip1a*), monoacyl glycerol lipase (*mgll*), alpha/beta-hydrolase domain containing 6 (*abhd6a*), alpha/beta-hydrolase domain containing 12 (*abhd12*), diacylglycerol lipase alpha (*dagla*), diacylglycerol lipase beta (*daglb*), fatty acid amide hydrolase (*faah*), N-acyl phosphatidylethanolamine phospholipase D (*nape-pld*), glycerophosphodiester phosphodiesterase 1 (*gde1*) (Oltrabella et al., 2017). Other relevant eCB-related genes can be found in Supplementary Table S.1. In our study, we will compare the mRNA expression profile of *cnr1* and *cnr2* in various zebrafish organs. To date, the mRNA expression profile via quantitative PCR (qPCR) of the receptors in zebrafish has only been done once (Oltrabella et al., 2017). Furthermore, we aim to confirm the expression of *cnr1* in the gonads prior to the assessment of breeding competency test.

As mentioned, the zebrafish is a facile model organism, and it has been used to study various functions of the ECS. For instance, the role of the CNR1 and CNR2 in zebrafish locomotor and neuronal development has been studied, in addition to examining CNR1 expression during embryogenesis (Migliarini & Carnevali, 2009a; Sufian et al., 2019; Watson et al., 2008). CRISPR-cas9 KO lines of *cnr1* and *cnr2* have also been generated to examine zebrafish behaviour (Acevedo-Canabal et al., 2019; Luchtenburg et al., 2019). Other research studies have involved examining the role of endocrine disruptors such as di-isononyl phthalate and bisphenol A on ECS homeostasis (Forner-Piquer et al., 2017, 2020). Overall, these studies suggest the wide applications of utilizing zebrafish to better understand the ECS.

Table 1.1. Cannabinoid receptor genes to be studied, their protein name, function, and localization in adult zebrafish. A list of other relevant ECS genes and their function are listed in Supplementary Table S.1.

Gene	Protein	Function	Localization	References
cnr1	Cannabinoid receptor 1	Receptor activated by endogenous and exogenous cannabinoid ligands; mainly localized to the CNS, also found on other organs such as uterus and ovaries	Brain, eyes, ovaries, class III and IV oocytes, testes, skin, muscles, kidney	Yazulla and Studholme, 2001; Migliarini and Carnevali, 2009; Piccinetti et al., 2010; Oltrabella et al., 2017
cnr2	Cannabinoid receptor 2	Receptor activated by endogenous and exogenous ligands; mainly localized to the immune system	Brain, eyes, heart, intestine, gills, muscle, spleen, kidney, testes, ovaries, liver, heart, skin, muscles	Rodriguez-Martin et al., 2010; Oltrabella et al., 2017

1.8 CRISPR-Cas9 gene editing

Genome editing is a powerful method that allows scientists to modify an organism's DNA, and it also has useful applications. In the context of biological research, this is especially useful as it provides an *in vivo* method to determine the functional role of a particular gene of interest. Particularly, CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) has become a popular tool in genome modification. CRISPR-Cas9 gene editing manipulates the immune defense systems of bacteria and archaea organisms. Interestingly, the distinctive repetitive spacer sequence (CRISPR sequence) in bacteria such as *E. coli* originates from foreign viruses and plasmids (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Furthermore, genes encoded in DNA repair are found adjacent to the CRISPR sequences and are named "Cas" genes. Upon infection from invading viruses, the bacteria can obtain pieces of the viral DNA and insert them into their own genome to create repetitive sequences named CRISPR arrays. The CRISPR sequences allow the bacteria to create an immune response upon further exposure to the virus. Now, the bacteria can transcribe CRISPR RNA (crRNA) from the CRISPR sequence to cleave the invading virus/plasmid via the Cas enzyme (Barrangou et al. 2007; Brouns et al. 2008; Garneau et al. 2010; Jansen et al. 2002; Makarova et al. 2002; Marraffini and Sontheimer 2008). As such, it was concluded that CRISPR and Cas9 were used as an immune defense mechanism for viruses and archaea.

It was later suggested that the principles of this immune system could be modified to manipulate the genome of eukaryotic organisms, such as mammals. This revolutionary methodology was developed by Charpentier and Doudna's team (Jinek et al., 2012). Here, they identified a dual RNA structure that directs the Cas9 protein to induce double-stranded breaks in the DNA. Specifically, this dual RNA structure was composed of the crRNA and a trans-activating

crRNA (tracrRNA). The crRNA, one of the two types of guide RNA, is complementary to the target DNA. The DNA target for the CRISPR-Cas9 system depends on the crRNA sequence. tracrRNA, the second component of the CRISPR RNA guides the Cas9 protein towards the target DNA (Jinek et al., 2012). Additionally, protospacer-adjacent motifs (PAM) are short DNA sequences (5'-NGG-3'). N is any nucleotide, followed by two Guanine nucleotides. Furthermore, the PAM sequence is downstream of the CRISPR-binding region. Interestingly, it was found that Cas nuclease will not cut the target site unless the PAM sequence is present downstream (Jinek et al., 2012). As a result, the PAM sequence is essential for DNA cleavage. The findings from this paper suggested that the tracrRNA:crRNA dual structure can be modified as a single RNA molecule to delete DNA target sites. The discovery led by Charpentier and Doudna's team paved the way for the next generation of genome modification—CRISPR-Cas9 gene editing. It manipulates the viral immune defense system and allows researchers to target DNA sites with more simplicity than transcription activator-like effector nucleases (TALENs) or zinc finger nucleases (ZFNs), for instance (Doudna & Charpentier, 2014). To date, the CRISPR-Cas9 system has had significant implications for genomic engineering and has become a conventional tool for biological research. Following the DNA breaks, two repair mechanisms can occur (Zimmer et al. 2019). Homology-directed repair (HDR) requires homologous regions to rejoin DNA that has been broken. On the other hand, non-homologous end joining (NHEJ) does not require homologous strands to repair the DNA. NHEJ can introduce indels (insertions or deletions), or frameshift mutations which can induce a premature stop codon in the open reading frame of the gene (Ran et al., 2013). In zebrafish, the injection of the CRISPR-Cas9 ribonucleoprotein mix will generate mosaic F_0 fish with a loss of function mutation (Cornet et al., 2018). The F_0 will be reared until sexual maturity, then bred again to generate F_1 fish through outcrossing. Lastly, the F_1 fish will be

in-crossed to generate F_2 homozygous fish. The resultant goal would be generating a loss of function mutation. Ultimately, the strength of this KO line can be determined experimentally. It will be beneficial to generate a "mutant" line of the major cannabinoid receptor (CNR1) to comprehend the role of the ECS in fertility outcome. The term mutant refers to an organism with a change in their DNA sequence because of a mutation. Figure 1.3. summarizes the mechanism underlying CRISPR-Cas9 targeted gene-deletion.

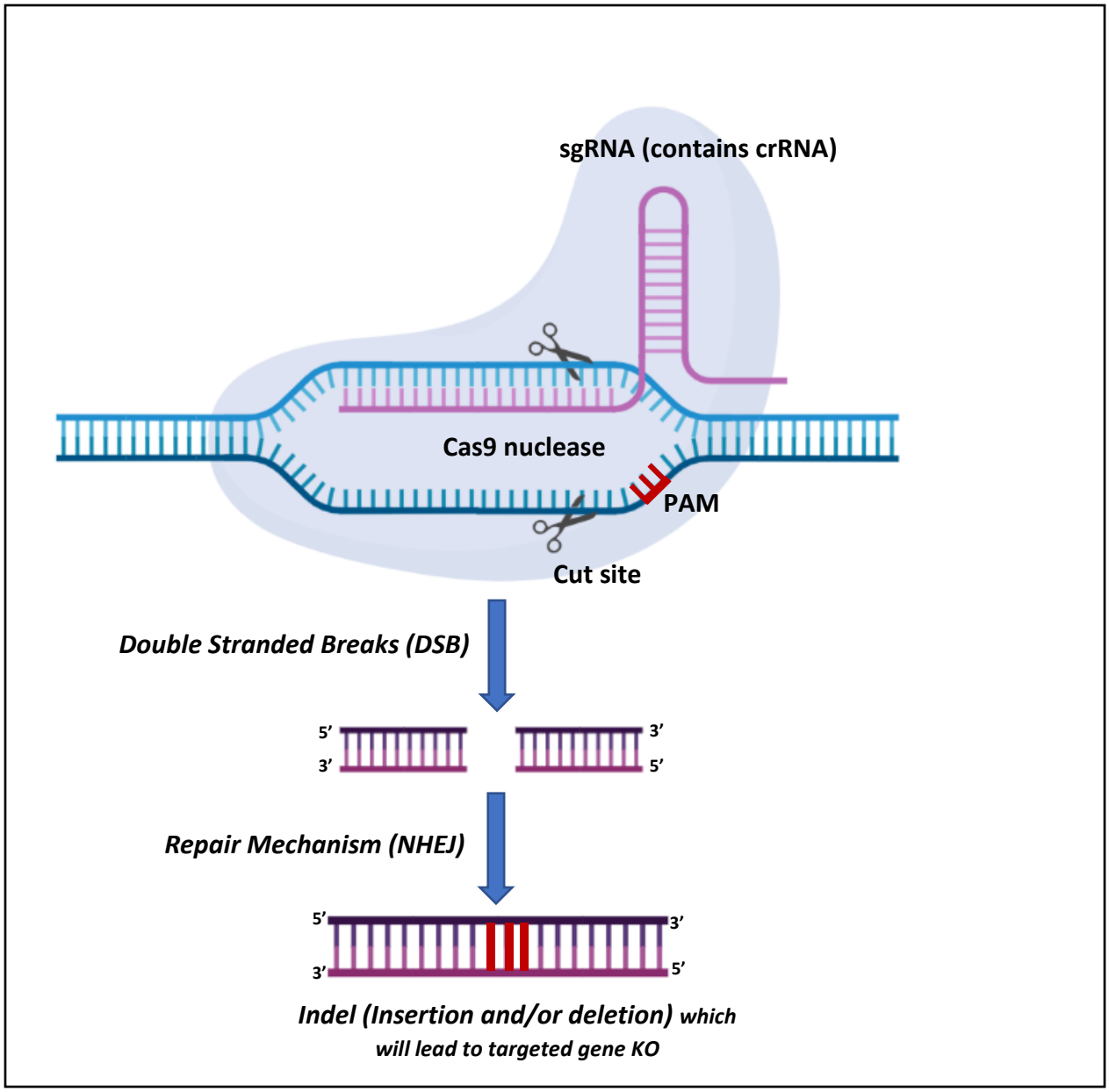

Figure 1.3. Mechanism of CRISPR-Cas9 targeted mutagenesis. The sgRNA guides the Cas9 enzyme and identifies the target sequence (i.e., gene of interest). Specifically, the crRNA is complementary to the target DNA. The Cas9 enzyme then makes double-stranded breaks (DSB) upstream of the protospacer adjacent motif (PAM). Afterwards, double-stranded repair mechanisms will occur. Ideally, in the event of targeted gene KO, non-homologous end-joining (NHEJ), the most predominant repair mechanism, will occur. NHEJ will repair DSBs through joining the DNA fragments. This error-prone mechanism can result in insertions/deletions (indels). Indels can lead to a frameshift mutation, resulting in a targeted gene KO. This figure in part was generated in part via Bio-Render.

1.9 Research objectives and hypotheses

The endocannabinoid system is an important biological pathway implicated in physiological processes including reproductive health. Interestingly, zebrafish have only recently emerged as a model for ECS research. For example, studies determining the expression profiles of the cannabinoid receptors and eCB signalling genes have been conducted in larval and adult zebrafish (Liu et al. 2016; Migliarini and Carnevali 2008; Oltrabella et al. 2017). Moreover, studies involving toxicological effects of exogenous cannabinoids and behavioural studies have also been examined (Acevedo-Canabal et al. 2018, 2019; Colon-Cruz et al. 2018; Forner-Piquer et al. 2017, 2018, 2020; Luchtenburg, Schaaf, and Richardson 2019; Martella et al. 2016; Sufian et al. 2019). However, there is no research clarifying the functional significance of the ECS in reproductive health in zebrafish. While both cannabinoid receptors have been identified in the zebrafish ovaries, there is little understanding of how CNR1 affects fertility outcome in female zebrafish (Migliarini & Carnevali, 2008). For this reason, my thesis will focus on generating a *cnr1*$^{-/-}$ KO line and assessing the breeding competency in *cnr1*$^{+/-}$ heterozygous females. Generating an mRNA expression profile will also aid in characterizing the ECS in the zebrafish species and confirm the expression of the receptors in the gonads. **It is hypothesized that there will be a decrease in fertility and fecundity in *cnr1* mutants. I also hypothesize there will be a difference between *cnr1* and *cnr2* expression, and the expression will differ between the organs.**

Aim 1: To develop a cnr1 knockout line via CRISPR-Cas9

We generated a KO line of *cnr1* using the novel genomic engineering method CRISPR-Cas9. The KO line will first be validated at the genomic level via Sanger sequencing, and later a protein assay such as Western blot will be conducted. After confirmation of the mutant group, the mutant line will be examined to assess the role of CNR1 in breeding competency.

Aim 2: Determine physiological role of cannabinoid receptor 1 in breeding competency

The generation of a *cnr1* mutant line makes it possible to study the *in vivo* role the major cannabinoid receptor in zebrafish. At this point, there is little understanding of CNR1's functional role in fertility. A *cnr1* mutant line was generated via the CRISPR-Cas9 method, and heterozygous *cnr1*$^{+/-}$ female fish breeding competency will be determined. To be specific, fertility, fecundity, survival rate, hatching rate, and phenotypic characteristics such as gonadosomatic index and condition factor will be assessed between the heterozygous mutant and non-mutant groups.

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1.11 Supplementary Tables

Supplementary Table S.1 ECS-related genes, their protein name, function, and localization in adult zebrafish.

Gene	Protein	Function	Localization	References
napepld	N-acyl phosphatidyl ethanolamine p hospholipase D	Catalyzes formation of N-acylethanolamine from N-acylphosphatidylethanolamine for AEA synthesis	Brain, skin, muscles, kidney, heart, intestines, liver, eyes, ovaries, testes	Oltrabella et al., 2017
faah	Fatty acid amide hydrolase	Degrades fatty acid amides, namely AEA	Brain, skin, eyes, muscles, kidney, testes, heart, liver	Yazulla and Studholme, 2001; Oltrabella et al., 2017
dagla	Diacylglycerol lipase alpha	Hydrolyzes DAG, releasing fatty acid and monoacylglycerol	Brain, skin, muscles, kidney, heart, intestine, spleen, eyes, ovaries, testes	Watson et al., 2008; Oltrabella et al., 2017
daglb	Diacylglycerol lipase beta	Hydrolyzes DAG, releasing fatty acid monoacylglycerol	Brain, muscles, kidney, heart, intestine, liver, spleen, eyes, ovaries, testes	Oltrabella et a., 2017
mgll	Monoacyl glyc erol lipase	Hydrolyzes 2-AG to arachidonic acid and glycerol	Brain, muscles, kidney, heart, intestines, liver, spleen, eyes, ovaries, testes	Thisse et al., 2001; Oltrabella et al., 2017

Chapter II:

Generation of *cnr1* knockout line via CRISPR-Cas9 and assessment of breeding competency and compensatory regulation in *cnr1* knockout zebrafish

2.1. Chapter summary

Mammalian studies suggest the influential role of the endocannabinoid system in reproductive function. Interestingly, the impairment in the ECS leads to compromised function in fertility in females. However, in zebrafish (*Danio rerio*), it is unclear to what extent the ECS affects reproduction, as studies have been very limited. In this study, we generated a line zebrafish *cnr1*$^{-/-}$ KO fish via CRISPR-Cas9 to determine the functional role of the receptor in breeding competency. Analysis of Sanger Sequencing revealed a 5 base pair deletion, resulting in a frameshift mutation and an early stop codon in Exon 2 of the CNR1 protein. It was predicted that the stop codon would result in a truncated protein. As a result, we confirmed the KO of *cnr1* at the genomic level, however we will need to conduct protein analysis to confirm the KO of the protein. We also assessed the mRNA expression level of major cannabinoid receptors *cnr1* and *cnr2* via ddPCR in zebrafish. Specifically, the findings revealed no significant interactive effects between the organ and gene. Furthermore, it was hypothesized that in the KO fish, there will be a decrease in mutant fertility and fecundity. For the first stage of the assessment, *cnr1*$^{+/-}$ female fish were examined. During 15 breeding events, spawning frequency, cumulative egg production, survival rate of offspring, hatching rate, and standard body length of offspring were compared between heterozygous mutant and wild-type siblings. Our analyses revealed no significant difference in breeding competency between the two genotypes. The possibility of compensatory regulation in

the heterozygous mutants was further examined, in which *cnr1* and *cnr2* mRNA expression was compared. There were no interactive effects revealed between genotype and gene. Overall, these results are inconclusive in determining the functional role of CNR1 in fertility and reproductive competency. However, the results from our study are necessary steps towards characterizing the endocannabinoid system in the zebrafish species. My data are the first of its kind in attempting to examine the involvement of the major cannabinoid receptor, CNR1 in zebrafish fertility and fecundity. Moving forward, we will utilize the findings from the heterozygous KO to examine the effect of a homozygous $cnr1^{-/-}$ KO on breeding competency.

2.2 Introduction

CRISPR-Cas9 has enabled researchers to study the function of individual genes in the vertebrate model, which is especially useful for endocannabinoid research. The gene editing technology is more advantageous than pharmacological investigations because off target effects can occur. In particular, CRISPR-Cas9 is advantageous because it enables researchers to determine the function of a targeted gene *in vivo* using a simpler and more efficient method than ZFN or TALEN (Doudna & Charpentier, 2014). Several studies have already examined the effect of pharmacological inhibition on the ECS in the zebrafish model, but there are a lack of genetic knockout studies in the zebrafish model (Khara, Amin, and Ali 2022; Sufian, Amin, and Ali 2021; Sufian et al. 2019; Sufian et al. 2018). Furthermore, most of the studies largely focus on behaviour and neurology. For instance, in 2017, Fin and colleagues examined the role of cannabinoid receptor interacting protein 1 (*cnrip1*), which interacts with the C-terminus of CNR1, and found no effect on morphological or behavioural phenotypes in *cnrip1* mutants (Fin et al., 2017). Later, a *cnr2*-KO line was generated and anxiety-like behaviour was assessed (Acevedo-Canabal et al., 2019). Overall, *cnr2*-mutant larvae exhibited behaviour that resembled anxiety.

The studies mentioned suggest the importance of the cannabinoid receptor in modulating various physiological functions. However, its relevance in zebrafish reproduction, the focus of my thesis, is unknown. Generating a mutant line of *cnr1* via CRISPR-Cas9 would be advantageous because it will provide novel *in vivo* data demonstrating its potential function in zebrafish reproduction. As a result, utilizing CRISPR-Cas9, we will generate a full KO of *cnr1*. I will also demonstrate the validation of this KO genotype via Sanger Sequencing and prediction of the protein sequence.

It has been suggested that the endocannabinoid system plays an important role in fertility processes in vertebrate species. The major cannabinoid receptors CNR1 and CNR2 have been detected in mammalian female reproductive organs, as well as metabolic enzymes for eCB (El-talatini et al., 2009; Paria et al., 1995). Furthermore, in mammals, the endogenous cannabinoid (eCB) AEA and its fluctuating levels have been implicated in fertility outcome, such that higher levels during the implantation stage resulted in miscarriages (Guo et al., 2005; Habayeb et al., 2021; Mauro Maccarrone et al., 2000). In particular, when the ECS is interrupted, it interferes with embryo development (Das et al., 1995). Together, these studies suggest the relevance of the ECS in reproduction. Regardless, the mechanisms by which this system operates is not completely clear.

For some time, mammals such as mice have been the preferred animal models for ECS research. However, zebrafish are recently proving themselves to a facile model organism for a multitude of reasons. For instance, they are inexpensive, can be housed in larger numbers, have a short developmental time, and have high homology to other vertebrate organisms (Hoo et al. 2016; Li and Ge 2020; Segner 2009). In the context of reproductive research, they are especially useful because they can produce a large amount of offspring in a single spawning event, contain bilateral gonads, and reach sexual maturity within a short timeframe (Li and Ge 2020).

More recently, zebrafish have been utilized as a model organism for ECS research. Such research studies involved characterizing the system and examining the effect of toxic contaminants on the ECS (Forner-Piquer et al. 2017, 2020; Migliarini and Carnevali 2009b; Sufian et al. 2019; Watson et al. 2008). Despite the interest in zebrafish and ECS research, there is still little information clarifying the role of the ECS in reproduction in zebrafish. For instance, it is unclear to what extent the cannabinoid receptors influence breeding competency in zebrafish. However, in mammals, KO and knockdown studies have exemplified the functional role of the cannabinoid

receptors in fertility outcome. For instance, *cnr1*$^{-/-}$, *cnr2*$^{-/-}$, and *cnr1*$^{-/-}$/*cnr2*$^{-/-}$ mice suffer impaired pregnancy. Mice missing the CNR1 and/or CNR2 suffer from various complications including but not limited to delayed blastocyst growth, ectopic pregnancy, reduced sex hormone levels, lower ovarian volume, reduced ovarian follicles, and early-term birth (Li et al. 2019, 2020; Paria, Wang, and Dey 2002; Wang et al. 2004; Wang, Xie, and Dey 2008; Wenger et al. 2001). These studies indicate the importance of the ECS in reproductive function in mammals.

Studies examining the effect of pharmacological inhibition of CNR1 and CNR2 have been examined in zebrafish, in addition to select *cnr1* and *cnr2* KO studies via CRISPR-Cas9 (Acevedo-Canabal et al. 2018, 2019; Colon-Cruz et al. 2018; Fin et al. 2017; Khara et al. 2022; Sufian et al. 2021; Sufian et al. 2019; Sufian et al. 2018). These studies suggested that the inhibition or KO of *cnr1* and/or *cnr2* had negative or altered effects in swimming behaviour, anxiety, and sensorimotor function. Unfortunately, these KO studies have been limited in that there has been no KO study clarifying the role of the ECS in breeding capacity in zebrafish. As a result, our project will shed novel findings regarding the role of CNR1 in breeding capacity. Such information will be crucial for establishing a well-defined model of the zebrafish in cannabinoid research. Moreover, since zebrafish shares genetic homology with mammalian species including humans, the data gathered from my project can be extrapolated to better understand the mechanisms of the cannabinoid system in higher vertebrates (Bailone et al., 2020; Howe et al., 2013).

In my study, *cnr1* KO fish were obtained by designing a single guide-RNA (sgRNA) target for CNR1 and subsequently performing microinjections with Cas9 into the one-cell stage of embryos. The mosaic F_0 fish carrying a loss of function mutation were crossed to generate heterozygous F_1 fish, and then finally F_2 fish that were homozygous. It was hypothesized that there will be a decrease in mutant fertility and fecundity in the mutant group. Due to the limited number

of homozygous females present at the time, the first stage of this breeding competency test involved only heterozygous females. Later, *cnr1* and *cnr2* mRNA were quantified in the WT and mutant organs via droplet digital PCR to detect a possible compensatory response from either receptor.

2.3 Materials and Methods

2.3.1. Animals

Zebrafish (*Danio rerio*) adults of the strain Tüpfel long fin (TL) were obtained from the Fish Vivarium at York University. The zebrafish were maintained in a recirculating aquarium (Aquaneering, CA, USA) at 28ºC and pH 7.4. Adult fish were subjected to 14 hours of light and 10 hours of dark. Adult fish were fed three times daily, twice with brine shrimp and again with commercial high-protein pellets (Zeigler, PS, USA).

2.3.2. Generation of *cnr1* mutant line using CRISPR-Cas9 technology

a) Breeding and microinjection

sgRNA and microinjection preparation

Utilizing CRISPR-Cas9, a KO line of *cnr1* was generated. The sgRNA was prepared prior to CRISPR-Cas9 microinjections. The sgRNA was designed using CHOPCHOP (Labun et al., 2019), and targeted Exon 2 with the sequence (5' ACCTCCGGCCTGCAGTACAT 3'). It was upstream of the PAM sequence (5' CGG 3') in the DNA. The sgRNA was prepared via a polymerase chain reaction (PCR) with the guide-constant oligo, gRNA primer, and short-guide oligo (Table 2.1). The PCR program was as follows: initial denaturation at 95°C for 30 s; denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min (for 35 cycles); and a final extension at 68°C for 10 min; followed by a hold at 10°C. After, the PCR products were visualized on a 1.5% agarose gel. The PCR reactions were then pooled and purified using the Monarch® PCR & DNA Cleanup Kit, following the manufacturer's protocol. The products were quantified using Synergy™ LX Multi-Mode Reader from BioTek. Afterwards, the sgRNA was prepared by *in vitro* transcription, using the NEB HiScribe™ T7 High Yield RNA Synthesis Kit. The reaction is listed in Table 2.2, and amplification primers and genotyping

primers in Table 2.3. On the day of injection, the injection mix was prepared using the following recipe: 1μL H_2O, 1μL of 200 ng/μL sgRNA, 1 μL of 20 μM cas9 (NEB), 0.6 μL of 2 M KCl, and 0.4 μL phenol red (from 0.5% stock). The mix was incubated at room temperature for 5 minutes, then kept on ice until loading into the microinjection needle.

b) Embryo injections

Several breeding traps for adult zebrafish were set up the evening before injections. The female:male ratio of each trap was 2:1. Females and males were separated overnight prior to removing the divider for injections. Microinjection was performed within the one-cell stage (20-30 min post fertilization). Roughly 200 embryos were injected during this process. Mutants for the tyrosinase enzyme (gene *tyr*) were also generated as a positive control. The mutation for this gene resulting in pigmentation loss was confirmed to have occurred by 48 hours post fertilization (data not shown).

c) Genomic DNA collection and genotyping methods

The genomic DNA (gDNA) was collected at 1-day post fertilization. The protocol for genomic DNA extraction involved collecting 10 embryos per genotype and placing each one in a PCR reaction tube. 20 μL of 50 mM NaOH was added to each sample. The samples were then heated to 95°C for 10 min, and vortexed. After, the samples were cooled on ice. 2 μL of 1 M Tris-HCL (pH 8) was added to each mixture. Then, the samples were centrifuged. The gDNA was then subjected to PCR to amplify the CRISPR-Cas9 target region. The PCR reaction was as follows: 94°C for 3 min; then 94°C for 30 s, 55°C for 30 s, 68°C for 1 min (repeated 39x); 68°C for 10 min; and then left on hold at 10°C. The PCR samples were denatured and renatured again to another round of PCR. Finally, the PCR products were visualized on a 4-6% agarose gel to

visualize hetero and homo-duplexes of the CRISPR-Cas9 target region (Bhattacharya and Van Meir 2019).

To prepare samples for sequencing, several PCR products of the microinjected samples, and 1 wildtype sample were collected at random. The PCR products were purified using Monarch® PCR & DNA Cleanup Kit. Sequencing samples were prepared in PCR strips, diluted to 10 ng DNA. 7 μM of the reverse primer was added to each purified DNA template. Finally, the samples were sent to the DNA Sequencing and Synthesis Facility at Sick Kids to confirm the presence of a genetic mutation. The genetic mutation for the F_0 microinjected samples was determined by the presence of multiple nucleotide peaks starting at the site of mutation.

The founder (F_0) fish were reared until sexual maturation, then bred with another WT fish to yield the F_1 generation. F_1 fish were genotyped by anaesthetizing them at 0.168 g/L of ethyl 3-aminobenzoate methanesulfonate (tricaine or MS-222). A small section of the tailfins was excised with a scalpel while the fish were anaesthetized. To keep track of fish identity, they were organized into a 24-chamber tank. The section of the tailfin was used to collect gDNA and to perform downstream genotyping methods as previously described (Bhattacharya and Van Meir 2019). The *cnr1*$^{+/-}$ F_1 fish were grouped based on which fish had the same base pair deletions/additions. Then, the *cnr1*$^{+/-}$ F_1 fish were in-crossed to generate *cnr1*$^{-/-}$ F_2 fish. Two online tools, which include The Nucleotide database generated by NCBI and the ExPASy Translate tool were utilized to determine the nucleotide sequence and expected protein sequence of the WT and mutant fish.

Table 2.1. PCR mix for gRNA preparation.

Ingredient	Volume (μL)
Water	34.25
5x Buffer	10
gRNA primer 1 (10μM)	1.25
gRNA primer 2 (10μM)	1.25
Short-guide oligo (1μM)	1
Guide-constant oligo (1μM)	1
dNTP (10mM)	1
OneTaq	0.25

*Total volume – 50μL; 4 reactions made per gRNA preparation

Table 2.2. Reaction mix for *in vitro* transcription of gRNA.

Nuclease-free water	X μL	Concentration
10x Reaction Buffer	1.5 μL	0.75X final
NTP	1.5 μL each	7.5 mM each final
Template DNA	X μL	200 ng PCR product
T7 RNA Polymerase Mix	1.5 μL	
Total Reaction Volume	20 μL	

Table 2.3. Primers utilized to generate sgRNA and sequencing PCR product for genotyping.

Primer Name	Sequence (5'—3')
gRNA primer 1	GCGTAATACGACTCACTATAG
gRNA primer 2	AAAGCACCGACTCGGTGCCAC
Guide-constant oligo	AAGCACCGACTCGGTGCCACT TTTTCAAGTTGATAACGGACTAG CCTTATTTTAACTTGCTATTTCT AGCTCTAAAAC
Short-guide oligo (cnr1 gRNA)	GCG*TAATACGACTCACTATAGG***ACCTCCGG CCTGCAGTACATCGG**GTTTTAGAGCTAGAA*
cnr1 sequence primer FWD	ATCTTTGCATCTACCAGGCTT
cnr1 sequence primer REV	GCTTTCCACAGGATAAGAGCA

*Italicized letters indicate the T7 promoter sequence. Bolded letters indicate sequence specific to *cnr1* target gene, and underline is PAM sequence

2.3.3. RNA extraction, polymerase chain reaction (PCR), and agarose gel electrophoresis

The RNA was extracted from zebrafish adults. 3-4 replicates for male brain, female brain, and gonads (ovaries and testes) were collected for ddPCR analysis. Each replicate consisted of 3-4 organs collected from different fish. RNA extraction was conducted using the Monarch® Total RNA Miniprep Kit from New England Biolabs$^{Ltd.}$, according to the manufacturer's purification protocol. RNA yield was quantified from the Synergy™ LX Multi-Mode Reader from BioTek. Then, 1µg of total RNA was utilized for complementary DNA synthesis and was produced using the 5x iScript™ reverse transcriptase Supermix (Bio-Rad).

cDNA synthesis was performed on the C1000TM Touch™ Thermal Cycler (Bio-Rad) machine: priming for 5 min at 25°C, then reverse transcription (RT) for 20 min at 46°C, and 1 min at 95°C. To check for the specificity of the primers, a PCR test was performed. The cDNA was first diluted to 1:20 with nuclease free water. PCR products synthesized using the same machine followed: 95°C for 3 min, 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds, and 72°C for 5 min. PCR products were subjected to agarose-gel electrophoresis to confirm the expected size. The gene products were identified via gel electrophoresis on a 1% agarose gel, using a UV-gel imager. The primer sequences corresponding to the gene of interest are in Table 2.4. After size determination, PCR products were purified and then sent for sequencing to confirm the specificity of the amplicons.

2.3.4. ddPCR analysis of *cnr1* and *cnr2* mRNA

Samples for ddPCR analysis were prepared using the QX200™ddPCR™ EvaGreen® supermix (Bio-Rad), according to the manufacturer's protocol. The samples were separated into nanolitre-sized droplets using the QX200™ ddPCR™ droplet generator (Bio-Rad). The samples were then amplified using a thermocycler (Bio-Rad). The PCR conditions were enzyme activation

for 5 min at 95ºC, 40 cycles of 30 s at 95ºC and 1 min at 60ºC. Afterwards, signal stabilization was performed at 4ºC for 5 min and then at 90ºC for 5 min. The concentration of both negative and positive droplets was measured using the QX200™ droplet reader (Bio-Rad), in copies/µL. The mRNA expression levels of *cnr1* and *cnr2* were normalized to that of the geometric mean of ribosomal protein S18 (*rps18*, mRNA transcript) and ribosomal protein L13a (*rpl13a*, mRNA transcript), and the data were expressed relative to the brain for the expression profile data and then relative to the wildtype ovaries for the detection of compensatory regulation. Primer sequences used are summarized in Table 2.4.

Table 2.4. Primer sets utilized for droplet digital PCR analysis.

Gene	Accession Number	Forward Primer (5'-3')	Reverse Primer (5'-3')
cnr1	NM_212820.2	CTGTTCAAGCTAGGAGGCGT	AAGGCGATCACTGCTTTCGT
cnr2	NM_212964.3	CTGACACGCACTAGAGCCAA	CGTACGGGAAGAGACGTGAG
rps18	NM_173234.1	CCCTCGTCATCCCAGAGAAGT	CGCCTTCCAACACCCTTAATAG
rpl13a	NM_212784.1	GTATTTGGCTTTCCTCCGCA	ACCATGCGCTTTCTCTTGTC

2.3.5. Assessment of breeding competency with heterozygous genotype (*cnr1*$^{+/-}$) and phenotypic characterization of mutant adults

The breeding competency test was conducted by breeding heterozygous mutant females with WT males in a 1:1 ratio every other day, for 15 breeding events. Fish were paired, either as a wildtype female with a wildtype male (as a control), or heterozygous mutant female with wildtype male. Firstly, wildtype males were confirmed to be fertile. Afterwards, heterozygous mutant females, wildtype females and wildtype males were separated for 1 week. Moreover, fish were siblings (i.e., identical parents) and size matched. Breeding was conducted every other day, from 7:00am-11:00am. Eggs were collected twice, once at 9:00am and again at 11:00am. During the

breeding competency test, males were swapped after 30 minutes if spawning did not occur. Embryos were collected and a maximum of 50 embryos were placed in a 50 mL Petri dish at a time. Upon collection, they were immediately cleaned with egg water, which is 0.1% methylene blue and sea salt (concentration 60 µg/ml) mixed into 1L of fresh system water. After breeding, fish were fed with brine shrimp and then pellets at 1:00pm. The water in the Petri dish was changed daily until 72 hours post-fertilization (hpf), upon which the larvae would be sacrificed. Spawning frequency (% females who spawn), cumulative egg production, survival rate at 24 hpf, hatching rate at 48 hpf, and standard body length at 72 hpf (length measured from the tip of the snout to the posterior end excluding the caudal fin) was measured during this time. The breeding assessment methodology is briefly summarized in Figure 2.1. There were 6-8 breeding pairs (i.e., n = 6-8), due to the limited number of heterozygous mutant females present. Furthermore, the phenotypic characterization of the heterozygous mutants and wildtype fish was assessed. The gonadosomatic index (GSI), condition factor (Kn), standard body length, and body weight were recorded. Relevant formulae are listed.

GSI:

$$\frac{gonad\ wet\ weight(mg)}{wet\ body\ weight(mg)} \times 100$$

Kn:

$$\frac{wet\ body\ weight(mg)}{length(mm)^{3}} \times 100$$

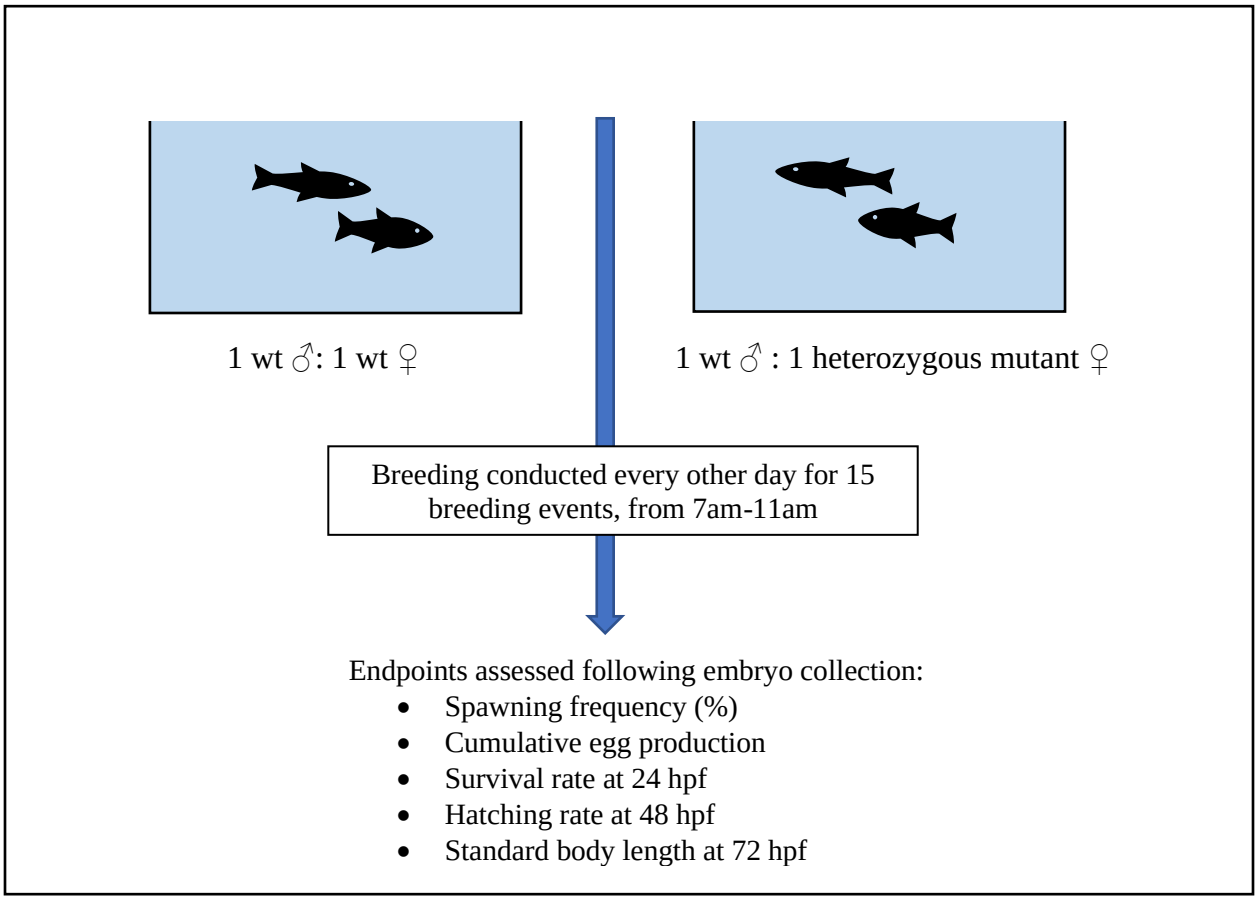


Figure 2.1. Methodology of breeding assessment assay of *cnr1*$^{+/-}$ mutant females. Zebrafish were paired in 1:1 ratio, with 1 WT male and 1 WT female (control) OR 1 WT male and 1 heterozygous mutant female (6-8 breeding traps for each cross, with each trap considered as n=1). Breeding was conducted every other day for 15 breeding events, and zebrafish bred from 7:00am-11:00am. Upon egg collection, spawning frequency, cumulative egg production, survival rate at 24 hours post-fertilization (hpf), hatching rate at 48 hpf, and standard body length at 72 hpf were measured.

2.3.6. Statistical analysis

All statistical analyses were conducted on Sigmaplot® (Version 14). Data were either analyzed by an unpaired Student's *t*-test, or two-way ANOVA, followed by a post-hoc Holm-Sidak test. All data were presented as means with ± SEM (standard error of the mean). Percentage data underwent arcsine transformation prior to statistical analysis. Numbers set to $p<0.05$ were used as values of significance. If the data did not meet assumption of normality or equal variance, values were transformed to log or square root before further statistical analysis, or non-parametric tests such as the Mann-Whitney Rank Sum Test were run.

2.4. Results

2.4.1. mRNA expression levels of *cnr1* and *cnr2* in zebrafish brain, ovaries, and testes

Utilizing ddPCR, the mRNA expression levels of *cnr1* and *cnr2* were determined in the male and female brain, ovaries, and testes. Two-way ANOVA results indicated that were was a significant interaction between the receptors and organ type for the females ($p<0.01$) (Fig. 2.2A). According to a post hoc test, *cnr1* expression was significantly higher than *cnr2* expression in the female brain. There was no significant interaction between receptor expression and organ type for the males (Two-way ANOVA, $p>0.05$) (Fig. 2.2B). See supplementary table S2.1 for two-way ANOVA results.

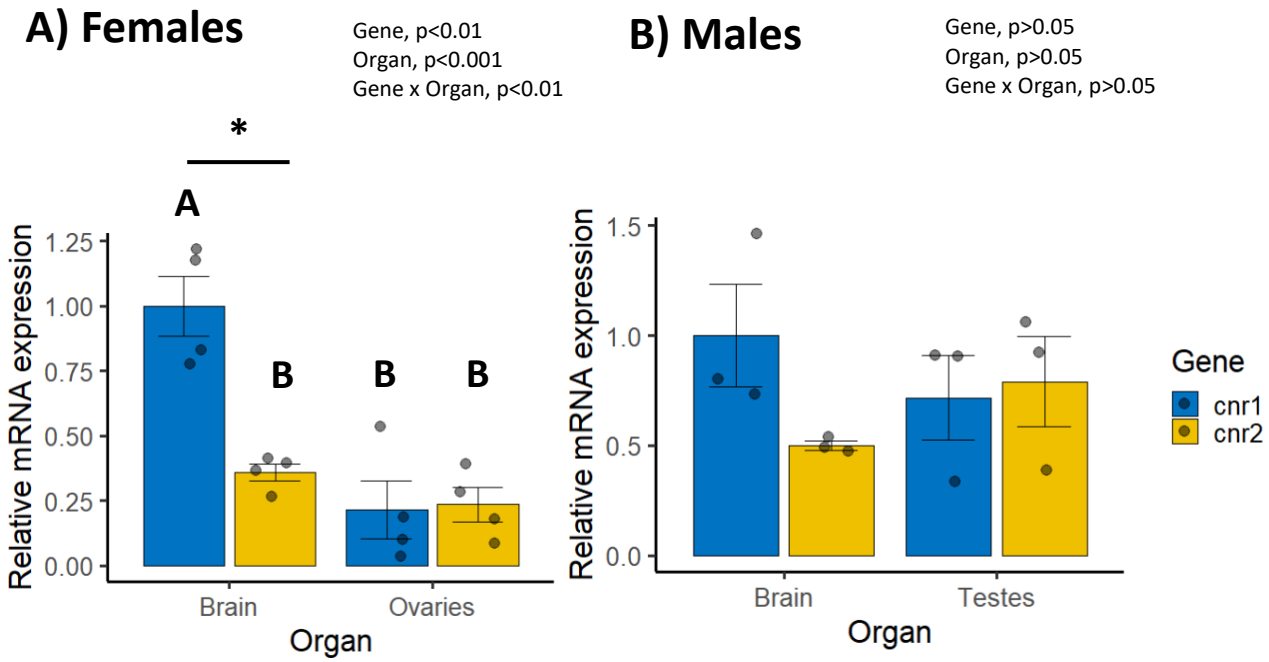
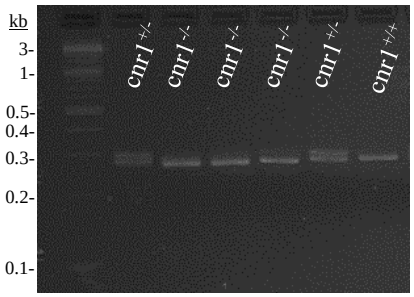


Figure. 2.2. Relative mRNA expression levels of *cnr1* and *cnr2* in adult zebrafish organs via ddPCR analysis. Expression of *cnr1* and *cnr2* in **(A)** female brain and ovaries, and in **(B)** male brain and testes. Values represented as mean ± SEM, n=3-4. Each replicate consisted of 3-4 organs collected from different fish. The mRNA expression levels were normalized by the geometric mean of *rpl13a* and *rps18* and expressed relative to the female or male brain. **A)** There was a significant interaction between *cnr1* and *cnr2* expression in the females (Two-way ANOVA, $p<0.05$, followed by a Holm-Sidak test). The bars labelled with different letters indicate a statistical difference between organs of the same gene (Holm-Sidak test, $p<0.001$), and the bars labelled with an asterisk indicate a statistical difference between the genes of the same organ (Holm-Sidak test, $p<0.01$). **B)** There was no significant interaction between *cnr1* and *cnr2* expression in the males (Two-way ANOVA, $p>0.05$)

2.4.2. Generation of *cnr1* mutation in zebrafish via CRISPR-Cas9

Using CRISPR-Cas9, a *cnr1* mutation was created in zebrafish. A sgRNA specific to *cnr1* was designed for the gene editing of *cnr1*. The mutation was generated by injecting a Cas9 enzyme and gRNA into the one-cell stage of zebrafish embryos. Sanger sequencing of the PCR products determined that there were 5 base-pair deletions, leading to a frameshift mutation and a truncated protein. The frameshift resulted in a stop codon on Exon 2 (Fig.2.3).

Homozygous and heterozygous mutants were obtained by in-crossing male and female *cnr1*$^{+/-}$ siblings. Roughly 50% of the offspring were heterozygous, 25% were homozygous, and 25% were wildtype. An additional line of *cnr1*$^{-/-}$ fish were further obtained by in-crossing male and female *cnr1*$^{-/-}$ fish.

A) 

B)

Wild-type allele

gRNA target sequence

aaaccacct tcagaaccat cacctccggc ctgca**gtaca** tcgg

Mutant allele (Mutant Group 1)

aaaccacct tcagaaccat ccacctccggc ctgca----- tcgg **5nt deletion**

C) **Translation of predicted protein sequence for mutant**

1 atg ctg ttc ccg gcc tca aag tcc gat gtt aaa tct gtc ctg gac gga gtg gcg gaa acc 60

M L F P A S K S D V K S V L D G V A E T

61 acc ttc aga acc atc acc tcc ggc ctg cat cgg ctc caa **tga** 103

T F R T I T S G L H R L Q -

Representative image of CRISPR-Cas9 targeted deletion

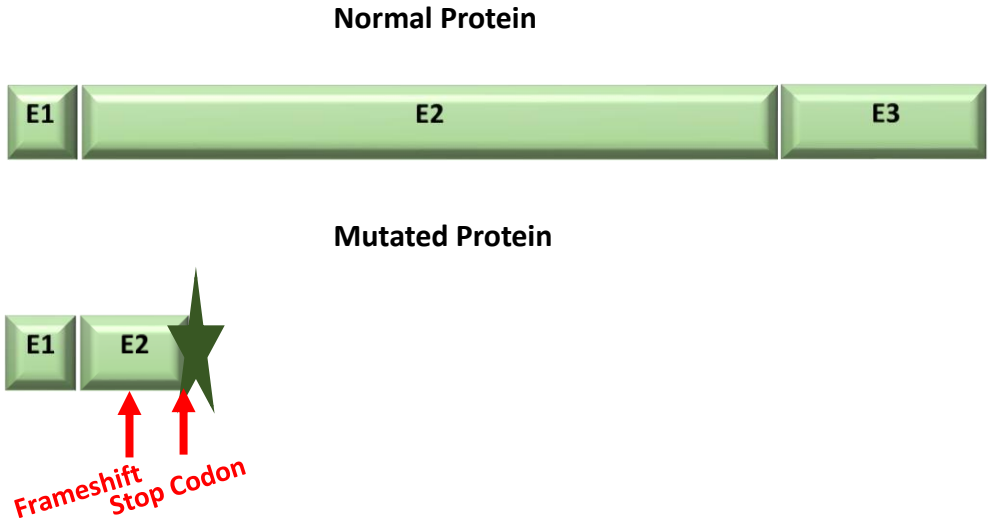


Figure 2.3. Confirmation of *cnr1* deletion in adult zebrafish (*Danio rerio*). (A) Multiplex PCR products from F_2 adults. Genomic DNA was collected from F_2 adults prior to genotyping, amplified via polymerase chain reaction, denatured, and underwent agarose gel electrophoresis (4-6%). Heterozygous fish were identified by double-bands, and homozygous fish (either wild-type or homozygous mutants) by single bands. **(B)** Nucleotide alignment of WT *cnr1* sequence and mutant alleles. Leftover PCR products were sent for Sanger sequencing, and their chromatograms were analyzed for specific indels. The nucleotides highlighted in gray is the gRNA target sequence. The nucleotides underlined are deleted nucleotides. Overall, the mutant group of interest had a 5-

nucleotide deletion. **(C)** Predicted protein sequence of *cnr1*$^{-/-}$ mutants and CRISPR/cas9 target deletion of *cnr1*. The translation of this modified nucleotide sequence resulted in an early stop codon, in which Exon 2 was the target for CRISPR-Cas9 deletion of *cnr1* in zebrafish.

2.4.3. Breeding competency in female *cnr1*$^{+/-}$ zebrafish

Overall, there was no significant difference in breeding competency in female *cnr1*$^{+/-}$ compared to *cnr1*$^{+/+}$ zebrafish (Fig.2.4.). The spawning frequency, cumulative egg production, survival rate, hatching rate, and standard body length was not statistically different between the wildtype and heterozygous mutants (using a Student's *t*-test). See Supplementary Table S2.2 for the full *t*-test results.

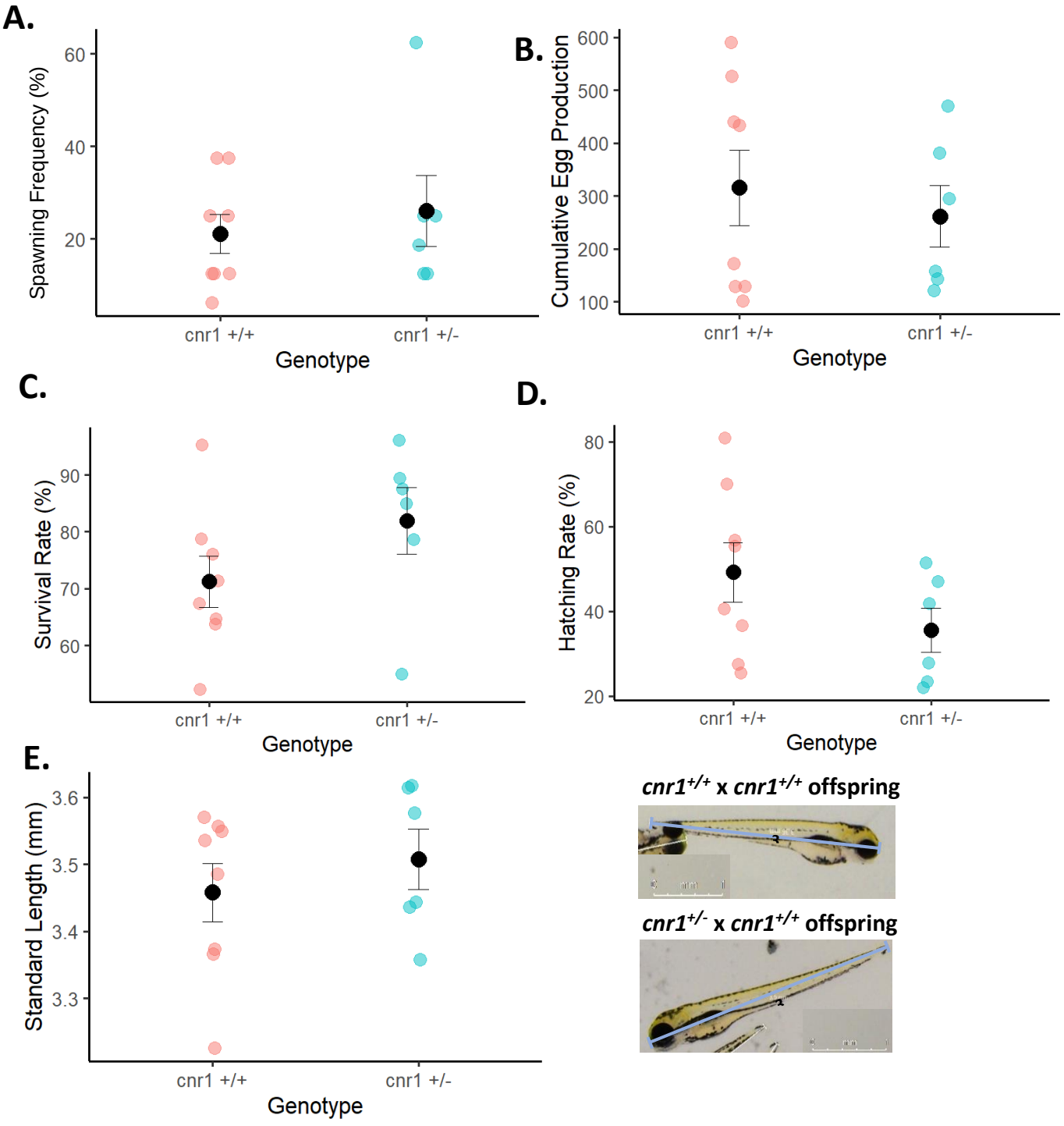


Figure. 2.4. Assessment of breeding competency in *cnr1*$^{+/+}$ and *cnr1*$^{+/-}$ females over 15 breeding events. (A) Average spawning frequency per fish (%). **(B)** Cumulative egg production. **(C)** Survival rate (%) at 24 hours post fertilization (%). **(D)** Hatching rate (%) at 48 hours post fertilization (%). **(E)** Average standard body length per fish at 72 hours post fertilization (mm). Each value represented as mean ± SEM, n = 6-8 breeding pairs (Student's *t*-test, p>0.05).

2.4.4. Phenotypic characterization of male and female *cnr1*$^{+/-}$ fish

The phenotypic characterization of *cnr1*$^{+/-}$ fish was assessed in adult zebrafish. The GSI, Kn, standard body length, and body weight was compared between the wildtype and heterozygous mutant fish. In the females, there was no significant difference between these parameters (Fig.2.5.). Representative images of the morphology of *cnr1*$^{+/-}$ fish are included, in which there is no detectable difference (Fig2.5A and Fig2.6A). On the other hand, a Student's *t*-test determined that the GSI of the *cnr1*$^{+/-}$-mutant males was significantly higher than the wildtype males (Fig.2.6B). For other parameters such as Kn, standard body length, and body weight, there was no difference (Fig.2.6C-E). See Supplementary Table S2.3 and S2.4 for the *t*-test results.

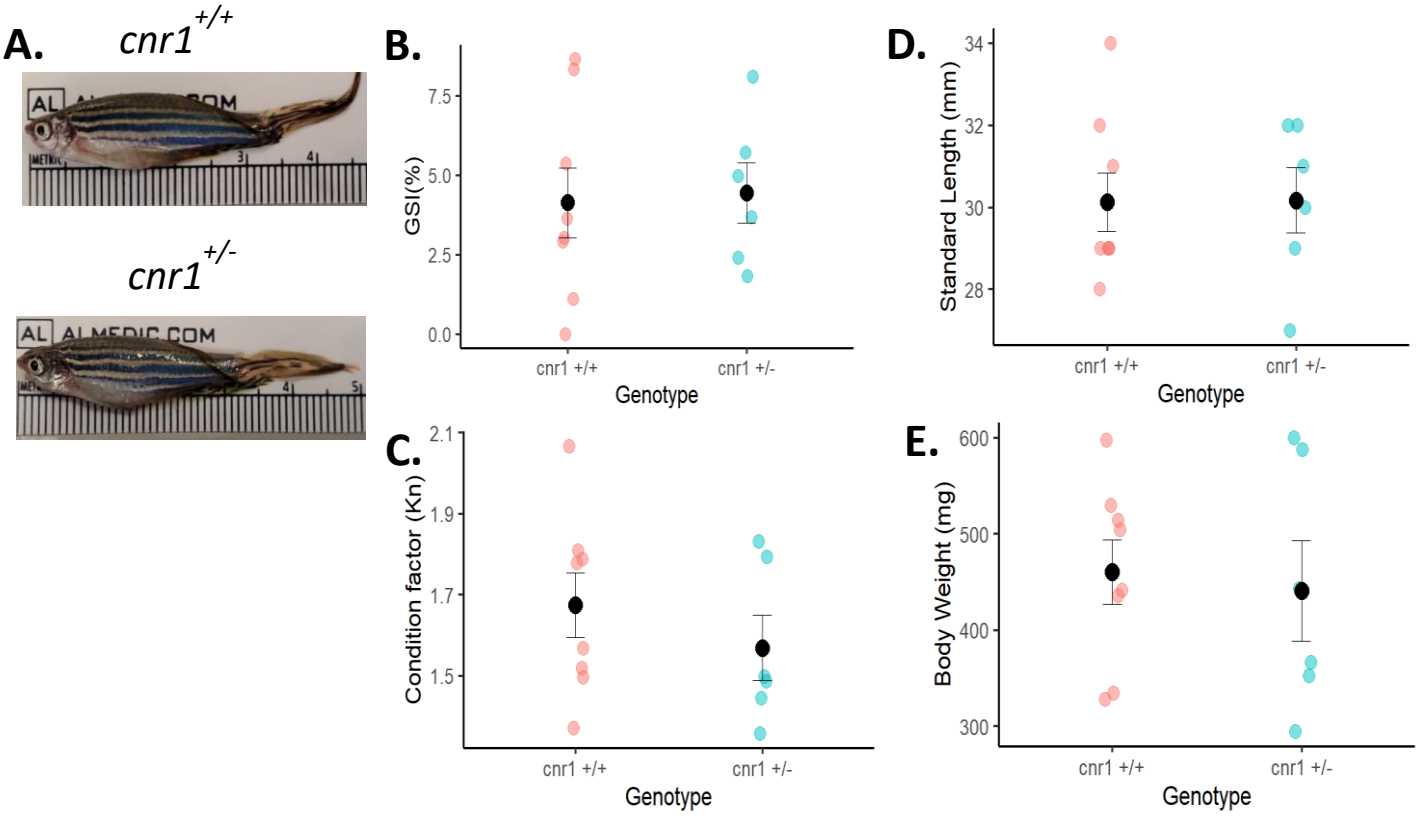


Figure. 2.5. Phenotypic characterization of the *cnr1*$^{+/-}$ female zebrafish. (A) Morphology of *cnr1*$^{+/+}$ and *cnr1*$^{+/-}$ female. **(B)** Gonadosomatic index (GSI%), **(C)** Condition Factor (Kn), **(D)** Standard body length (mm), and **(E)** Body weight of the fish (mg). Each value represented as mean ± SEM, n = 6-8. (Student's *t*-test, $p<0.05$).

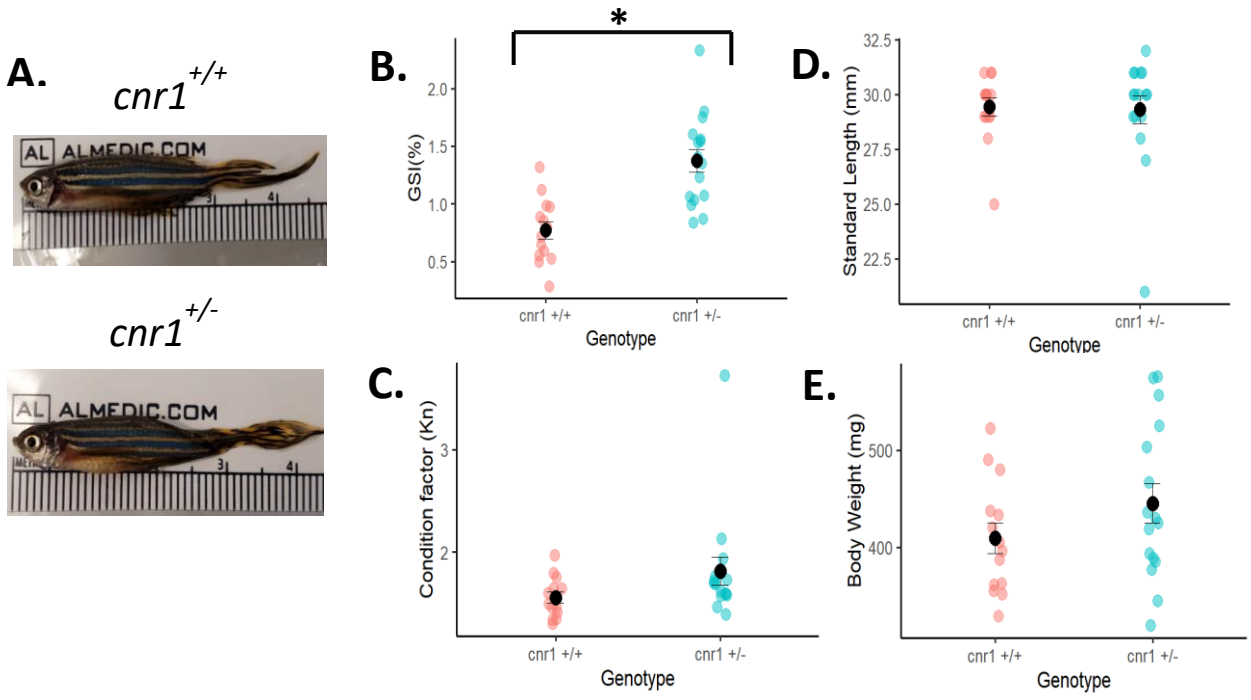


Figure. 2.6. Phenotypic characterization of the *cnr1*$^{+/-}$ male zebrafish. (A) Morphology of *cnr1*$^{+/+}$ and *cnr1*$^{+/-}$ male. **(B)** Gonadosomatic index (GSI%), **(C)** Condition Factor (Kn), **(D)** Standard body length (mm), and **(E)** Body weight of the fish (mg). Each value represented as mean ± SEM, n =14-16. The jitter plots labelled with an asterisk indicate a statistical difference between the *cnr1*$^{+/+}$ and *cnr1*$^{+/-}$ genotypes. (Student's *t*-test, $p<0.05$).

2.4.5. Assessing compensatory regulation in *cnr1*$^{+/-}$ mutants

Since there was mostly no significant difference in breeding competency and phenotype in the heterozygous mutants, the mRNA expression levels of *cnr1* and *cnr2* were assessed in adult organs via ddPCR to detect a possible compensatory response. According to two-way ANOVA for *cnr1* expression, there was no interactive effect between the genotype and organ (Fig.2.7.). A post-hoc test revealed a significant difference in *cnr1* expression between the testes and ovaries for both genotypes. However, for *cnr2* expression, there was a statistically significant interaction between genotype and organ (two-way ANOVA, $p<0.05$). A post-hoc test revealed a significant difference in *cnr2* expression for the *cnr1*$^{+/+}$ genotype for all organs, and a significant difference in *cnr2* expression between the *cnr1*$^{+/+}$ and *cnr1*$^{+/-}$ genotype in the ovaries. Overall, there was no evidence of compensatory increase in *cnr1* and *cnr2* mRNA abundance in the heterozygous *cnr1*$^{+/-}$ genotype. See Supplementary Tables S2.5 and S2.6 for detailed statistical results.

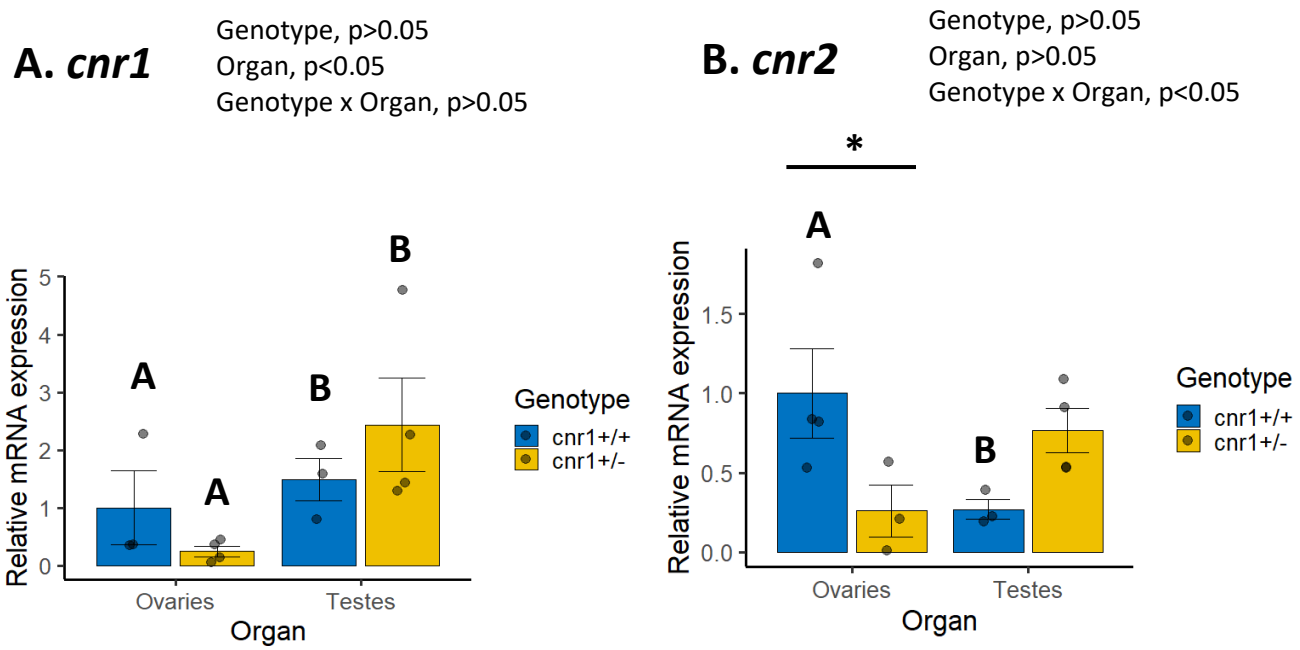


Figure. 2.7. Relative mRNA expression levels of *cnr1* and *cnr2* in *cnr1*$^{+/+}$ and *cnr1*$^{+/-}$ gonads to detect possible compensatory response. Expression level of **(A)** *cnr1* and **(B)** *cnr2* in wild-type and heterozygous mutant ovaries and testes. The mRNA expression levels were normalized by the geometric mean of *rpl13a* and *rps18* plotted relative to wildtype ovaries. Values represented as mean ± SEM, n=3-4. Each replicate consisted of 3-4 organs collected from different fish. **(A)** There was no interactive effect between genotype and organ for *cnr1*. A post-hoc test revealed a significant difference in *cnr1* expression between the testes and ovaries for both genotypes (two-way ANOVA, $p>0.05$, followed by a Holm-Sidak test). The bars labelled with different letters indicate a statistical difference between the organs (Holm-Sidak test, $p<0.05$). **(B)** There was an interactive effect between the genotype and organ for *cnr2*. A post-hoc test revealed a significant difference in *cnr2* expression for the *cnr1*$^{+/+}$ genotype for all organs, and a significant difference in *cnr2* expression between the *cnr1*$^{+/+}$ and *cnr1*$^{+/-}$ genotype in the ovaries (two-way ANOVA, $p<0.05$, followed by a Holm-Sidak test). The bars labelled with different letters indicate a statistical difference between organs of the same genotype (Holm-Sidak test, $p<0.05$), and the bars labelled with an asterisk indicate a statistical difference between the genotype of the same organ (Holm-Sidak test, $p<0.05$).

2.5. Discussion

2.5.1. Overview

In the present study, we combined functional genetics, breeding assessment, and gene expression analysis to characterize the endocannabinoid system (ECS) in the zebrafish model. Specifically, we utilized the next generation genome editing technology, CRISPR-Cas9, to KO cannabinoid receptor 1 (CNR1). For the first stage of breeding assessment in the mutants, we obtained heterozygous female mutants and assessed their breeding competency. The results indicated that there was no statistical difference in breeding competency between *cnr1*$^{+/-}$ and *cnr1*$^{+/+}$ females. We also evaluated the mRNA expression levels of the major cannabinoid receptors (CNR1 and CNR2) across various organs, in which there was no detectable difference of the gene expression level between cannabinoid receptor and organ. Overall, the results from my study provide important groundwork for future research towards characterizing the involvement of the endocannabinoid system in reproductive function in zebrafish. Moving forward, we will utilize the findings from the heterozygous KO to examine the effect of a homozygous *cnr1* KO on breeding competency.

2.5.2. mRNA expression profile of cannabinoid receptors in adult zebrafish

We generated an analysis of the ECS in the adult zebrafish organs, firstly by identifying the relative mRNA expression of major cannabinoid receptors in select organs. Ideally, the analysis of the ECS in the zebrafish could bridge knowledge gaps on their function. We examined the male and female brain, ovaries, and testes. Studies examining the mRNA expression levels between *cnr1* and *cnr2* in the zebrafish has been largely limited to organs pooled from both sexes (Lam, Rastegar, and Strähle 2006a; Migliarini and Carnevali 2009b; Sufian et al. 2019; Watson et al. 2008). Interestingly, both zebrafish and mammalian studies have found higher levels of *cnr1* in

the brain, and very little in the other organs such as the gonads (Herkenham et al., 1991; Marsicano & Kuner, 2008; Oltrabella et al., 2017). Such findings were similar to our results with the adult females, in which *cnr1* expression was higher in the brain and lower in the ovaries. Furthermore, our results indicated that *cnr1* expression was higher than *cnr2* in the female brain. In mammals, *cnr2* is less expressed in the brain, and more so in other organs such as the spleen and kidney (Galgglje' et al., 1995; Gong et al., 2006; Munro et al., 1993). Interestingly, there was no statistical difference in the male organs. As a result, our findings are not completely in line with mammalian studies which report that the expression between the cannabinoid receptors to varies among organs, and that there is a difference between *cnr1* and cnr*2* expression. It would be worthwhile to explore the expression of the receptors in different organs, and other ECS-related genes such as those involved in metabolism. Furthermore, it is worthwhile to note that according to the power analysis, the minimal sample size for male organs was $n \geq 4$, and the data for the testes had high variation. For male brain and testes, the results should be interpreted cautiously. It would be worthwhile to increase the sample size to reduce the variation in data. The low sample size may contribute towards a Type II error, in which one wrongly determines there is no difference in effects while there is one (Akobeng, 2016).

To my knowledge, only one other zebrafish study has reported significantly higher levels of *cnr1* and *cnr2* expression in the testes compared to the ovaries (Oltrabella et al., 2017). Mammalian studies have also explored the involvement of the ECS in the testes. CNR1, CNR2 and its associated metabolic enzymes have been detected in sperm cells of human and rat testes (Migliaccio et al., 2018; Nielsen et al., 2019). Furthermore, cannabinoids have been suggested to affect various sperm functions, influencing sperm motility, spermatogenesis, and at elevated levels can reduce sperm germ cell proliferation (Gérard et al., 1991; M Maccarrone et al., 2002; Rossato

et al., 2005). While our studies largely focused on the physiological role of the CNR1 in female zebrafish reproduction, it would be worthwhile to further explore the receptor's role in male reproduction as well.

The ECS is expressed throughout the vertebrate organism—present in the CNS and other organs such as the cardiovascular system, gastrointestinal tract, liver, immune system, muscles, bones, and skin (Mechoulam 2014). The function of the ECS in these organ systems is of interest and continues to be examined extensively. For instance, in the cardiovascular system, it is suggested that elevated cannabinoid levels improve cardiac performance in individuals suffering from cardiac disease (Kashina, 2016; Wagner et al., 2001). eCBs are also involved in immune cell migration, and influence lymphocyte proliferation (Almogi-Hazan & Or, 2020; Pandey et al., 2009). Furthermore, in the hepatic system, inhibiting the CNR1 has therapeutic implications for those suffering from metabolic steatosis, reversing the condition (Cluny et al., 2010; Jourdan et al., 2010; Mallat et al., 2011; Tam et al., 2010). These are just a few examples of potential physiological functions of the ECS in the vertebrate organism.

2.5.3. Generation of *cnr1* knockout via CRISPR-Cas9

Utilizing CRISPR-Cas9, we generated a KO of *cnr1* zebrafish. In comparison to other genomic engineering methods, CRISPR-Cas9 is beneficial because it is cost effective, simple to design, and operates at a high efficiency compared to other genotyping methods such as TALEN and ZFN (Doudna & Charpentier, 2014). Genomic engineering is particularly useful because it allows us to determine the functional role of many genes of interest. In our case, we were examining the role of *cnr1* in fertility and fecundity.

The aim of CRISPR-Cas9 is to generate indels (insertions/deletions) in the genome, and the resultant effect is a frameshift mutation that disrupts the open reading frame and/or introduces

a premature stop codon (Lalonde et al., 2017). Currently, we have confirmed the KO at the genomic level. We determined that our mutant group of interest has a 5bp deletion, resulting in a frameshift mutation near the N-terminal of the amino acid. The translation of this modified nucleotide sequence also resulted in a premature stop codon at the beginning of Exon 2, leading to the formation of a truncated protein and elimination of protein domains which encode for G-protein signalling, ligand binding sites, and various structural motifs. Nevertheless, moving forward, we will have to assess the protein level of the CNR1 further validate the KO. Protein expression analysis can be performed via a Western immunoblot (Estep et al., 2016). We have obtained human *cnr1* polyclonal antibodies commercially available from Sigma-Aldrich (Cat#: SAB4500345), as *cnr1* zebrafish antibodies for protein analysis are not currently available. According to protein database search programs, the immunogen range designed for this antibody has 94% sequence similarity to the zebrafish *cnr1*. We will utilize this antibody to perform Western blotting analysis to confirm the KO of *cnr1*.

2.5.4. Assessment of breeding competency and phenotypic characterization of *cnr1*$^{+/-}$ mutants

After validation of the *cnr1* KO genotype, we grouped together *cnr1*$^{+/-}$ female fish and assessed their breeding competency. Due to the low number of homozygous females, only heterozygous females were evaluated for the fertility assay. Overall, the results showed that the heterozygous KO has no effect on fertility in female *cnr1*$^{+/-}$ zebrafish. However, with a heterozygous mutation, it is possible that the wildtype allele is up-regulated in order to compensate for the mutated allele (Basilicata & Valsecchi, 2021). Furthermore, a compensatory response would result in similar breeding competency between the wild-type and heterozygous mutants. This possibility was further investigated in our study, by comparing *cnr1* and *cnr2* expression between wild-type and heterozygous mutants. Since there was no difference in fertility observed

in the heterozygous mutant genotype, we suspected that there may be an upregulation in the other major cannabinoid receptor, *cnr2*. Our results showed that there was no significant decrease in *cnr1* expression in the heterozygous mutants. This is because of the presence of the wild-type allele in the heterozygous fish. It is also noteworthy that the frameshift mutation in the *cnr1* gene would not affect the transcription process.

The results from the fertility assay are currently inconclusive, as it is not possible to determine the functional role of CNR1 from just heterozygous females. It would be worthwhile to explore the effect of a full *cnr1* KO on fertility in zebrafish. Studies comparing the effect of a homozygous versus a heterozygous mutation have been limited, but it appears the CNR1 is still functional in heterozygous mutants. Only a half reduction of CNR1 were observed in heterozygous KO mice, and mortality rate in mice pups was similar to wild-type mice (Breivogel et al. 2001; Zimmer et al. 1999). However, studies with mammalian models have shown that a full *cnr1* KO has deleterious effects in reproductive function (Li et al. 2019, 2020; Paria et al. 2001; Wang et al. 2004; Wang, Xie, and Dey 2008). As stated previously, there have been no other studies examining the functional role of CNR1 in zebrafish reproduction. Moving forward, it would be important to examine the effect of a full KO in zebrafish.

We also examined the physiological conditions of the heterozygous mutants. In the females, there was no difference observed in GSI, K, body weight, or standard body length. However, in males, the GSI was significantly higher in the heterozygous mutants. In fish, the GSI is a metric utilized to demonstrate the organism's reproductive capabilities (Rizzo & Bazzoli, 2020). A high GSI is also an indicator of well-developed gonads, i.e. higher sexual maturity (Tagarao et al., 2020). As a result, the male heterozygous mutants appeared to be more sexually developed than their wild-type siblings. In contrast, pharmacological inhibition of the cannabinoid

receptor via marijuana exposure results in testicular atrophy leading to a lower testicular weight (Banerjee et al., 2011; Fujimoto et al., 1982; Harclerode et al., 1979). Further, KO studies with *cnr1*$^{-/-}$ mice have demonstrated that they exhibit testicular atrophy (Bilkei-Gorzo et al., 2012). These results suggest that disturbing CNR1 impairs testicular development, which can have negative consequences for sexual maturity. It would be worthwhile to further explore the mechanism underlying my contrasting results which suggested a higher GSI in male heterozygous mutants.

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2.7. Supplementary tables

Supplementary Table S2.1. Results from two-way analysis of variance for *cnr1* vs. *cnr2* mRNA abundance for females and males

Females

Source of Variation	Degrees of Freedom	F-value	P-value
Organ	1	26.601	<0.001*
Gene	1	12.358	0.004*
Organ x gene	1	14.055	0.003*

*Multiple comparison procedure (Holm-Sidak method) demonstrated that *cnr1* vs. *cnr2* in female brain is statistically significant ($p<0.001$), and female brain vs. ovaries is significant ($p<0.001$).

Males

Source of Variation	Degrees of Freedom	F-value	P-value
Organ	1	0.000313	0.986
Gene	1	1.376	0.274
Organ x gene	1	2.468	0.155

Supplementary Table S2.2. Results from Student's *t*-test for spawning frequency, cumulative egg production, survival rate, hatching rate, and standard body length.

Measurement	*t*-value	Degrees of Freedom	95% 2-tailed confidence interval for difference of means	2-tailed P value
Spawning frequency	-0.622	12	-0.265-0.147	0.546
Cumulative egg production	0.552	12	-158.153-265.403	0.591
Survival rate	-1.481	12	-0.335-0.0640	0.165
Hatching rate	1.454	12	-0.0715-0.358	0.172
Standard body length	Non-parametric test run instead (Mann-Whitney Rank Sum test) since normality test failed. P > 0.05, no statistical difference between variables			

Supplementary Table S2.3. Results from Student's *t*-test for GSI, Kn, body weight, and standard body length in *cnr1*$^{+/-}$ females.

Measurement	*t*-value	Degrees of Freedom	95% 2-tailed confidence interval for difference of means	2-tailed P value
GSI	0.185	11	-2.952-3.495	0.8563
Kn	0.9201	11	-0.145-0.357	0.378
Body weight	0.3364	11	-109.105-148.947	0.7424
Standard length	0.0387	12	-2.39-2.30	0.9698

Supplementary Table S2.4. Results from Student's *t*-test for GSI, Kn, body weight, and standard body length in *cnr1*$^{+/-}$ males.

Measurement	*t*-value	Degrees of Freedom	95% 2-tailed confidence interval for difference of means	2-tailed P value
GSI	4.7465	28	-0.867 to -0.344	<0.0001
Kn	1.4779	25	-0.232-0.038	0.1519
Body weight	1.3748	28	-89.145-17.541	0.1801
Standard length	0.1478	28	-1.49-1.72	0.8835

Supplementary Table S2.5. Results from two-way analysis of variance for *cnr1* mRNA in wildtype vs. heterozygous mutants

Source of Variation	Degrees of Freedom	F-value	P-value
Organ	1	5.485	0.041*
Genotype	1	0.0288	0.869
Organ x genotype	1	2.216	0.167

*Multiple comparison procedure (Holm-Sidak method) demonstrated that testes vs. ovaries $p<0.05$

Supplementary Table S2.6. Results from two-way analysis of variance for *cnr2* mRNA in wildtype vs. heterozygous mutants

Source of Variation	Degrees of Freedom	F-value	P-value
Organ	1	0.319	0.585
Genotype	1	0.371	0.556
Organ x genotype	1	9.609	0.011*

*Multiple comparison procedure (Holm-Sidak method) demonstrated that expression of *cnr2* was significantly different in cnr$1^{+/+}$ vs. *cnr*$1^{+/-}$ ovaries, and *cnr2* expression was different between *cnr*$1^{+/+}$ ovaries and testes.

Chapter III:

General Discussion

3.1. Implications and significance of thesis

In this study, we combined experiments involving functional genetics, breeding assessment, and gene expression analysis to explore the role of the ECS in zebrafish reproduction. My results provide important groundwork for understanding the involvement of CNR1 in zebrafish fertility and fecundity. Currently, our results are inconclusive in determining the functional significance of CNR1 in reproductive function. We determined no statistical difference in breeding competency observed in heterozygous mutant *cnr1*$^{-/-}$ females compared to their wildtype siblings. Interestingly, we observed a significant increase in male GSI heterozygous mutants.

Studying the endocannabinoid system is of value because of its wide presence in the body—with CNR1 being highly populated in the central nervous system, to CNR2 prevalent in the immune and peripheral organs (Silver, 2019). The presence of the ECS in the vertebrate organism varies in function as well. For instance, in the CNS, the ECS is implicated in learning and memory, nervous system development, synaptic plasticity, and nociception (Skaper & Di Marzo, 2012). In the immune system, the ECS can affect the immune response, trigger apoptosis in immune cells, and immune cells can even help synthesize eCBs (Pandey et al., 2009). Interestingly, in the cardiovascular system, CNR1 can worsen cardiac conditions, whereas CNR2 can be protective in function (Maccarrone et al. 2015). Finally, in the gastrointestinal tract, eCB signalling is influential in determining food intake and energy balance. CNR1 and CNR2 are also located in enteric nerves (Maccarrone et al., 2015). These are several examples of the ECS' role in various organ systems. Clearly the system has a diverse yet essential involvement in regulating countless processes in the body.

Manipulating the cannabinoid system also has therapeutic implications for disease (Aizpurua-Olaizola et al., 2017). For instance, cannabinoid therapies can enhance the effect of cancer treatment, inducing apoptosis in cancer cells (Costa et al., 2016). Neurological disease can also be targeted, whereby THC and CBD improve physical symptoms associated with Multiple Sclerosis or Huntington's disease (Iannotti et al., 2016). Manipulating the ECS is also useful in the context of fertility. Some infections can induce miscarriages, however manipulating the CNR1 can reduce the rate of embryo resorption and help maintain progesterone levels in the mouse (Wolfson et al., 2015).

3.2. Conclusion and future studies

In the present study, we utilized CRISPR-Cas9 gene editing technology to generate a cnr*1* KO. We have first confirmed the KO at the genomic level by Sanger Sequencing and predicted the translation of a truncated protein. However, these results are not enough to confirm the KO of the CNR1. We will need to further confirm the KO at the protein level via a Western blot. Once we obtain confirmation of the KO from the western blot, we can validate that this KO has successfully occurred. Furthermore, the generation of a *cnr1* KO line will provide a powerful tool to investigate the role of this receptor not only in reproduction, but also in other physiological processes. CNR1 is an essential component of the endocannabinoid system, with receptors being implicated throughout the vertebrate organism (Silver, 2019). Studies also suggest that the dysregulation of the ECS can result in impairments in reproductive function, including processes involved in oocyte maturation, folliculogenesis, and embryo development (Walker et al., 2019). Moving forward, we plan to assess the functional role of CNR1 by assessing the breeding competency in $cnr1^{-/-}$ mutants.

Our findings also demonstrated that *cnr1* and *cnr2* mRNA are expressed in the gonads, suggesting their role for the ECS in regulating reproduction. Moving forward, it would be worthwhile to compare the receptor expression in other organs and examine other ECS-related genes, such as those involved in eCB metabolism. Clarifying the distribution of these fundamental ECS-related genes will aid in defining the system within the zebrafish species.

Although we have for the first time acquired data to explore the reproductive ability of zebrafish in the context of the ECS, there is still much to uncover. To explain, our breeding competency results are from the heterozygous KO. These results are insufficient to ascertain the functional role of the CNR1 in fertility. Therefore, we cannot conclude the role of CNR1 in regulating female fertility. Due to the limited number of homozygous females present, we had decided to examine only the heterozygous mutants. Moving forward, we plan to examine the breeding competency in homozygous mutants. As a result, we may not be able to confidently state that there is no difference in breeding competency *cnr1*$^{+/-}$ fish. Regardless, establishing a mutant line provides us with a unique opportunity to produce a variety of functional studies. We know that CNR1 plays roles in the cardiovascular, gastrointestinal, central nervous, and immune system (Mauro Maccarrone et al., 2015; Pandey et al., 2009; Skaper & Di Marzo, 2012). As a result, we can examine the role of the ECS in various organ systems.

Our study focused on the role of the CNR1 in female reproduction only. However, studies strongly support that the ECS is functional in male fertility as well. Firstly, Sertoli cells, necessary in sperm production in males, contain CNR2, and are suggested to influence sperm motility in mammals (Agirregoitia et al., 2010; Maccarrone et al., 2003). CNR1 is also expressed in the testes, vas deferens, and located on the tail of spermatozoa in humans (Agirregoitia et al., 2010; Gye et al., 2005; Tambaro et al., 2005). Furthermore, in mammals, it is known that CNR2 is also

expressed in the male testes, and may even contribute towards maintaining spermatogenesis (Di Giacomo et al. 2016; Liu et al. 2009). Interestingly, based on the study conducted by Oltrabella and colleagues and our findings, both CNR1 and CNR2 are detected at higher levels in the male gonads compared to female (Oltrabella et al., 2017). It would be interesting to explore the specific role of the cannabinoid receptor in male fertility and fecundity. Future experiments can involve generating a fertility assay using similar methods in my study, whereby we compare the breeding competency in *cnr1* KO males.

I have also been in the process of generating a *cnr2* KO line. Currently, I have mosaic F_0 mutant fish that will need to be further bred to obtain F_1 heterozygous fish. Future studies can examine the functional role of CNR2 in combination with CNR1, to obtain a wider understanding of the receptors' role in reproduction.

Overall, the findings from my study have been vital steps towards characterizing the role of the ECS in female reproduction in the zebrafish species. We aim to use our findings to further clarify the role of the essential and ubiquitous endocannabinoid system. This system is conserved in all vertebrate species, therefore unlocking its mechanisms has high implications for animal physiology.

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