# Elucidating the roles of endocannabinoid signalling pathway in motor neuron and locomotor development in zebrafish early life

by

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## *Abstract*

The major endocannabinoids (eCBs), anandamide (AEA) and 2-arachidonoylglycerol (2-AG) mediate their effects through their actions on cannabinoid receptors (CBRs), mainly via CB1R and CB2Rs. The predominant catabolic regulators that degrade AEA and 2-AG are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL also known as MGLL), respectively. Although the eCB signalling pathway is present from very early stages of development, little is known about their mechanisms that underlie embryonic growth, and the onset of locomotion and motor neuron development. In my thesis, I sought to investigate the role of the eCB system in zebrafish embryonic development by pharmacologically inhibiting the CB1 and CB2 receptors (with AM251 and AM630, respectively) for the first two days of post-fertilization (dpf). Selective inhibition of CB1R and CB2Rs upon treatment with AM251 and AM630 resulted in embryos with axial malformations, shorter trunks, pericardial edema and reduced heart rates, particularly at higher concentrations at 2 dpf. Locomotion studies at 5 dpf revealed a change in their activity, swim velocity, the number of swim bouts and the cumulative duration of swim bouts. The morphological and locomotor deficits observed upon inhibition of the CBRs during the first 48 hours of development led me to examine in more detail the role of CB1R and CB2R during development. Therefore, I further investigated the role of the CBRs by pharmacologically inhibiting CB1R and CB2R (with AM251 and AM630, respectively) in either the first or second day of development. Motor neuron morphology and neuromuscular outputs were then examined and quantified in 5 dpf larvae. Although blocking both CB1R and CB2R resulted in gross morphological deficits and reductions in heart rate, the effects of CB2Rs inhibition were more pronounced when compared to

CB1Rs. Blocking CB1Rs from 0 to 24 h post-fertilization (hpf) resulted in an increase in the number of secondary and tertiary branches of primary motor neurons, whereas blocking CB2Rs had the opposite effect. Both treatments resulted in reduced levels of swimming. Additionally, blocking CB1Rs resulted in greater instances of non-inflated and partially inflated swim bladders compared with AM630 treatment, suggesting that at least some of the deficits in locomotion may result from an inability to adjust buoyancy.

Finally, I studied the effects of inhibiting the enzymes (FAAH and MAGL) that break down the eCBs, in the early part of development. My study analyzed the functional roles of increased accumulation of AEA and 2-AG levels upon inhibition of FAAH and MAGL, and examined their effects on development. *In vivo* application of a dual FAAH/MAGL inhibitor, JZL 195, resulted in a reduction in primary and secondary motor neuronal axon branching, as well as in a reduction of nicotinic acetylcholine receptors (nAChRs) expression at neuromuscular junctions (NMJs). Application of the specific inhibitor of the FAAH enzyme, URB 597, also resulted in a decrease in motor neuron branching of the primary motor neurons only. However, secondary motor neuron branching and nAChR expression remained unaffected following URB 597 treatment in the first 24 hrs of development. Furthermore, I also confirmed that the effects of JZL 195 and URB 597 were mediated through CB1Rbecause co-treatment with the selective CB1R inhibitor, AM 251, prevented the aberrant branching of motor neurons and reduced nAchRs expression. Interestingly, JZL 184, a selective MAGL inhibitor, showed no effects throughout the study when examining motor neuron development and nAChRs expression at NMJs. Locomotion studies revealed that any disruption of FAAH or MAGL activity in the first 24 hrs of development by JZL 195,

URB 597 and JZL 184 can reduce larval swimming activity. Although CB1R inhibition with AM 251 attenuated JZL 195 and URB 597 induced behavioural changes, it was unable to block the effects of JZL 184. Therefore, my study strongly indicates that accumulation of AEA preferentially acting through CB1R at early life is responsible for motor neuronal changes associated locomotor deficiency.

## *Preface*

This thesis contains all the original works of Md Shah Sufian. The research project, of which this thesis is a part, received research ethics approval from University of Alberta Research Ethics Board, Project name "Synaptic maturation in the fish embryos", AUP00000816.

Some of the research conducted for this thesis forms part of a collaboration between Dr. Declan Ali and Dr. William Ted Allison at the University of Alberta.

Results presented in chapter 3 and parts of the discussion in chapter 6 have been published as M.S. Sufian, J. Waldon, R. Kanyo, W. T. Allison, and D. W. Ali, "Endocannabinoids in Zebrafish are Necessary for Normal Development and Locomotion," *Ashdin Publ. J. Drug Alcohol Res.*, vol. 7, p. 9, 2018.

The results in chapter 4 and parts of the discussion in chapter 6 have also been published as M. S. Sufian, M. R. Amin, R. Kanyo, W. Ted Allison, and D. W. Ali, "CB1 and CB2 receptors play differential roles in early zebrafish locomotor development," *J. Exp. Biol.*, vol. 222, no. 16, Aug. 2019.

The work in the publications from chapter 3 and 4 were conceptualized by M.S. Sufian, D.W. Ali and W.T. Allison. The data collection and analysis were performed by M.S. Sufian and R. Kanyo. The manuscript writing, review and editing were done by M.S. Sufian, R. Kanyo, W.T. Allison and D.W. Ali.

The data analysis and concluding results in chapter 5 are my original work, as well as the literature review in chapter 2.

## *Acknowledgements*

First, I would like to express my sincere gratitude to my research supervisor Dr. Declan William Ali. It is my honour to get the opportunity to work under his supervision. His invaluable guidance, kindness and patience made my journey easier here. He supported me at my difficult times and always helped me with writing and future directions.

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## *List of abbreviations*

AEA = Anandamide

AG = Arachidonoylglycerol

AMC = 7-amino-4-methylcoumarin

AMC-AA = 7-amino-4-methylcoumarin arachidonamide

BSA = Bovine serum albumin

BTX = Bungarotoxin

CBD = Cannabidiol

CBN = Cannabinol

CBR = Cannabinoid receptor

CB1R = Cannabinoid receptor type 1

CB2R = Cannabinoid receptor type 2

CDTA = Calcium dependent transacylase

CNS = Central nervous system

DAGL = Diacylglycerol lipase

DMSO = Dimethyl sulfoxide

dpf = days post-fertilization

eCB = Endocannabinoids

EW = Egg water

FAAH = Fatty acid amide hydrolase

hpf = hours post-fertilization

JNKs = Jun N-terminal kinase

LTD = Long term depression

MAGL = Monoacylglycerol lipase

MAPK = Mitogen activated protein kinase

MS222 = Tricaine methane sulfonate

nAChRs = Nicotinic acetylcholine receptors

NAPE-PLD = N-acyl phosphatidylethanolamine phospholipase D

NMJs = Neuromuscular junctions

NT = Neurotransmitter

PBS = Phosphate buffer saline

PFA = Paraformaldehyde

PMN = Primary motor neuron

PNS = Peripheral nervous system

PTU = Phenylthiourea

SEM = Standard error of the mean

SMN = Secondary motor neuron

THC = Tetrahydrocannabinol

TRP = Transient receptor potential channels

VGCC = Voltage gated calcium channels

WHO = World health organization

# Chapter 1. Introduction and Literature Review

## 1.1 The discovery of cannabis and cannabinoid system

Cannabis use has a history of more than 5000 years as a therapeutic compound during religious functions or recreational activities in different parts of the world [1]. Although cannabis cultivation continued for many years, the first investigational studies to explore their pharmacological profiles were reported only at the end of the 19th century[2]. Cannabis extracts are mainly composed of more than400 different chemical compounds, around 104 compounds which have already been identified as cannabinoids (CBs) [3]. The first isolated compound from cannabis plant was cannabinol (CBN) [4], and muchlater, Mechoulam and Shvo extracted another compound, which was named cannabidiol (CBD) [5]. The main psychoactive component of cannabis, $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) was first isolated and identified the following year in 1964[6]. More recently it has been reported that a wide range of mental health-related disorders might arise from cannabis consumption, particularly when consumed at a young age [7]. Due to the psychotropic side effects, cannabis compounds are believed to be associated with a high risk of abuse and thus currently are not approved for any disease treatment. Now, cannabis has an annual consumption rate of approximately 147 million people worldwide, reported by the World Health Organization (WHO)[8]. However, a growing number of states in the USA (The United States of America) are reconsidering the scientific claims supporting the medicinal benefits of cannabis[9]. Despite some presumable benefits, it is important to investigate the public data to review the safety profiles and abusive potential of the drugs that contain cannabis extracts. Cannabis research reached another milestone when

$\Delta^9$-THC binding sites were identified successfully, which was followed by the discovery of the CB receptors [10][11]. Disclosure of the presence of CB receptors provoked further investigations into the existence of the endocannabinoid (eCB) system and physiological cannabinoids [12][13]. The crucial breakthroughs and advancement in the pharmacological research on cannabinoid system encouraged current researchers to explore further the benefits of medicinal cannabis. In this study, I focused on demonstrating the relevance of eCBs and eCB pathway physiology in early life stages.

## 1.2 Endocannabinoid (eCB) signalling pathway

The endocannabinoid (eCB) signalling network can regulate numerous physiological functions, including pain, neuroprotection, motor movement, memory and cognition [14][15][16][17]. An integral component of the eCB system is the cannabinoid (CB) receptors, of which CB1R and CB2R are the two most abundant forms that belong to the G-protein coupled receptor (GPCR) family of receptors. The two main endogenous eCBs are N-Arachidonoylethanolamine, also referred to as anandamide (AEA), and 2-Arachidonoylglycerol (2-AG). AEA and 2-AG are highly lipophilic substances that are released into the extracellular space when the appropriate signals are received. They are thought to be produced on-demand as a result of increased cellular activity and they bind to and activate the G-protein coupled receptors, CB1R and CB2R [18]. However, they may also interact with other receptors such as GPR55, GPR18, the serotonin receptor, 5-HT2 and even transient receptor potential channels (TRPV1) channels [19][20]. CB1Rs and CB2Rs are metabotropic receptors that are intracellularly coupled to the Gi/o subset of G-proteins, and their activation leads to the downregulation of cAMP levels [21][22]. CB1Rs are mainly localized to the CNS, while CB2Rs are

primarily found outside of the CNS, associated with the immune system, the reproductive system and the digestive system [23]. Anandamide is thought to be synthesized via N-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD) and metabolized by fatty acid amide hydrolase (FAAH). 2-AG is synthesized via the action of diacylglycerol lipase (DAGL) and is metabolized via monoglyceride lipase (MAGL) (**Fig 1.1**).

The eCB signalling pathway differs considerably from that of other neurotransmitter signalling pathways, such as for cholinergic, aminoacidic, and monoaminergic neurotransmission [16]. During classic neurosignalling, neurotransmitters are synthesized presynaptically and are released into the synaptic cleft upon the arrival of action potential at presynaptic neurons [24]. However, eCBs are synthesized and released from the*postsynaptic* compartments and act in a retrograde manner on CBRs that are located presynaptically. It allows the eCBs to act as a feedback mechanism to restrain presynaptic neurons from releasing neurotransmitters (**Figure 1.1**)[25]. Ligand binding studies showed that anandamide is capable of inhibiting adenylate cyclase activity in membranes that express CB1Rs [26][27], but it shows significantly less efficacy at CB2Rs, suggesting that anandamide has differential actions on CB1 versus CB2 receptors. In contrast, 2-arachidonoylglycerol (2-AG) appears to be a full agonist at both CB1Rs and CB2Rs [28].

### 1.2.1Cannabinoid receptors and their relevance

The activation of CBRs mainly leads to an inhibition of adenylyl cyclase and voltage-gated calcium channels (VGCCs) activity. CBR agonism can also activate mitogen-

activated protein kinases (MAPK) and inward potassium channel rectifiers. Therefore, activation of CBRs can substantially impact cellular functions, including synaptic activity, cell motility and, gene transcription [29]. Both CB1R and CB2R are coupled to $G_{i/o}$ and activation of different MAPK isoforms, such as extracellular signal-regulated kinase, ERK1, ERK2, p38 MAPK and Jun N-terminal kinases (JNKs) or inhibit AC and cyclic AMP-PKA signalling [30].

CB1Rs are highly expressed in axon terminals and pre-terminal axon segments in certain CNS areas, especially in the cortex, basal ganglia, hippocampus and cerebellum [31][32]. Outside the CNS, CB1Rs are present in peripheral tissues in vasculature, heart, bladder and intestine. In chicks and mice, CB1R protein expression first occurs before neuronal development [33] and increases in a region-specific manner [34]. Whereas CB2Rs are present at a lower number in CNS, but are found to be primarily associated with microglia and resident macrophages [35][36]. Reports suggest that the deletion of CB2Rs can increase the risk of schizophrenia onset; however, a diversified role of neuronal or microglial CB2R is not fully elucidated yet[37][38]. CB2Rs have been found to be highly inducible following injury or inflammation, where their expression can increase up to 100 fold [39]. Similar to CB2Rs, CB1 receptor can get up-regulated during cardiovascular dysfunctions favouring disease progression (Montecucco F 2012).Altogether, CB receptors are considered to be responsible for maintaining a delicate balance between cellular and physiological functions to ensure a normal growth during early development.

## 1.2.2 Endocannabinoids (eCBs) and their physiological functions

eCBs and eCB-like compounds mediate their underlying actions largely through CB1R compared to CB2R. Although the major distinctive features among AEA and 2-AG have already been identified, their full spectrum of actions are not well understood [40].

One of the major disparities among the major eCBs is AEA and 2-AG modulation of synaptic plasticity. Electrophysiological studies revealed that 2-AG is one of the primary mediators of CB1-induced synaptic plasticity [41][42][43]. AEA regulates long term depression (LTD) via acting on presynaptic CB1R [44] and postsynaptic TRPV1 [44][45].

Another distinctive feature between eCBs is the existence of their selective catabolic and metabolic enzymes, which make it possible to preferentially target AEA and 2-AG synthesis and degradation with pharmacological tools. Furthermore, differential localization of the enzymes at the cellular level offers control over eCB specific activity. Researchers are now showing a high interest in evaluating the consequences of AEA or 2-AG depletion or accumulation during development by perturbing their synthesis or degradation pathway [16].

It is well known that cannabinoids (CBs) can bind both CB1R and CB2R; however, most of the neurobehavioral effects of CBs are due to their binding with CB1Rs [46]. Direct activation of CB1R by exogenous cannabinoids offer beneficial activities, such as during analgesia, but can also exhibit undesirable side effects, like locomotor and cognitive impairment [47][48]. Recently, to avoid unwanted psychotropic side effects, indirect manipulation of eCB pathway becomes more appealing to target pain management and other CNS related problems [49].

### 1.2.3 eCB signalling role during development

Endocannabinoids, their receptors and the enzymes that produce and degrade them are highly expressed in cerebellar cortex in humans and rodents. The eCB signalling pathway is also important for normal development of cerebellum in perinatal mouse. The same study also showcased an association of the eCB system with neurodevelopment of forebrain and midbrain [50]. Endocannabinoids can efficiently facilitate neuronal [51] and glial differentiation [52]and they determine neurite extension and growth cone development [51][53]. CB1Rs regulate axonal fasciculation of brain cortices [54], and CB2Rs promote differentiation of oligodendrocytes [55].

Expression of the AEA synthesizing enzyme NAPE-PLD, increases in rats during the early post-natal period [56], and thus, an increased endogenous accumulation of AEA is present during that period [57]. Considering the developmental expression pattern of CBRs in rodents, CB1Rs are highly present in young animals, and decrease with age[58][59]. In monkeys, CB1Rs are highest at 1 week of postnatal development that drops for the following 2 months and stays unchanged until they reach adulthood [60]. Studies in humans ranging from early life to 50 years have shown that expression of CB1Rs but not CB2Rs slowly decreases over time [61]. Expression of MAGL enzyme mRNA also remains high during early life [62] and is highly localized with presynaptic CB1Rs [63]. Importantly, pharmacological manipulation of FAAH and MAGL enzymes was successful in prolonging eCB bioavailability [64]. Research has found that plasma AEA levels can be a higher risk factor that leads to miscarriage during pregnancy[65]. As similar to AEA, 2-AG can also be present in uterus and blastocytes to help with embryo implantation [66].

## 1.3 Zebrafish as ananimal model

Zebrafish (*Danio rerio)* has become an important vertebrate models to study brain development and disease progression *in vivo*[67]. The key advantages for using zebrafish are rapid embryonic development which occurs outside of the mother in an egg chamber, or semi-transparent chorion, and therefore it is easier to follow the developmental progress. Zebrafish research can be low cost and high throughput when compared with other vertebrate models. Moreover, a wide range of studies can be performed with ease and, in a very short period of time. Since fetal development does not require a maternal-placenta interaction, the concentration and time course of a drug exposure studies in zebrafish can be accurately controlled. It has been estimated that testing a drug in rodent models can be 50 times more costly compared with zebrafish assays. Furthermore, zebrafish studies often allow the flexibility to complete the studies in days rather than weeks or months [68].However, one of the main disadvantages is the zebrafish egg chorion can possibly interfere with the permeability of drugs during their exposure (Shaukat Ali 2011).

Zebrafish are native to the southern parts of Asia and are known to mainly inhabit slow moving river water near rice fields [69]. Zebrafish are relatively smaller in size (<120 mm length), and a light to dark stripes are present in their skin [69]. Zebrafish offspring reach their sexual maturity by the age of 3 months, and an adult female is capable of laying a couple of hundred eggs at a time. Egg size is roughly around 0.7 mm and transparent at the time of fertilization. However, most of the major organs are developed within 2 days post fertilization (dpf) (**Figure 1.2**), and by day 5, zebrafish larvae show food-seeking behaviour [69][70]. All the stages and important events that occur in zebrafish during embryonic development are briefly studied by Kimmel *et al.*

*1995* (**Figure 1.2**). The transparency of zebrafish during embryonic stages makes it possible to closely observe the development simply by using microscope or immunostaining technique. Zebrafish embryos can be easily raised and maintained in a laboratory environment at 28.5º C for consistent development. Also, zebrafish genome sequencing has revealed a genetic composition that is similar to higher vertebrate models. Therefore, zebrafish can be a very simple yet powerful animal model to investigate cellular and physiological functions during embryogenesis.

### 1.3.1 Zebrafish embryonic developmental stages

Zebrafish embryogenesis is separated into seven different stages: zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching(Figure 1.2) [70]. The zygote period represents newly fertilized eggs and is followed by the blastula stage. The blastula is present between 2.25 hpf to 5.25 hpf when cell divisions and epibol1y start. Epiboly continues with the initiation of gastrula (5.25 hpf- 10 hpf). Muscle and notochord precursors start to develop and the tail bud forms at the end of the epiboly [70]. The next stage is defined as segmentation (10 hpf-24 hpf) when somites develop, the embryos elongate and tail buds become more prominent. Skeletal muscles arise from somites [71] and at around 16 hpf, eyes are formed and brain subdivisions rising from neuromeres can be distinguished.

Mauthner neurons, one of the important reticulospinal neurons which play a key role in the C escape response in zebrafish, start receiving synaptic inputs from sensory neurons, like Rohon beard (RB) cells at around 18 hpf. Mauthner neurons then synapse on spinal primary motor neurons [72][73]. At 19 hpf, primary motor neurons extend

axons into the periphery, which then contact muscle fibres and form synapses with target cells to ensure a robust and coordinated spontaneous muscle contraction [72][73]

In the pharyngula (24-48 hpf) stage, the tail continues to elongate, no longer curved, and pigmentation occurs in retinal epithelium. Around 27 hpf, embryo can respond to touch stimuli by exhibiting a swimming escape pattern. Zebrafish embryos hatch at around 48-72 hpf, and by this time more pigmentation occurs. By the end of the 3 dpf, most embryos have hatched and are referred to as larvae [70].

### 1.3.2 Zebrafish motor neurons

Zebrafish have two different kinds of motor neurons based on the origin, location and innervation type, primary motor neurons (PMN) and secondary motor neurons (SMN). PMNs are born earlier around 9-16 hpf and are larger in size (11.3 ± 1.4 μm) compared to SMN. SMNs first appear 5-6 hours after the growth of first PMNs, and the soma size of SMNs are smaller, around 6.7 ± 1.0 μm in diameter [74]. The motor neurons are highly organized, where each myotome presents a similar organizational pattern of PMNs and SMNs. Each segment generally possesses 3 to 4 PMNs and approximately 20-24 SMNs.

The PMNs are categorized into three groups- caudal (CaP), middle (MiP) and rostral (RoP) neurons depending on their location in the spinal cord (**Figure 1.3**) [74]. Ca PMN growth cones exit the spinal cord at 17 hpf and grow ventrally along the horizontal myoseptum [75]. MiP MNs are located between CaP and RoP cell bodies, and their growth cones initially follow the path of CaP MNs. However, MiP MNs then extend caudally and dorsally to reach the dorsal myotome and never extend beyond

horizontal myoseptum. RoP MNs are located at the rostral end of the hemi segment, and the growth cones caudally exit the spinal cord. RoP growth cones follow MiP neurons to reach horizontal myoseptum.

Secondary motor neurons (SMNs)- dorsally projecting (dS), ventrally projecting (vS) and dorsoventrally projecting (dvS) MNs are classified based on their muscle innervation pattern [76]. dS MNs innervate dorsal muscles of the cell bodies while vS MNs are located dorsally to dS and innervate ventral muscles only. In contrast, dvS MNs are the largest SMNs and normally can branch toward both dorsal and ventral axons.

When leaving the spinal cord, SMNs can form nerve bundles and follow the path already set by PMNs to synapse at muscle fibers [77]. Findings also identified several key axonal guidance molecules, sema3A1, sema3A2, GDNF, neurolin, which can possess significant roles during primary and secondary motor neuronal growth [76].

### 1.3.3 Locomotion in embryonic zebrafish

Development of NMJ components are indispensable in establishing the locomotor pathway in organisms. In zebrafish, two distinguishable patterns of locomotion, a slower swimming behaviour and a rapid C escape movement can be seen from the very early stages. Both types of locomotion behaviours can be easily traced and quantified in zebrafish larvae. The type of locomotion changes during various stages of development [78]. Locomotor responses first develop with spontaneous tail contractions at 17 hpf, and by 21 hpf, embryos show responses to touch. Although swimming starts at 27 hpf in zebrafish, it is infrequent until they hatch. A spontaneous swim movement is seen

only after swim bladder development at 5 dpf[79]. Different locomotor behaviours require a proper development of neural and neuromuscular circuits in an orderly fashion. nAChR expression at NMJs can be detected at around 17 hpf, particularly when primary motor neurons innervate muscle fibers. It is a clear indication of a link between NMJ development and embryo coiling at 17 hpf. Starting from 27 hpf, embryos can exhibit a full coiling response followed by a brief swimming and correction of their orientation to complete escape response [78]. Also, the touch response that occurs after touching either the posterior or anterior portions of the fish can determine the activation of different sensory circuits, such as head stimuli activate trigeminal neurons and stimulus at tail can activate Rohn Beard neurons [79]. However, touching the head or the tail can both activate M cells, which are the largest reculospinal interneurons located in hindbrain (Kimmel 1981). The pattern of swimming changes remarkably by day 5 when both spontaneous swimming (slow response and short distance) and escape behaviours (fast response and long distance) are well developed. Zebrafish larvae use red muscle fibers during slow swimming and use both red and white muscle fibers to complete fast swimming [80].

### 1.3.4 Zebrafish as an animal model to study eCB role during development

Zebrafish is a well-established vertebrate animal model in current research, and numerous studies have already benefitted from this model while investigating cannabinoid biology. Zebrafish possesses the key components of the eCB system, CBRs and other eCB related genes, and the functional genes linked with addiction, development, anxiety, immune functions, learning and memory [81]. Several studies

have investigated the effects of the main components of cannabis extracts, THC and CBD, on adult and larval zebrafish models of nociception, epilepsy and anxiety[82][83]. Over the last couple of decades, zebrafish models have successfully been used to test the efficacy of drugs, their toxicology and effects on development.

The zebrafish eCB system shares a genetic homology of mammals. Zebrafish CB1Rs sequencing reveals a 69% nucleotide and 73.9% amino acid identity similar to human [84]. In zebrafish,CB1R expression occurs as early as the 3 somite stage and by 48 hpf, can widespread into preoptic area, dorsal telencephalon, tegmentum, anterior hindbrain [84][85]. Zebrafish express two CB2R orthologs that show 98% genetic similarity between them and 39% amino acid similarity with human CB2Rs [86]. Although CB2R expression is low in brain area, it is present highly in intestine, retina, gill, muscle, spleen. The genes responsible for eCB synthesis and degradation, including NAPE-PLD, DAGL, FAAH and MAGL are also expressed at a very early age of around 1 hpf [85]. These enzymes are distributed widely in brain, reproductive area, muscle, eye, heart and intestine [85].

It is reported that the eCB system contributes to brain development through axonal guidance, myelination, cell proliferation and synaptic growth [87]. During early development, CB1R and DAGL have been found to be expressed at around the same time, confirming the larval ability to synthesize and perform 2-AG actions [88]. Importantly, CB1R morpholino knockdown can result into aberrant axon formation [88], and similar to that DAGLα knockdown was also found to exhibit axonal deformities in midbrain-hindbrain area and differential behaviour patterns. It has been suggested that 2-AG can regulate axon formation and can control movement perception during development [89]. Another study has found the role of eCB signalling in liver

development and function in zebrafish embryos [90]. However, not much is known about the specific roles AEA plays in zebrafish during embryonic development, although the metabolic enzymes of AEA are present from the beginning of birth. Together, these findings show that the eCB system plays important roles in early embryonic development and that the components of eCB signalling remain an important avenue that can be explored further to better understand their roles in developmental aspects.

## 1.4 Research objectives and hypothesis

The main purpose of this study is to examine the roles of eCB signalling pathway into the development of neuronal systems related to locomotor aspects in zebrafish embryos. I will discuss how a perturbation of eCB components can affect embryogenesis in zebrafish resulting in alterations of their morphology, motor neuron morphology and locomotion. Although previous studies performed a wide range of investigations on the functional physiology of cannabinoid receptors (CBRs), there is still a scarcity of information available on discerning a comprehensive role played by CB1Rs and CB2R. My study will extend further looking into the metabolic enzymes of eCBs (AEA and 2-AG), particularly the degrading enzymes, FAAH and MAGL. I will attempt to uncover the roles played by both FAAH and MAGL enzymes, by applying their pharmacological inhibitors, JZL 195, URB 597 or JZL 184. I will also determine if cells involved in locomotion are affected or any developmental delay occurs during motor neuron growth upon FAAH and MAGL inhibition in embryos. To find out if an increased bioavailability of AEA and 2-AG are responsible for exerting the detrimental effects and acting through eCB signalling pathway, I will preferentially block CB1R or

CB2R from 0-24 hpf. The study will help us to unravel if the increased AEA and 2-AG underlying effects are CBRs mediated, or they might also work CBR independently.

Therefore, in this study, I sought to investigate questions regarding the role of the eCB during embryogenesis. My three specific aims are the following:

*Aim 1*: To explore the roles of cannabinoid receptors (CBRs) in first 48 hrs of development to determine locomotion in zebrafish.

*Aim 2*: To differentiate the roles of CB1R and CB2R in early development of zebrafish motor neurons and locomotion.

*Aim 3*: To investigate the functional roles of endogenous AEA and 2-AG toward motor neuron and locomotor development via CBRs.

***I hypothesize*** **that pharmacological inhibition of CBRs for the first 48 hrs of fertilization upon application of the pharmacological inhibitors, AM 251 and AM 630 will affect zebrafish embryo development in a dose dependent manner**. Despite some general roles played by CBRs during locomotion, it is also quite important to understand the distinguishable features of CB1R and CB2R. Since a distinctive embryonic expression pattern exists between CB1R and CB2R gene, ***I hypothesize*** **that CB1R and CB2R will play differential role during developmental stages. Therefore, it is highly likely that CBR inhibition at various selected time points will diversely affect motor neuron growth and development of locomotor movement**. Not only CBRs but also eCB catabolic enzymes, FAAH and MAGL inhibition will also alter embryo morphology, and motor neuron growth and locomotion. **Therefore,** ***I also hypothesize*** **that FAAH and MAGL interruption from 0 to 24 hpf will disrupt motor neuron and locomotor development by**

**elevating AEA and 2-AG. And also, an inhibition of CB1R or CB2R will attenuate AEA and 2-AG mediated actions.**

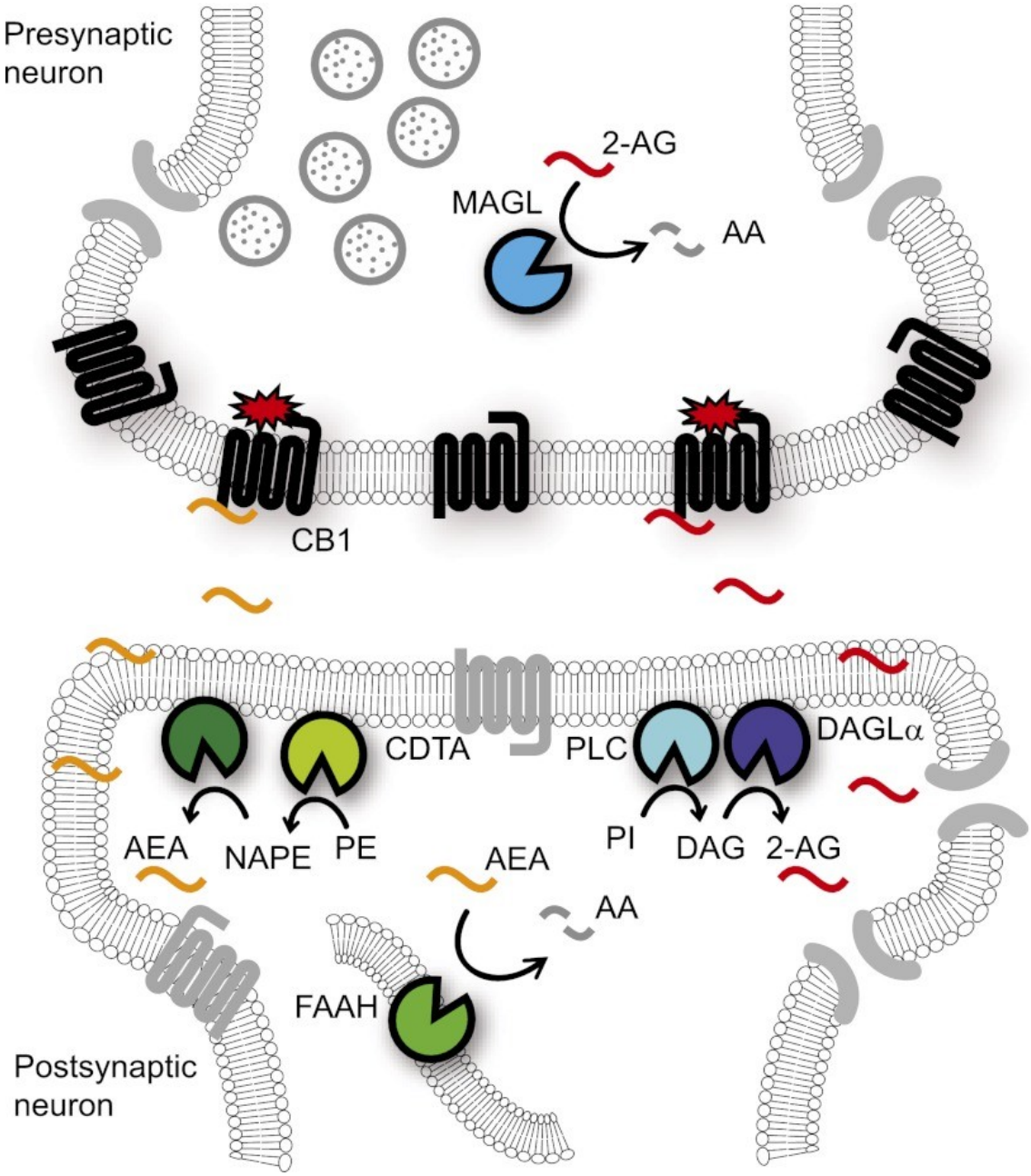
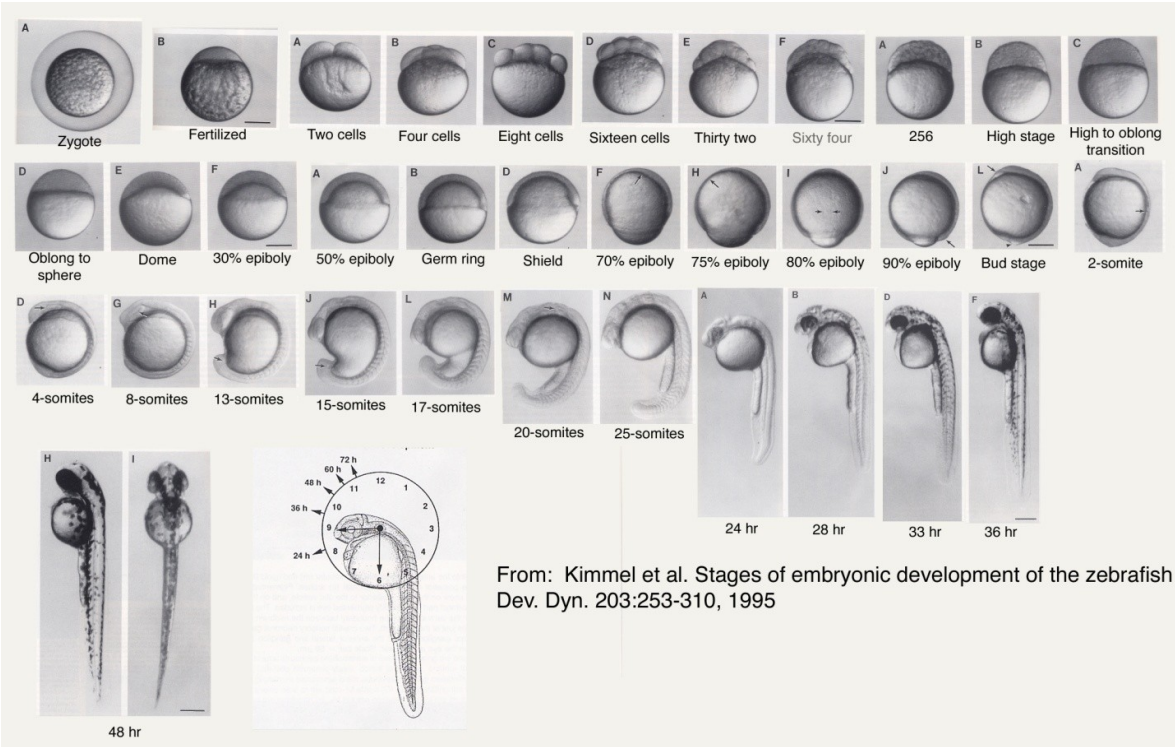


**Figure1.1. A Schematic showing endocannabinoid synthesis and metabolism by Blankman JL *et al2013*** [16]**.** The eCBs, AEA and 2-AG are both synthesized in postsynaptic neurons. NAPE acts as a precursor for AEA synthesis by NAPE-PLD. However, NAPE generation through calcium-dependent transacylase (CDTA) enzyme is not well characterized yet. 2-AG synthesis occurs via the action of DAGL from phosphatidylinositol (PI) precursor. Following a rapid synthesis, eCBs enter synaptic cleft and can act on presynaptically located CBRs. CBR activation enhances $G_{i/o}$ signalling, which can lead to the inhibition of neurotransmitter (NT) release from presynaptic neurons. In the final step, AEA is rapidly metabolized by post-synaptically located FAAH enzyme, whereas 2-AG metabolism occurs by pre-synaptically located MAGL enzyme. Both AEA and 2-AG metabolism can give rise to an accumulation of arachidonic acid (AA).




From: Kimmel et al. Stages of embryonic development of the zebrafish Dev. Dyn. 203:253-310, 1995

**Figure 1.2 Different stages of embryonic development in zebrafish as depicted in Kimmel *et al* 1995**[70]. The diagram shows key stages of development during embryogenesis and each stage highlights the changing spectrum that takes place during first 48 hours of development. Live embryos were analyzed using a dissecting microscope to distinguish the morphological features occurring at each step. The transparent feature of live embryo makes it accessible to examine deeper structures and characterize each event solely by using a compound microscope.

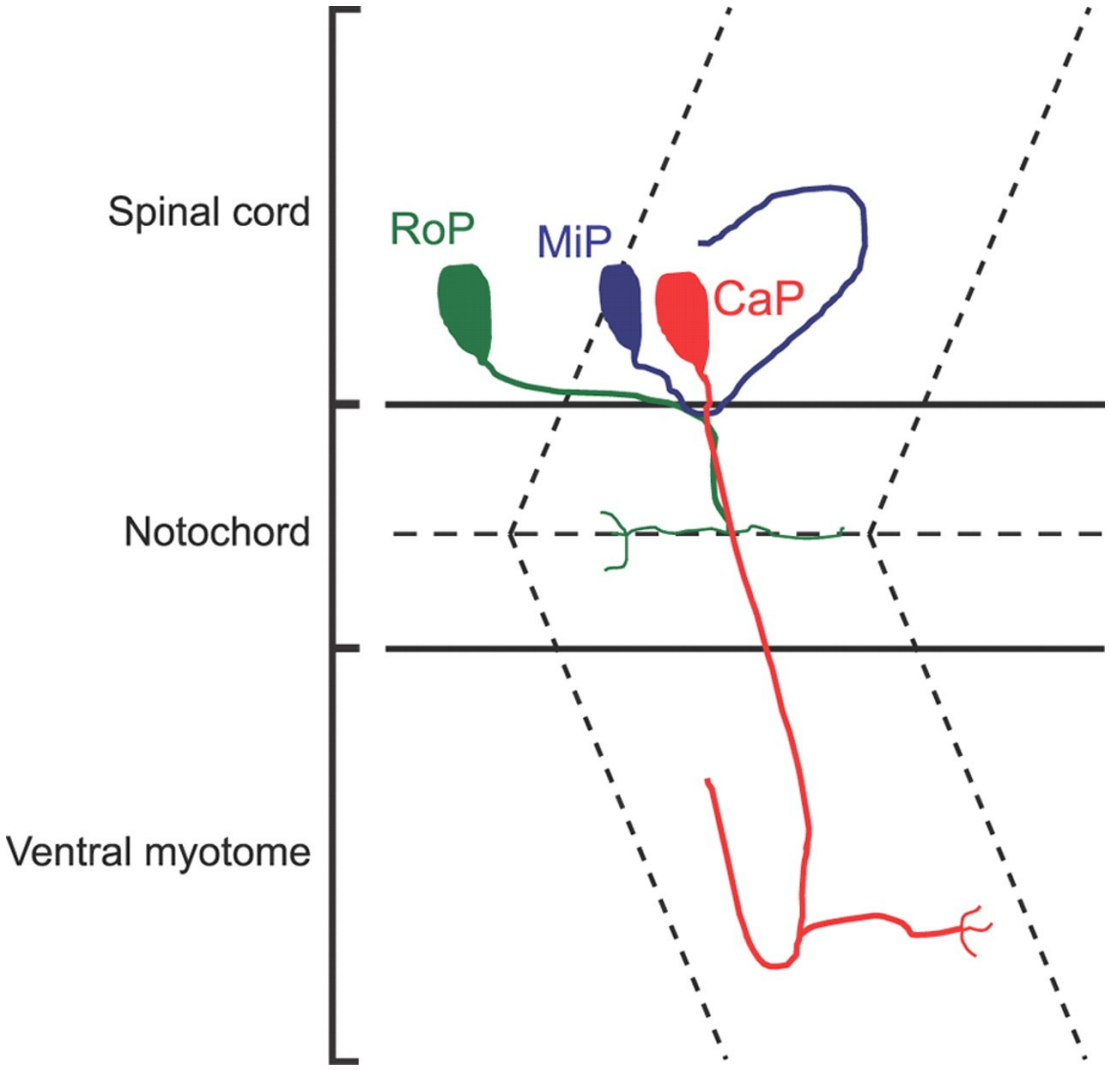

Spinal cord
RoP
MiP
CaP
Notochord
Ventral myotome

**Figure 1.3.Schematic illustration displays spinal cord primary motor neuron as depicted in Myers *et al* 1986**[74]**.** The diagram shows three different stereotypical primary motor neurons, CaP (red), MiP (blue) and RoP (green) at 48 hours post development. The drawing represents a lateral view of spinal cord, notochord and ventral myotome. Also, it shows the axonal trajectories of one CaP, one MiP and one RoP neuron coming from an individual hemisegment. The dashed horizontal line represents horizontal myoseptum, and dashed chevrons indicate hemisegment boundaries.

# Chapter 2. Materials & Methods

## 2.1 Animal care and use

I used the Tubingen Longfin (TL) strain of wild type zebrafish (Danio rerio) in my study and all adult fish used for breeding were maintained at the University of Alberta Aquatic Facility. Animal housing and experimental procedures throughout the experiments were approved by the Animal Care and Use Committee at the University of Alberta (AUP #00000816), adhered to the Canadian Council on Animal Care guidelines for human and animal use. For breeding, 5 adult zebrafish consist of 3 females and 2 males were randomly selected and placed in a breeding tank the evening before eggs were required. The following morning, eggs were collected from the breeding tanks, usually within 30 mins of release by the females. A 12h light/dark (8am lights on/8pm lights off) cycle and 28.5°C temperature was set for housing the embryos and larvae in incubators to ensure a consistent growth and development.

## 2.2Drug treatments

The compounds tested in my study are: JZL 195, URB 597, JZL 184, AM630 (Adooq Bioscience, Irvine, CA, USA) and AM 251 (Selleck Chemicals, Houston, TX, USA). Drug compounds were dissolved in dimethylsulfoxide (DMSO). The DMSO final concentration in working solutions never exceeded 0.1%. Embryos were exposed throughout egg water (EW) containing appropriate drug concentration.

For the receptor antagonist studies (Chapter 3), embryos were exposed to CB receptor blockers AM251 (0.05 μM to 5 μM) and AM630 (0.2 μM to 10 μM) for 24 hours, at

which point the embryos were washed and then incubated in the antagonist solution for another 24 hours to complete the 48-hour incubation period.

In a separate study in chapter 4, I studied the distinctive properties of CBRs on motor neuron development and locomotion. Here, embryos were exposed to the CB receptor antagonists AM251 0.5 μM (Selleck Chemicals, Houston, TX, USA) or AM630 5 μM (Adooq Bioscience, Irvine, CA, USA) diluted in egg water (60 μg/ml Instant Ocean) either from 0 to 24 or 24 to 48 hpf. The experimental dose was selected after testing a range of concentrations of AM251 (0.05–5 μM) and AM630 (0.2–10 μM) and also were used in previous zebrafish studies.

And, in the last part of my study, I evaluated the actions of the FAAH and MAGL inhibition in development (Chapter 5).We exposed the embryos in egg water (EW) containing a selective or dual inhibitor of FAAH and MAGL enzyme (JZL 195, dual FAAH/MAGL inhibitor; URB 597, FAAH inhibitor; and JZL 184, MAGL inhibitor) or co-exposed with CB receptor inhibitors (AM 251: CB1 receptor antagonist; AM630: CB2 receptor antagonist), during their first 24 hours post-fertilization (0-24 hpf). The selected dose for each drug treatment in this study was chosen based upon testing a range of concentrations to avoid any toxic effect (data not shown). However, the concentrations are also in close range to previous literature [91][92][89][93][94][95][96].

Fresh solutions were prepared on the day of the experiment immediately before use. The drug stocks were diluted using egg water (EW; 60μg/ml Instant Ocean). After antagonist exposure, eggs were washed several times with egg water to remove the drugs and were then kept in normal egg water. The egg water was changed every morning until further experimentation at 2 or 5-days post fertilization. For

immunohistochemical studies, pigmentation was blocked by a single time application of phenylthiourea (PTU) at an optimal concentration of 0.003% into egg water at 24 hpf [97].

## 2.3 Embryo imaging and morphological observations

Photographs of embryos and larvae were taken using a Lumenera Infinity2-1R color camera mounted on a Leica DM2500 stereomicroscope under 2.5X (embryos or larvae full-length images) or 5X (swim bladder at 5 dpf) magnification. Embryos were placed in a 16-well plate with a single embryo per well and were anaesthetized using 0.02% MS-222. To obtain body length measurements, embryos were imaged with a Leica dissecting microscope, and then the images were analyzed offline using ProAnalyst software (Xcitex, Woburn, MA, USA). For heart rates, quantification was performed offline using video recordings of embryonic heart activity for a continuous 30 s and then multiplying it by a factor of 2.

## 2.4 Immunohistochemical staining

Treated embryos were manually dechorinated at 2dpf and then fixed with 2% paraformaldehyde (PFA) for approximately 2 hr. After fixation, samples were washed every 15 mins for 2 hr in 0.1 M phosphate buffer saline (PBS; 150 mM NaCl, 8 mM $Na2HPO_4$, 2 mM $NaH_2PO_4.2H_2O$, and pH 7.2). Embryos were then permeabilized using 4% Triton X-100 containing 2% Bovine Serum Albumin (BSA) and 10% normal goat serum (NGS) for 30 mins. Sample preparations were then incubated at 4ºC with their corresponding primary antibodies for 48 hrs.

Primary motor neurons and secondary motor neurons were visualized using mouse monoclonal anti-zebrafish Znp1 and Zn-8 (DSHB; Developmental Studies Hybridoma Bank) primary antibodies, respectively. Anti-znp1 (1:250, University of Iowa, deposited by B. Trevarrow) can target synaptotagmin-2 isoform, a protein highly localized in primary motor axons [98][99], whereas anti-zn8 (1:250, University of Iowa, deposited by B. Trevarrow) can target DM-GRASP, a protein highly present in the surface of secondary motor axons [100][101]. After 48 hrs primary antibody incubation, whole body embryo tissues were washed in PBS every 15 min for 3 hrs. After washes, samples were incubated in Alexa Fluor ® 488 goat anti-mouse IgG (1:1000, Molecular Probes, Life Technologies) secondary antibody for 4 hrs at room temperature. For nAChRs labelling, embryos were incubated with Alexa-488 conjugated α-bungarotoxin (100 nM, Molecular Probes, Invitrogen) for 4 hrs at room temperature.

Embryos were then washed with PBS every 30 mins for 7 hr and mounted in MOWIOL mounting media. A confocal Zeiss LSM microscope was used to take immunofluorescent images of embryos using a 40X objective. Multiple image stacks were photographed every 1μm z-section throughout the entire thickness of the embryo. While counting primary, secondary motor axon branches, the number and the largest size of α-bungarotoxin puncta, image stacks were compiled and tracked using Image J software (National Institutes of Health, Bethesda, MD, USA). For znp1 staining, primary, secondary and tertiary branches emanating from a single (middle) primary motor axon per sample were counted. During zn8 staining, the percentage of normal (non misshapen or non truncated) axonal branches emanating from three secondary motor axons per sample were counted and averaged. For α-BTX staining, the total

number and the largest size of puncta (per 2500 μm$^2$ area) were counted and averaged from three different boxes that were evenly placed (1 in dorsal, 1 in middle, and 1 in ventral).

## 2.5 Locomotor activity measurements

To track locomotor activities, individual 5 dpf larvae were placed in a single well of a 96-well plate and then video-recorded. The data were analyzed following previously published procedures [102][103]. Larvae were gently positioned in the centre of wells containing 150 μl egg water, pH 7.0 and the central 48 wells were used from a 96-well plate (Costar 3599). Prior to video recording, larvae were acclimated in the well plate for 60 min. Plates were placed on top of an infrared backlight source, and a Basler GenlCaM (Basler acA 1300-60) scanning camera with a 75 mm f2.8 C-mount lens (Noldus, Wageningen, The Netherlands) was used for individual larval movement tracking. EthoVision® XT-11.5 software (Noldus) was used to quantify activity (%of time active over 1h time period), velocity (mm/s, average velocity from active time period), swim bout frequency and cumulative duration of swim bouts for 1 h. To exclude background noise, ≥0.2 mm was defined as an active movement. The %of pixel change within a corresponding well between samples were defined as activity (motion was captured by taking 25 frames/s ) as reported previously [103].

## 2.6 FAAH and MAGL enzyme activity assay

An *in vitro* 96 well plate-based assay was carried out to assess FAAH and MAGL enzyme activity. Enzyme inhibitor screening assay kits were purchased from Cayman

Chemical (Ann Arbor, USA) to measure the enzyme activities. The assay was run following the manufacturer's protocol on protein extracts collected from treated embryos at 24 hpf.

The FAAH enzyme assay measures the fluorescence from 7-amino-4-methylcoumarin (AMC), upon FAAH enzyme cleavage of the non-fluorescent substrate, AMC-arachidonamide (AMC-AA) [104]. Fluorescence was detected and quantified at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. For MAGL enzyme assay, the substrate 4-nitrophenylacetate was hydrolyzed by MAGL enzyme yielding a yellow product, 4-nitrophenol, and the reading was recorded at an absorption wavelength of 410 nm.

Each well contained a reaction mixture of 30µg embryo protein extract, the enzyme assay buffer (either FAAH assay buffer: 125mM Tris-HCl with 1 mM EDTA, pH 9.0, or MAGL assay buffer: 10 mM Tris-HCl with 1 mM EDTA, pH 7.2), and was incubated for 5 minutes at 37ºC. Final reactions were then initiated by adding either 10 µl of FAAH or MAGL enzyme substrate (AMC-AA; 400µM or 4-nitrophenylacetate; 4.25 mM) and incubated for another 30 mins at 37ºC before plate reading. Control experiments were run every time with all other treatment groups. Control samples contained protein extracts collected from untreated embryos or human recombinant FAAH or MAGL enzyme. Also, blank wells were run each time to monitor any background fluorescence from each reading. All the sample reactions were run in triplicates.

## 2.7 Statistical analysis

GraphPad Prism software (Version 7, San Diego, CA) was used to perform all the data analysis and the values were reported as means ± SEM (standard error of the mean) here. For statistical analysis, one way ANOVA followed by Dunnett'sor Tukey's post-hoc multiple comparison test was run to determine the significance of the results ($p<0.05$). Dunnett's multiple comparisons test was used to identify the significance between every treatment group with the control. Tukey's pos-hoc test was chosen while comparing every treatment group with every other one. For locomotion experiments, outliers due to off tracking (undetected larval tracing) were identified using ROUT at Q=0.1 (Q = maximum desired false recovery rate) and removed objectively.

# Chapter 3. Results: Role of CBRs in Zebrafish embryo development and locomotion

In this study, I set out to determine if inhibition of the prototypical eCB receptors during the early stages of zebrafish development would alter normal development. To do this I used CB1R and CB2R antagonists to block the receptors during the first 48 hours of zebrafish development and examined the effects on factors such as overall morphology, heart rate, survival in the first 5 days of life, the extent to which hatching from the egg casing occurred and episodes of locomotion or swimming in 5 day old larvae. My results suggest that the CB1 and CB2 receptors have roles in early organismal development and can implicate the eCBs in a variety of developmental programs.

## 3.1 Gross morphology

The goal of my study was to determine the role of the eCB system in early development by pharmacologically blocking the CB1 and CB2 receptors. The effects on the gross morphology of 2 dpf embryos are shown in Figure 3.1. Treatment with the higher concentrations of AM251 and AM630 had a clear impact on embryonic morphology with animals showing smaller, malformed trunks and increased incidences of pericardial edema at 2 dpf (Figure 3.1).

Incubation with CB1 receptor blocker AM251 resulted in a dose-dependent and statistically significant reduction in hatching at 2-5 dpf. Untreated embryos and DMSO

controls hatch from the egg casing between 48 hpf and 72 hpf as expected for normal, healthy fish (Figure 3.2), whereas embryos exposed to AM251 (0.05 μM, 0.2 μM, 0.5 μM, 2 μM and 5 μM) hatched at rates of 88%, 84%, 76%, 60% and 30% respectively, by 3 dpf (Figure 3.2A; $p>0.0001$). By day 5 all embryos had hatched except for the 5 μM treated group in which the hatching rate was only 75% (Figure 3.2A) (N=5 experiments, n=20 embryos per treatment). Exposure to AM630 had a similar effect to that of AM251. For instance, embryos exposed to AM630 (0.2 μM, 0.5 μM, 2 μM, 5 μM and 10 μM) hatched at rates of 96%, 89%, 83%, 59% and 42% respectively by 3 dpf (Figure 3.2B; $p>0.0001$) (N= 5-6 experiments, n=20 embryos per experiment). Survival rates were significantly reduced in a dose-dependent manner following exposure to AM251 and AM630 (Figure 3.3). Interestingly, the effect stabilized within the first 24 hours of exposure. For instance, when treated with the highest concentration of AM251 (5 μM), the survival rate was reduced to 49% by day 1 but then remained relatively constant (46%) until day 5 despite continued incubation in the antagonist until day 2 (Figure 3.3A). This effect was essentially the same for all concentrations tested (Figure 3.3A). The effect of AM630 on survival paralleled that of AM251 except that survival rates were slightly different. For instance, 5 μM AM630 resulted in a survival rate of 62% by day 5, while 10 μM resulted in a survival rate of 34% by day 5 (Figure 3.3B) (N= 6 experiments, n=20 embryos per experiment).

To investigate gross morphology at 2 dpf, I manually removed embryos from the chorion and examined them for a number of morphological features such as body length, malformations of the trunk and pericardial edema. Treatment with the CB1 receptor blocker AM251 resulted in significantly shorter embryos, but only at the higher concentrations of 2 and 5 μM (Figure 3.4A). Statistical analysis revealed that

malformations of the trunk and instances of pericardial edema were significantly greater at 2 and 5 μM (Figure 3.4C, E) ($p>0.05$, $p>0.001$, $p>0.0001$) (N=5 experiments, n=62-96). Similar results were obtained following exposure to the CB2 receptor blocker AM630 (Figure 3.4B). Embryos exhibited shorter bodies, trunk malformations and pericardial edema, particularly when exposed to the higher concentrations of 5 μM and 10 μM (Figure 3.4D, F; $p>0.05$, $p>0.01$, $p>0.0001$) (N=5 experiments, n=57-94).

## 3.2 Cardiac activity

I also examined the heart rate of embryos treated with AM251 and AM630 (Figure 3.5). Embryos treated with AM251 had significantly lower heart rates when treated with 0.5-5 μM AM251 (Figure 3.5A; $p>0.0001$; N= 5 experiments, n=62-96) and 2-10 μM AM630 (Figure 3.5B; $p>0.0001$; N= 5 experiments, n=57-94) compared with both untreated and DMSO controls. The heart rate fell by as much as 50% at the highest concentrations used in this study. Taken together, blocking CB1 and CB2 receptors during the first 48 hours of development lead to small changes in morphological development, hatching rates and embryonic survival.

## 3.3 Locomotor assays

To determine if swim bouts were altered by treatment with AM251 and AM630, I transferred individual 5 dpf larvae to 96-well plates and video recorded their movements for an hour (Figures 3.6, 3.7). Figure 3.6A shows results obtained from a representative experiment following treatment with AM251. It should be noted that the control group (labelled CTRL) in Figure 3.6 represents untreated embryos, whereas

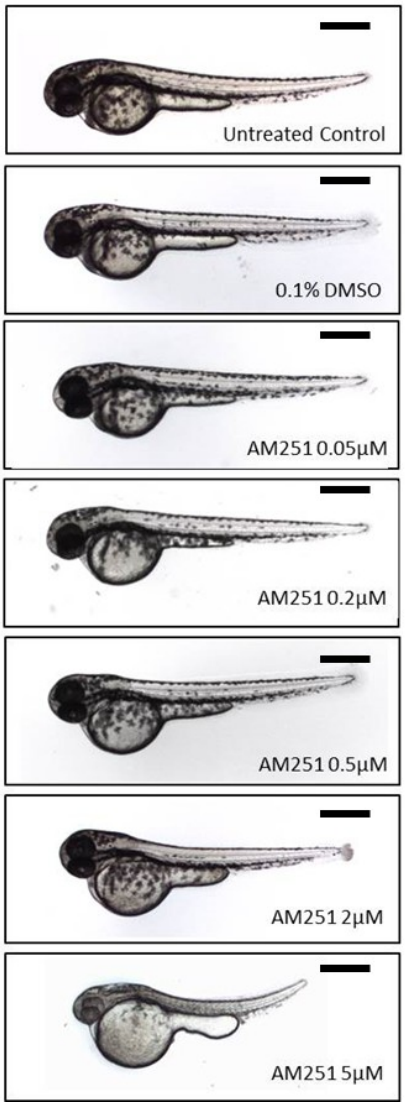
0.1% DMSO controls are shown in Figure 3.7 for simplicity. A schematic showing the movement path of 8 individual larvae per treatment, shows the variability within each group as well as the general trend towards a reduction in movement in larvae treated with higher concentrations of AM 251 (Figure 3.6A). Quantification and statistical analysis of the movement patterns is shown in Figure 6B-E. The change in activity (Figure 3.6B), the change in velocity (Figure 3.6C), the number of swim bouts (Figure 3.6D) and the cumulative duration of swim bouts (Figure 3.6E) were all significantly reduced in animals treated with 0.2-5 μM (N=3 experiments, n=22-26). In fact, the larvae ceased all detectable movement when treated with 5 μM AM251. Embryos treated with 0.2-2 μM AM630 showed no significant change in movement compared with controls, however embryos treated with 5-10 μM AM630 experienced reduced levels of activity, swimming velocity and bouts of swimming, (Figure 3.7B-E) (N=3 experiments, n=22-26).

Taken together these results suggest that inhibition of cannabinoid receptors CB1R and CB2R during the first 2 days of development altered a number of characteristics in developing zebrafish embryos including survival, hatching, morphology, heart rate and locomotion.

Table 1. Effects of CB1R and CB2R blocking for the first 48 hpf in zebrafish embryos (Chapter 3).

| Figures | Findings |
|---|---|
| Fig 3.1-3.5 | 1. AM 251 and AM 630 act dose-dependently during gross morphological studies (Rates of survival, hatching and deformities) |
| Fig 3.2-3.4 | 2. Survival and hatching rates decrease and malformation rate increases with both AM 251 and AM 630 |
| Fig 3.4-3.5 | 3. Body length reduces and heart rates get decreased with both AM 251 and AM 630 |
| Fig 3.6-3.7 | 4. Locomotion is reduced when CB1R and CB2R are inhibited. Locomotion is affected even at lower doses of AM 251 (0.2-5μM) while AM 630 act only at higher doses (5-10μM). |

A)


Untreated Control
0.1% DMSO
AM251 0.05µM
AM251 0.2µM
AM251 0.5µM
AM251 2µM
AM251 5µM

B)

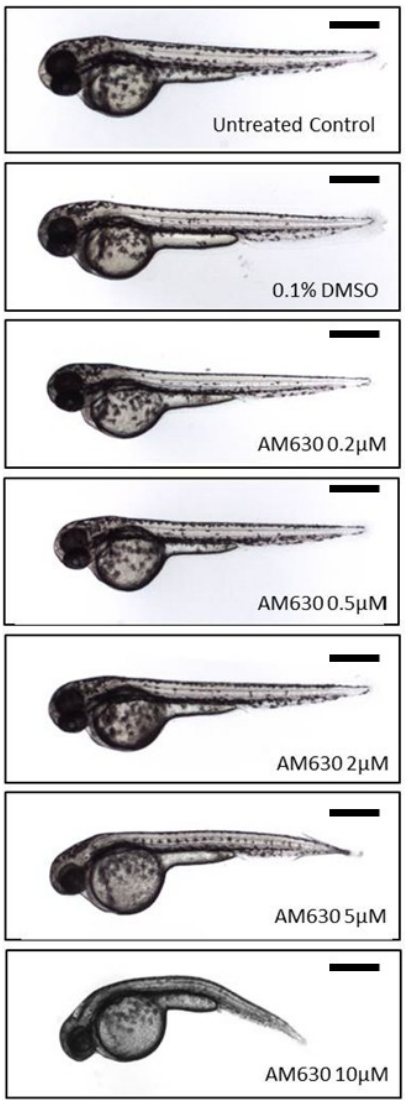
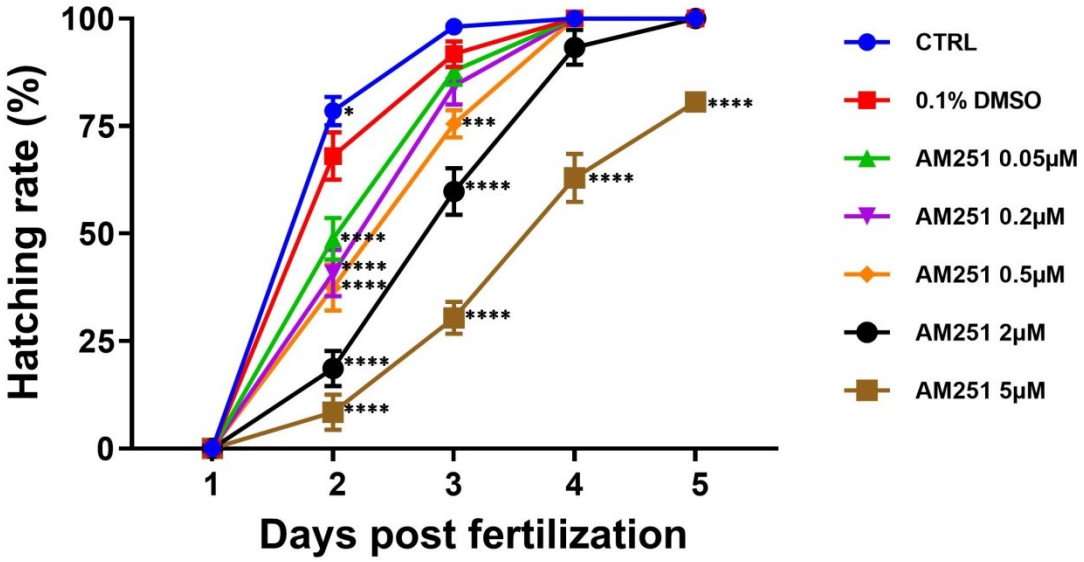

Untreated Control
0.1% DMSO
AM630 0.2µM
AM630 0.5µM
AM630 2µM
AM630 5µM
AM630 10µM

**Figure 3.1.AM251 and AM630 drug exposures on Zebrafish embryos and their subsequent effects.** Images showing embryos treated with AM251 (A) and AM630 (B) with their corresponding untreated control and vehicle control (0.1%DMSO). After 48 hours of treatment, embryos were allowed to develop in normal embryo media. Images were taken at 48-52 hpf. Scale Bar = 0.5 mm.

A)


Hatching rate (%)
100
75
50
25
0
1
2
3
4
5
Days post fertilization
CTRL
0.1% DMSO
AM251 0.05μM
AM251 0.2μM
AM251 0.5μM
AM251 2μM
AM251 5μM

B)

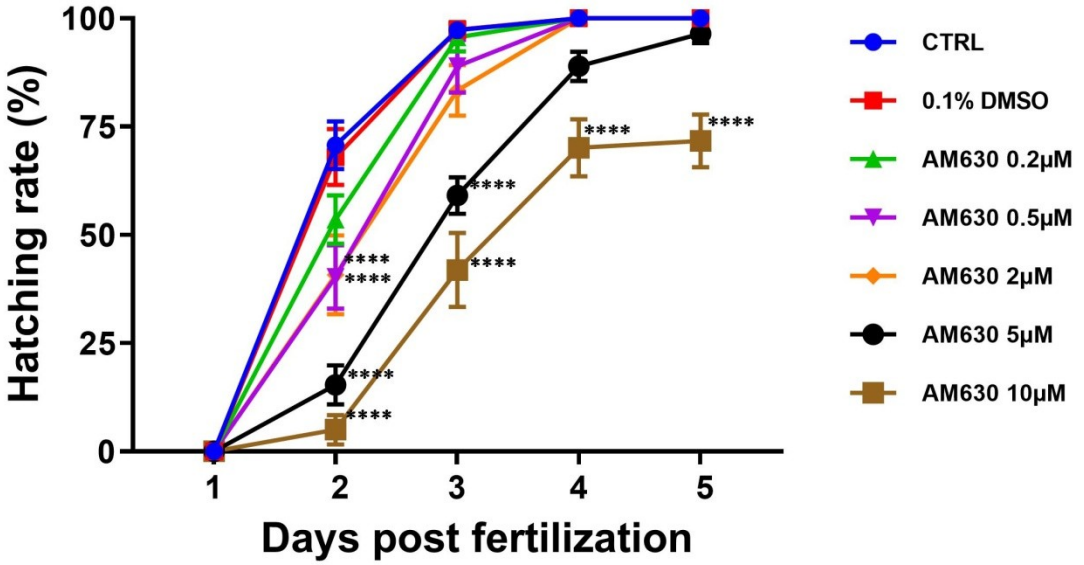
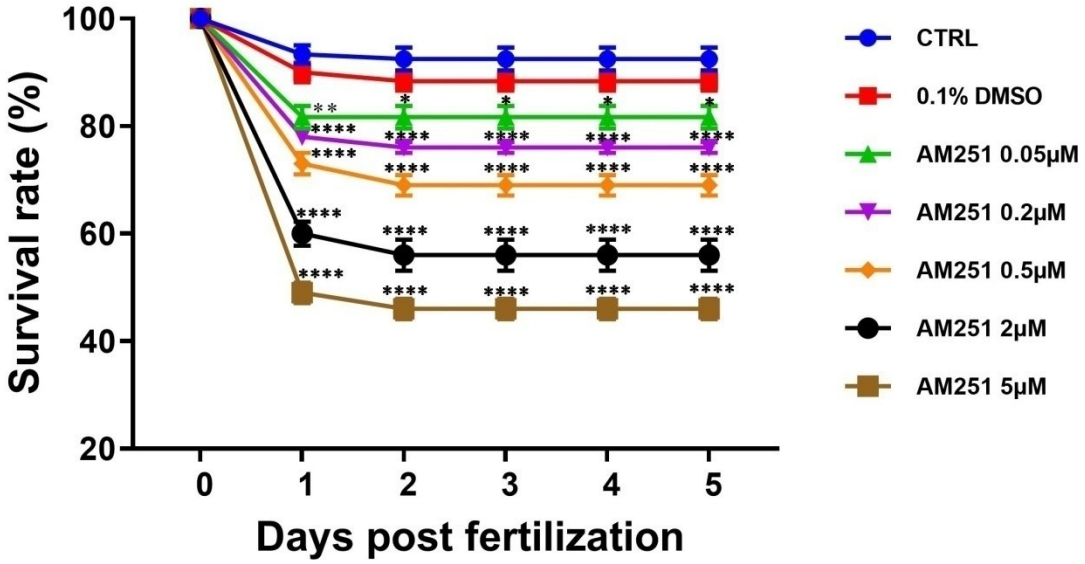

Hatching rate (%)
100
75
50
25
0
1
2
3
4
5
Days post fertilization
CTRL
0.1% DMSO
AM630 0.2μM
AM630 0.5μM
AM630 2μM
AM630 5μM
AM630 10μM

**Figure 3.2.AM251 and AM630 exposure has a dose-dependent effect on zebrafish hatching.** Hatching rate of zebrafish embryos within the first 5 days of post fertilization (dpf) after 48 hours of treatment with AM251 and AM630 (N=6 experiments and n=20 embryos per treatment) (A, B). The y-axis represents the mean ± SEM. **** significantly different from their vehicle control (0.1% DMSO) treated on the same day, $p<0.0001$, *** Significantly different from their vehicle control (0.1% DMSO) treated on the same day, $p<0.001$,* Significantly different from their vehicle control (0.1% DMSO) treated on the same day, $p<0.05$.

A)


Survival rate (%)
100
80
60
40
20
0
1
2
3
4
5
Days post fertilization
CTRL
0.1% DMSO
AM251 0.05μM
AM251 0.2μM
AM251 0.5μM
AM251 2μM
AM251 5μM

B)

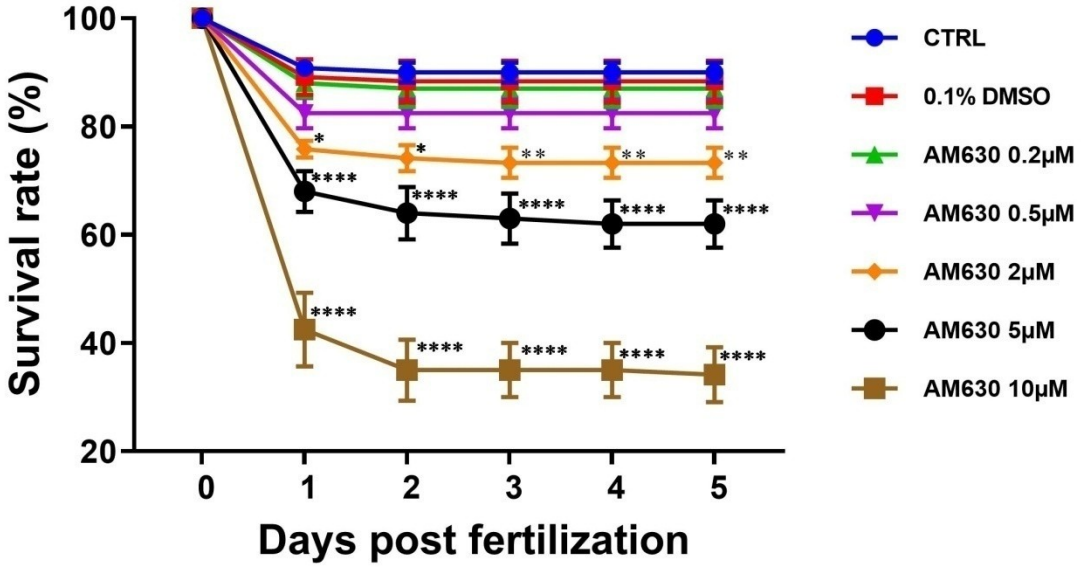

Survival rate (%)
100
80
60
40
20
0
1
2
3
4
5
Days post fertilization
CTRL
0.1% DMSO
AM630 0.2μM
AM630 0.5μM
AM630 2μM
AM630 5μM
AM630 10μM

**Figure 3.3.AM251 and AM630 exposure effect on zebrafish survival.** Survival rate of zebrafish embryos within the first 5 days of post fertilization (dpf) after 48 hours of treatment with AM251 and AM 630 (N=6 experiments and n=20 embryos per treatment) (A, B). **** Significantly different from their vehicle control (0.1% DMSO) treated on the same day, $p<0.0001$, ** represents $p<0.01$, * represents $p<0.05$.

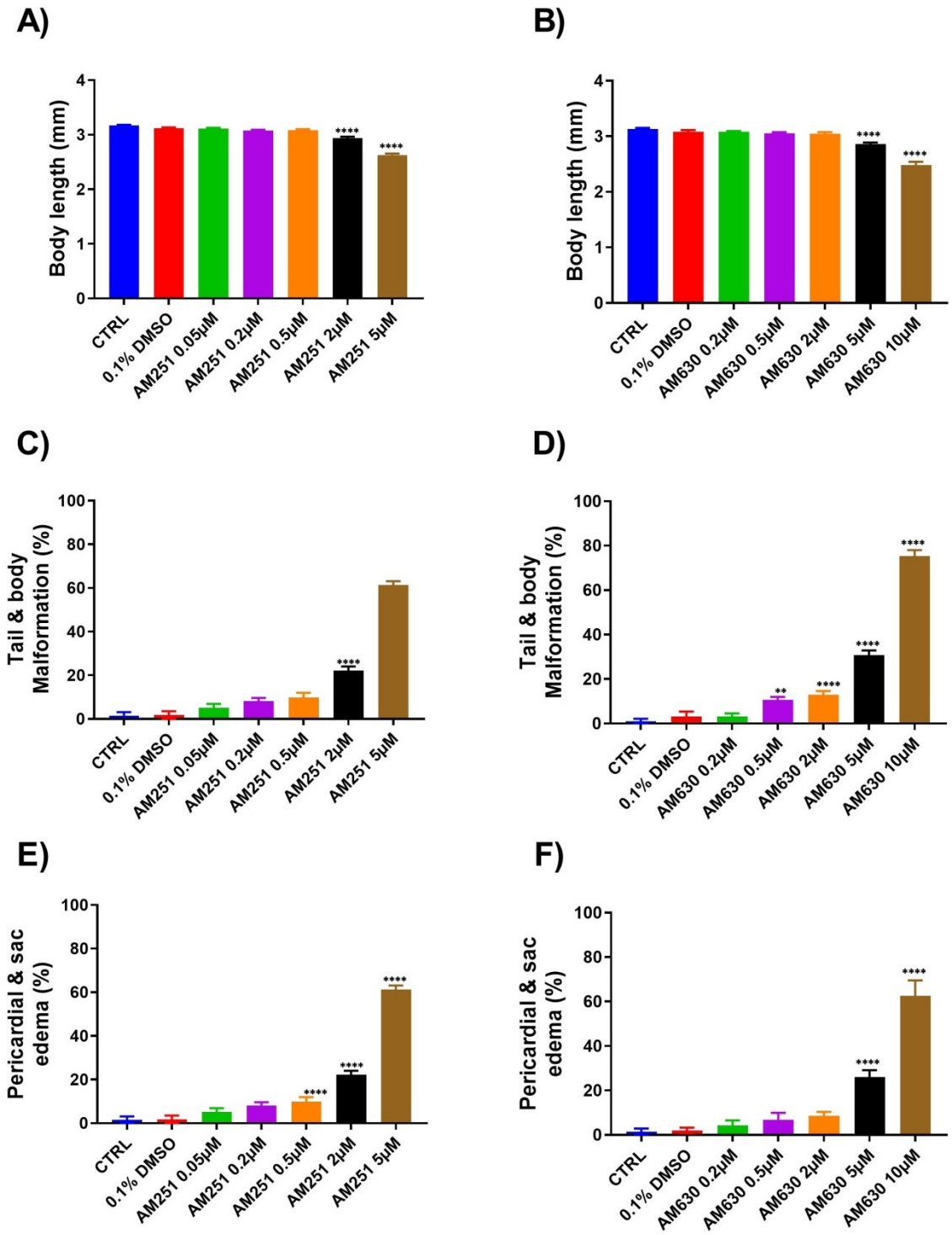
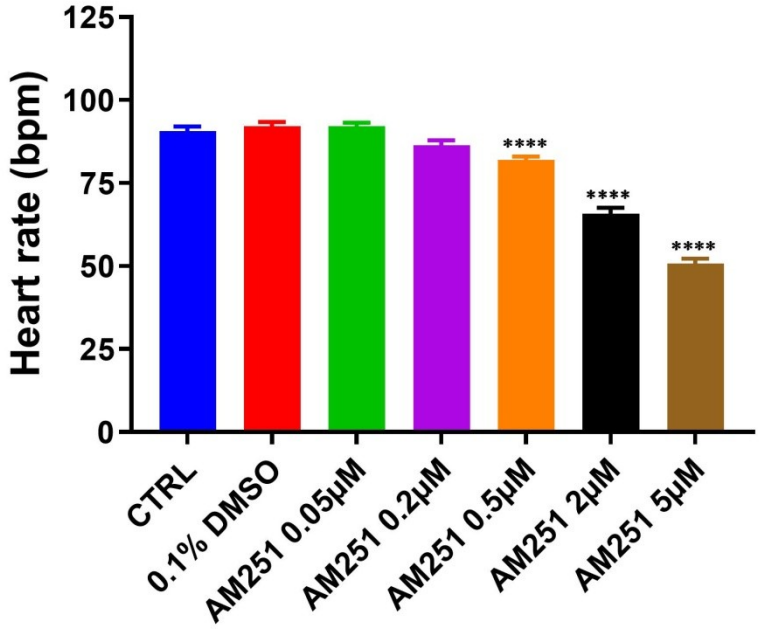

A)
Body length (mm)
CTRL
0.1% DMSO
AM251 0.05µM
AM251 0.2µM
AM251 0.5µM
AM251 2µM
AM251 5µM
B)
Body length (mm)
CTRL
0.1% DMSO
AM630 0.2µM
AM630 0.5µM
AM630 2µM
AM630 5µM
AM630 10µM
C)
Tail & body Malformation (%)
D)
Tail & body Malformation (%)
E)
Pericardial & sac edema (%)
F)
Pericardial & sac edema (%)

**Figure 3.4.AM251 and AM630 effect on body length and also tail-body malformation, pericardial-sac edema incidence rate in zebrafish embryos.** Bar graphs showing the effect of AM251 (0.05 μM – 5 μM; n=18-54) and AM630 (0.2 μM – 10 μM; n=21-52) on body length, tail and body malformations and pericardial edema. Data were collected from 48-52 hpf embryos. Controls consisted of untreated embryos (N=5 experiments, n=49-55) and embryos treated with 0.1% DMSO (N=5 experiments, n=28-36). **** Significantly different from their vehicle control (0.1% DMSO) treated on the same day, $p<0.0001$, *** represents $p<0.001$, ** represents $p<0.01$, * represents $p<0.05$.

A)


Heart rate (bpm)
125
100
75
50
25
0
****
****
****
CTRL
0.1% DMSO
AM251 0.05μM
AM251 0.2μM
AM251 0.5μM
AM251 2μM
AM251 5μM

B)

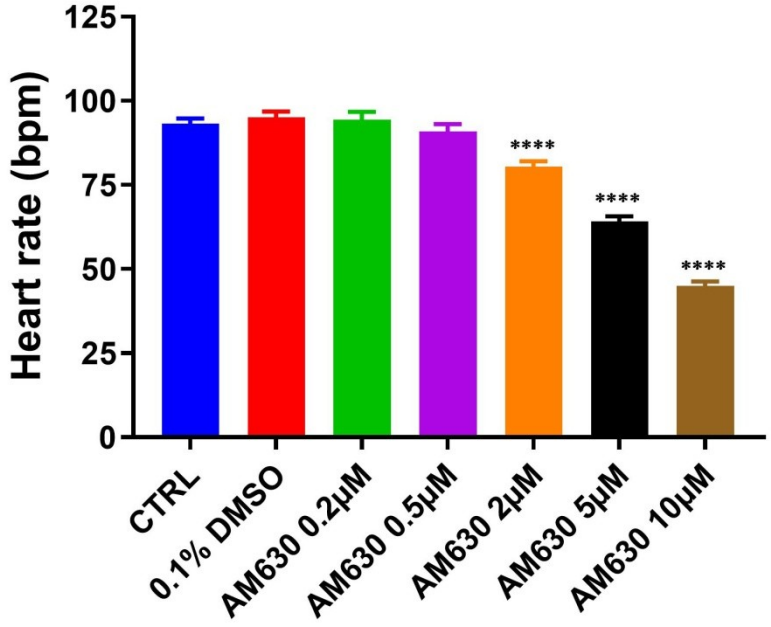

Heart rate (bpm)
125
100
75
50
25
0
****
****
****
CTRL
0.1% DMSO
AM630 0.2μM
AM630 0.5μM
AM630 2μM
AM630 5μM
AM630 10μM

**Figure 3.5.Heart rate variations in zebrafish embryos upon AM251 and AM630 exposure.** Bar graphs present the heart beat per minute in zebrafish embryos after 48 hours treatment with AM251 and AM630, and data were collected from 48- 52hpf. Treatments for heart rate measurement include untreated control (n=43), 0.1% DMSO (n=28), AM251 (0.05 µM; n=36, 0.2 µM; n=33, 0.5 µM; n=36, 2 µM; n=38 and 5 µM; n=18) and untreated control (n=45), 0.1% DMSO (n=29), AM630 (0.2 µM; n=21, 0.5 µM; n=39, 2 µM; n=46, 5 µM; n=48 and 10 µM; n=26) for AM251 and AM630 respectively (A,B). **** Significantly different from their vehicle control (0.1% DMSO), $p<0.0001$.

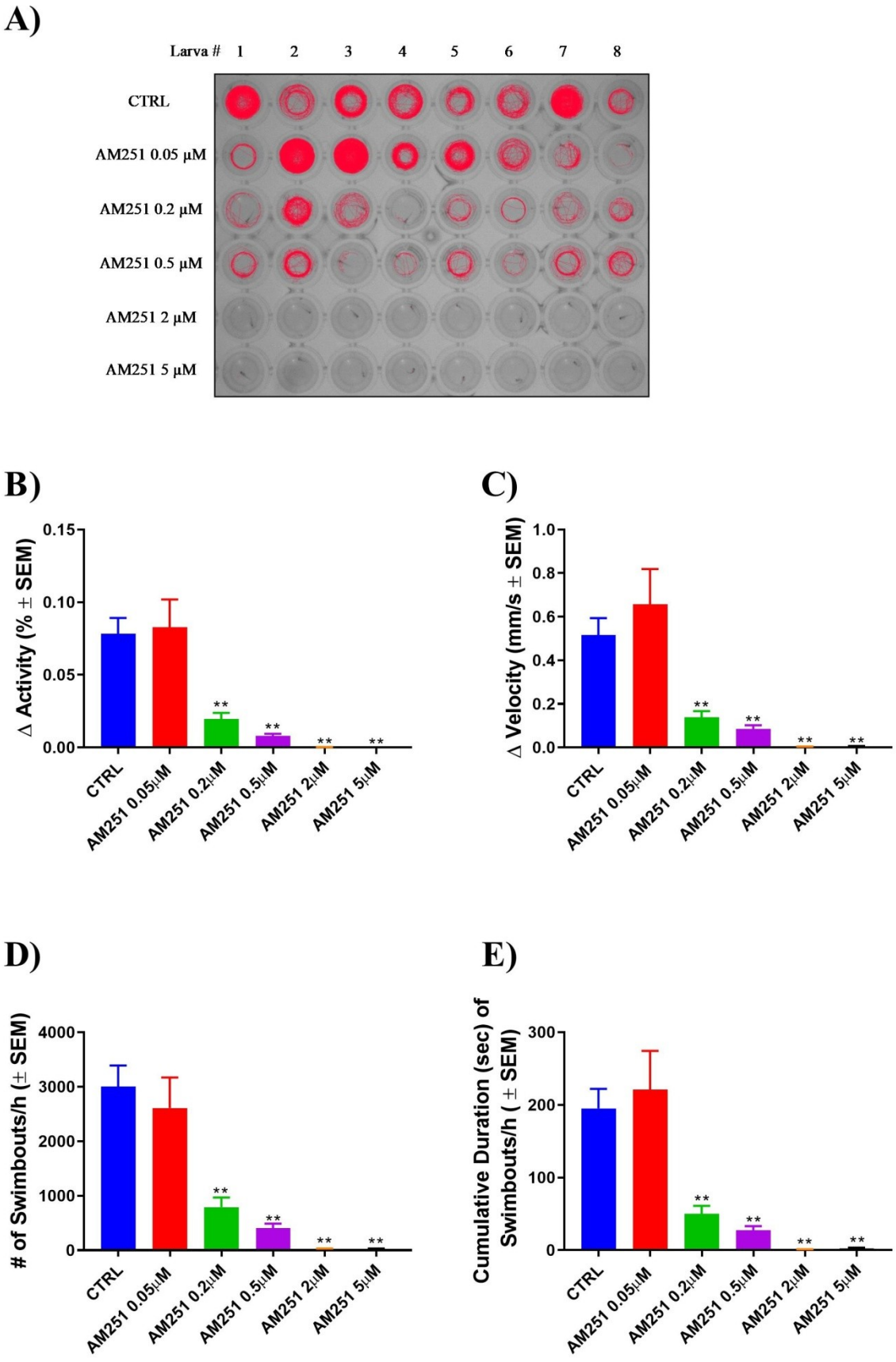

A)
Larva # 1 2 3 4 5 6 7 8
CTRL
AM251 0.05 µM
AM251 0.2 µM
AM251 0.5 µM
AM251 2 µM
AM251 5 µM
B)
Δ Activity (% ± SEM)
0.15
0.10
0.05
0.00
**
CTRL
AM251 0.05µM
AM251 0.2µM
AM251 0.5µM
AM251 2µM
AM251 5µM
C)
Δ Velocity (mm/s ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
D)
# of Swimbouts/h (± SEM)
4000
3000
2000
1000
0
E)
Cumulative Duration (sec) of Swimbouts/h ( ± SEM)
300
200
100
0

**Figure 3.6.Locomotor activity decreases upon AM251 exposure on zebrafish embryos.** Representative image of a portion of a 96-well plate, where each well contains an individual larva. Eight larvae per row are displayed, each representing a replicate at the dose indicated. Red lines represent movement of the fish during 60 minutes. Tracing was recorded at 5 dpf after 48hrs treatment with AM251 (A). Bar graphs display changes in embryos mean activity (% rate for one hour), mean velocity (in mm/s for one hour), frequency of swim bouts within one hour and cumulative duration of swim bouts (in seconds) for one hour. Embryos were treated with AM251 (0.05 μM; n=24, 0.2 μM; n=26, 0.5 μM; n=24, 2 μM; n=24 and 5 μM; n=22) with respect to their untreated control (n=26) (B-E). ** Significantly different from their untreated control, $p<0.01$.

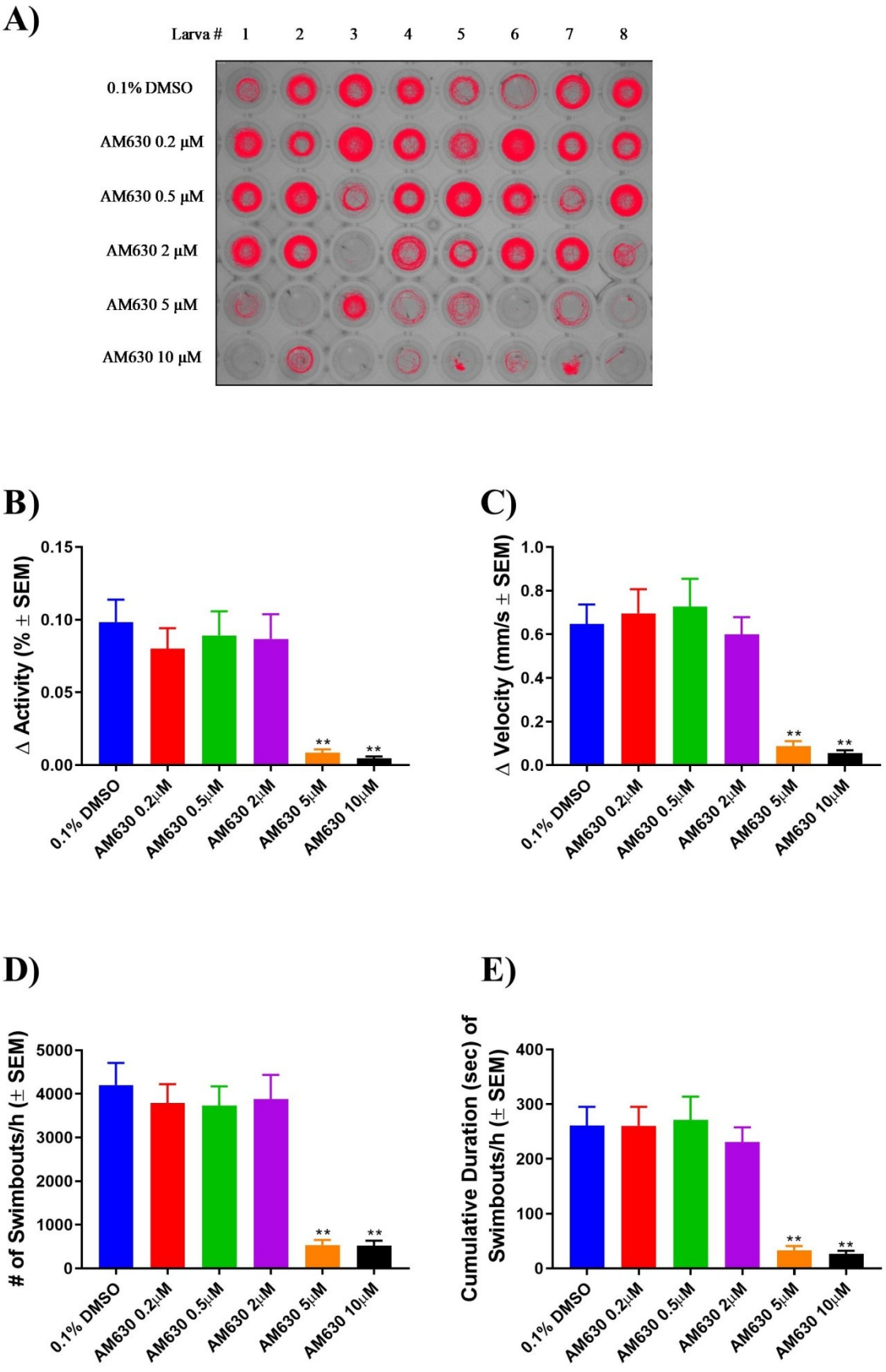

A)
Larva # 1 2 3 4 5 6 7 8
0.1% DMSO
AM630 0.2 µM
AM630 0.5 µM
AM630 2 µM
AM630 5 µM
AM630 10 µM
B)
Δ Activity (% ± SEM)
0.15
0.10
0.05
0.00
**
**
0.1% DMSO
AM630 0.2µM
AM630 0.5µM
AM630 2µM
AM630 5µM
AM630 10µM
C)
Δ Velocity (mm/s ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
**
**
0.1% DMSO
AM630 0.2µM
AM630 0.5µM
AM630 2µM
AM630 5µM
AM630 10µM
D)
# of Swimbouts/h (± SEM)
5000
4000
3000
2000
1000
0
**
**
0.1% DMSO
AM630 0.2µM
AM630 0.5µM
AM630 2µM
AM630 5µM
AM630 10µM
E)
Cumulative Duration (sec) of Swimbouts/h (± SEM)
400
300
200
100
0
**
**
0.1% DMSO
AM630 0.2µM
AM630 0.5µM
AM630 2µM
AM630 5µM
AM630 10µM

**Figure 3.7.Locomotor activity decreases upon AM630 exposure on zebrafish embryos.** Representative image of 48 individual larval tracing recorded at 5dpf after 48hrs treatment with AM630 (A). Bar graphs display changes in embryos mean activity (% rate for one hour), mean velocity (in mm/s for one hour), frequency of swim bouts within one hour and cumulative duration of swim bouts (in seconds) for one hour. Embryos were treated with AM630 (0.2 μM; n=24, 0.5 μM; n=24, 2 μM; n=24, 5 μM; n=24 and 10 μM; n=20) with respect to their vehicle control (n=26) (B-E). ** Significantly different from their vehicle control (0.1% DMSO), $p<0.01$.

# Chapter 4. Results: Identifying distinctive roles of CB1 and CB2 receptors in early zebrafish locomotor development

Since eCB receptors are expressed in a region-specific and development manner, I set out to determine whether inhibition of the prototypical eCB receptors CB1R and CB2R during the early stages of zebrafish development would alter normal development of cells in the locomotor system in a fashion that was receptor specific. I treated embryos with CB1R and CB2R antagonists during either the first 24hrs (0-24 hpf) or second 24 hrs (24-48 hpf) of development and examined a range of features associated with locomotion. My findings indicate that the eCB system plays a role in motor neuron pathfinding and branching, and in the development of normal locomotor activities.

## 4.1 Morphology and cardiac activity

In this study, I attempted to delineate the effects of the eCB system in zebrafish early development by blocking the cannabinoid receptors with the CB1R antagonist AM251 or the CB2R antagonist AM630 for the first or second 24 h of development as shown in Fig. 4.1A,B. My previous study indicated that blocking the CB1Rs and CB2Rs for a full 48 h before hatching resulted in morphological and locomotor deficits [105]. In the present study, I significantly expanded this work by blocking the eCB receptors either individually or in combination, for the first or the second 24 h of development, and by examining the effects on the development and morphology of primary and secondary motor neurons.

First, I determined dose-dependent effects of the antagonists by applying a range of commonly used concentrations (0.05–5 μM for AM251 and 0.2–10 μM for AM630)when testing survival, hatching, heart rates and morphological deficits[106][107][96][93]. Based upon these results, I used a single concentration for AM251 and AM630 that was 50–70% effective for the remainder of the study. The results indicate that blocking CB1Rs with AM251 at either time points (0-24 hpf or 24-48 hpf) can affect zebrafish gross morphology. However, blocking the CB2Rs in the first 24 h resulted in significant morphological defects (Fig. 4.1C,D), whereas blocking CB2Rs from 24 to 48 hpf had no obvious effects on morphology (Fig. 4.1C,D). To examine the effects of blocking both CB1Rs and CB2Rs simultaneously, I incubated fertilized eggs in concentrations of the blockers that were approximately 50–60% effective. The results of these combined blockers were intriguing because they showed little effect when used from 0 to 24 hpf, but had a greater effect when used from 24 to 48 hpf (Fig. 4.1C,D). Blocking the CB2R from 0 to 24 hpf significantly reduced body length ($P<0.05$; Fig 4.1E). In contrast, inhibition of CB1R from 24 to 48 hpf had a greater effect on body length compared with blocking of CB2R (Fig. 4.1F). Finally, treatment with both inhibitors simultaneously had greater effects when applied from 24 to 48 hpf, but not when applied earlier (Fig. 4.1E,F; $P<0.05$). Together, these results suggest that the CB2R plays a greater role in gross morphological development of zebrafish in the first 24 h after egg fertilization whereas the CB1R may play a comparatively greater role in the second 24 h of development.

An examination of gross morphological deficits indicate that blocking CB1R and CB2Rs in the first 24 h had a greater effect on pericardial edema, yolk sac edema, and tail and body malformations (Fig. 4.2) compared with blocking the receptors from 24 to

48 hpf. For instance, incubation in AM630 in the first 24 h resulted in rates of edema of 40±4% (n=62) compared with 14±1% (n=70) when incubated from 24 to 48 hpf (Fig. 4.2B,C). Similarly, blocking CB2Rs from 0 to 24 hpf resulted in a 43±2% (n=62) rate of tail and body malformations compared with 14±1% (n=70) when treated from 24 to 48 hpf (Fig. 4.2D,E). In both of these treatments, combining the CB1R and CB2R antagonists generally did not lead to a greater effect (Fig. 4.2).

Blocking CB2R in the first 24 h of development had the greatest effect on cardiac activity and resulted in heart rates of 70±2 beats min−1 (n=36 from 3 different experiments) compared with 99±1 beats min−1 (n=35 from 3 different experiments) in control animals (Fig. 4.2F). Blocking CB1Rs in either the first or second day of development reduced heart rate from 91 beats min−1 in controls to around 85 beats min−1 (Fig. 4.2F,G). Blocking CB2Rs from 24 to 48 hpf had a smaller effect on heart rate and resulted in rates of 86±1 beats min−1 (n=35) compared with 98±1 beats min−1 in vehicle controls (Fig. 4.2G). Exposure to the combined antagonists from 0 to 24 hpf during the second day decreased heart rate to an intermediate level compared with each individual blocker (Fig. 4.2F), whereas exposure to both blockers from 24 to 48 hpf had a significantly larger effect (Fig. 4.2G). Taken together, these findings implicate a more significant role for CB2Rs in early morphological development compared with CB1Rs.

## 4.2 Morphology of motor neurons

Because my research focus is directed towards understanding neurodevelopment associated with locomotion, I examined whether the eCB system might be involved in the development of motor neurons in zebrafish embryos. To do this, I performed

immunohistochemistry to image the morphology of primary motor neurons, specifically focusing on their branching patterns. Blocking CB1Rs in the first day of development had minimal effect on the primary branches emanating from the main axon (Fig. 4.3B,E), but it significantly increased the number of secondary and tertiary axonal branches from control values of 13±1 (n=7) to 23±2 (n=7) branches per ventral motor axon (n=6 from 3 different experiments, $p<0.01$). Combining the CB1R and CB2R antagonists resulted in an intermediate level of branching that was not significantly different from that of controls (n=7 from 3 different experiments, $P<0.92$; Fig 4.3F).

Blocking CB1Rs in the second day of development resulted in a significant increase in the number of secondary and tertiary branches from 11±1 (n=7 from 3 different experiments) in the controls to 19±1 in the treated group (Fig. 4.3L). However, application of AM630 from 24 to 48 hpf had no significant effect on the number of secondary and tertiary branches (n=7 from 3 different experiments, $P<0.92$; Fig 4.3L). Application of both blockers simultaneously resulted in branch numbers that were intermediate between the effect of CB1R and CB2R individually (Fig. 4.3L). These data provide some of our most interesting results and suggest that CB1Rs and CB2Rs play opposing roles with respect to the extent of motor neuron branching.

An examination of secondary motor neurons showed that exposure of the embryos to either the CB1R or the CB2R antagonist in the first 24 h resulted in disruption of the lateral branches to the extent that some branches were completely absent while others were truncated or misshapen (Fig. 4.4A–E). Interestingly, we found no alterations or deficits in the ventral branches of secondary motor neurons (Fig. 4.4A–D,F). Similar results were obtained when exposures occurred over the 24–48 hpf time frame. In those

experiments, we found that blocking the CB1Rs or CB2Rs altered the number and shape of the lateral branches without affecting the ventral branches (Fig. 4.4G–L).

## 4.3 Locomotor assays

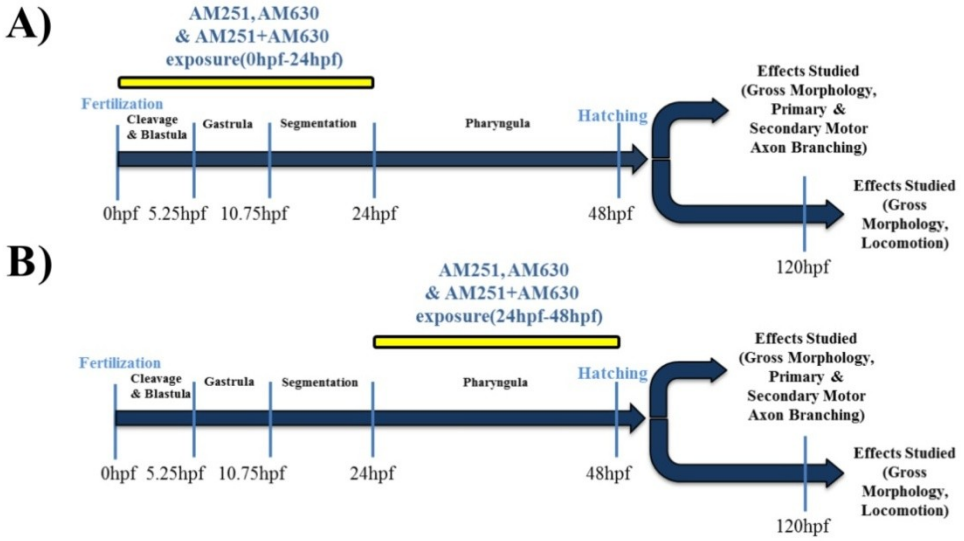
Since I identified alterations in the branching patterns of both primary and secondary motor neurons, I asked whether these deficits translated into functional changes in locomotion and movement. To address this, I allowed the fish to develop until they were 5 dpf, when they become more active. However, it was noted a significant number of morphological deficits in the treated groups, such as pericardial edema and trunk malformations, which might impact swimming. Quantification of these deficits showed significantly high levels of pericardial and yolk sac edema ($P<0.05$; Fig 4.5B) and tail malformation ($P<0.05$; Fig 4.5D) in fish treated with either antagonist. The proportion of animals exhibiting malformations was greater in animals treated in the first 24 h compared with the second 24 h (Fig. 4.5B–E).

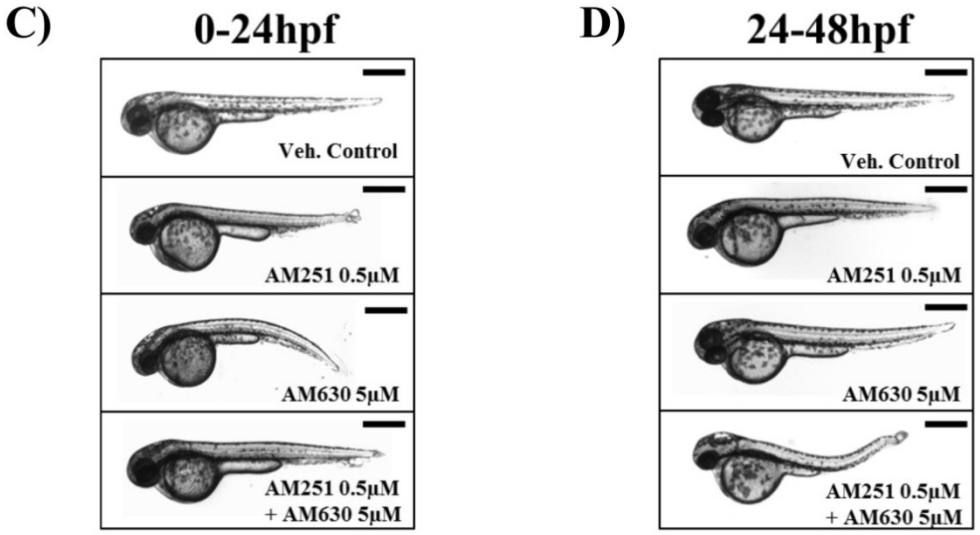
To examine larval locomotion, individual larvae was transferred to single well of a 96-well plate and allowed them to acclimate to their new environment for 60 min before filming their activity. I found that embryos treated with AM251 in the first 24 h exhibited approximately one-half to one-third the level of activity, swim velocity, number of swim bouts and cumulative duration of swim bouts compared with controls ($P<0.052$, n=19–28 from 3 different experiments; Fig. 4.6B–E). This was also evident when embryos were treated with AM251 from 24 to 48 hpf ($P<0.048$, n=16 from 3 different experiments; Fig. 4.6F–I). Exposure to the CB2R antagonist AM630 resulted in trends towards fewer and smaller swim bouts, but without significance (Fig. 4.6B–I).

It was noticed that animals exposed to AM251 tended to lie on the bottom of their holding dishes more often than animals exposed to AM630 or vehicle-treated controls. Therefore, I examined their swim bladders to determine whether they had developed properly (Fig. 4.7). It was found that treatment with AM251 resulted in a smaller percentage of animals with fully inflated swim bladders (Fig. 4.7B,E). For instance, only 36±5% of animals treated with AM251 in the first 24 h had fully inflated swim bladders, whereas 65±3% of animals treated with AM630 had fully inflated swim bladders, compared with control levels of around 90±1% (n=58–59 from 4 different experiments, $P<0.005$; Fig. 4.7A,B). Likewise, in the groups treated with AM251 from 24 to 48 hpf, 53±2% had fully inflated swim bladders, compared with 74±4% in the AM630-treated group and 92±1.56% in the controls (n=54–60 from 4 different experiments, $P<0.0055$; Fig. 4.7A,E). Concurrently, there were greater proportions of animals with partially inflated and non inflated swim bladders in the AM251-treated groups compared with the AM630-treated animals (n=54–60 from 4 different experiments; Fig. 4.7C,D,F,G). Thus, the deficits in swim bladder inflation could account for some or all of the deficits in locomotion. Together, my findings show that activity, locomotion and swim bladder development are largely influenced by activation of CB1Rs and CB2Rs.

Table 2. Effects of CB1R and CB2R blocking at either from 0-24 hpf or 24-48 hpf in zebrafish embryos (Chapter 4).

| Figures | Findings |
|---|---|
| Fig 4.1-4.2 | 1. CB1R inhibition at both 0-24 hpf and 24-48 hpf significantly affected the gross morphology and heart rate of zebrafish. Although CB2R inhibition at 0-24 hpf had a profound effect on zebrafish embryos, blocking CB2R from 24-48 hpf had minimal effects on their gross morphology and heart rates |
| Fig 4.3 | 2. Blocking CB1R at both 0-24 hpf and 24-48 hpf time points increase primary motor neuron branching. On the contrary, CB2R inhibition only at 0-24 hpf had effects, which is a reduction in primary motor neuron branching |
| Fig 4.4 | 3. CB1R inhibition decreased secondary motor neuron branching pattern, whereas CB2R disruption had no effects on secondary motor neuron growth |
| Fig 4.1-4.7 | 4. For few instances, co-application of CB1R and CB2R exhibited an augmented effects, but often the co-exposure showed effects that were intermediate compared to either antagonist alone, particularly when blocked at 0-24 hpf |
| Fig 4.6 | 5. CB1R inhibition reduced locomotion in zebrafish embryos, while CB2R inhibition showed no significant effects in activity |
| Fig 4.7 | 6. CB1R inhibition can greatly impact swim bladder development compared to CB2R |


A)
AM251, AM630
& AM251+AM630
exposure(0hpf-24hpf)
Fertilization
Cleavage
& Blastula
Gastrula
Segmentation
Pharyngula
Hatching
Effects Studied
(Gross Morphology,
Primary &
Secondary Motor
Axon Branching)
Effects Studied
(Gross
Morphology,
Locomotion)
0hpf
5.25hpf
10.75hpf
24hpf
48hpf
120hpf
B)
AM251, AM630
& AM251+AM630
exposure(24hpf-48hpf)
Fertilization
Cleavage
& Blastula
Gastrula
Segmentation
Pharyngula
Hatching
Effects Studied
(Gross Morphology,
Primary &
Secondary Motor
Axon Branching)
Effects Studied
(Gross
Morphology,
Locomotion)
0hpf
5.25hpf
10.75hpf
24hpf
48hpf
120hpf


C)
0-24hpf
Veh. Control
AM251 0.5μM
AM630 5μM
AM251 0.5μM
+ AM630 5μM
D)
24-48hpf
Veh. Control
AM251 0.5μM
AM630 5μM
AM251 0.5μM
+ AM630 5μM

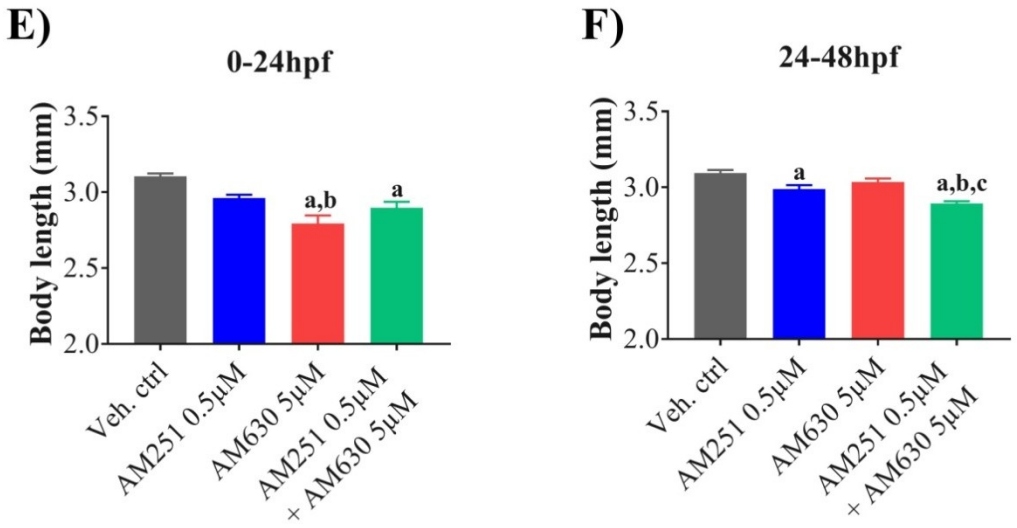

E)
0-24hpf
Body length (mm)
3.5
3.0
2.5
2.0
a,b
a
Veh. ctrl
AM251 0.5μM
AM630 5μM
AM251 0.5μM
+ AM630 5μM
F)
24-48hpf
Body length (mm)
3.5
3.0
2.5
2.0
a
a,b,c
Veh. ctrl
AM251 0.5μM
AM630 5μM
AM251 0.5μM
+ AM630 5μM

**Figure 4.1.Effect of the endocannabinoid receptor antagonists AM251 and AM630 on zebrafish embryo morphology.** (A-B) Schematic showing the timeline for drug exposure and when experimental measurements were made. (A-B) Drug exposure during the first 24 h of development (0–24 hpf) and drug exposure during the second 24 h of development (24–48 hpf) are highlighted by the yellow bar. Embryos were allowed to develop in normal egg water after each treatment. Primary or secondary motor neuron axonal branching was investigated between 48 and 52 hpf, while locomotion was recorded at 120 hpf (5 dpf). Gross morphological observations occurred at 2 and 5 dpf. The treatments include vehicle control (0.1% DMSO), AM251 0.5 μM, AM630 5 μM or AM251 0.5 μM +AM630 5 μM, either from 0 to 24 hpf or 24 to 48 hpf. (C,D) Representative images of treated embryos were taken at 48–52 hpf; scale bars: 0.5 mm. (E,F) Body length (mm) at 2 dpf development (n=33, 31, 40 and 35 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+5 μM AM630 treatments, respectively, from 0 to 24 hpf, and n=29, 27, 37 and 34 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+5 μM AM630 treatments, respectively, from 24 to 48 hpf ). Data are presented as means±s.e.m. [a] Significantly different from vehicle control, $P<0.05$; [b]significantly different from 0.5 μM AM251, $P<0.05$; ;[c] significantly different from 5 μM AM630, $P<0.05$ (one-way ANOVA followed by Tukey's post hoc multiple comparisons test).

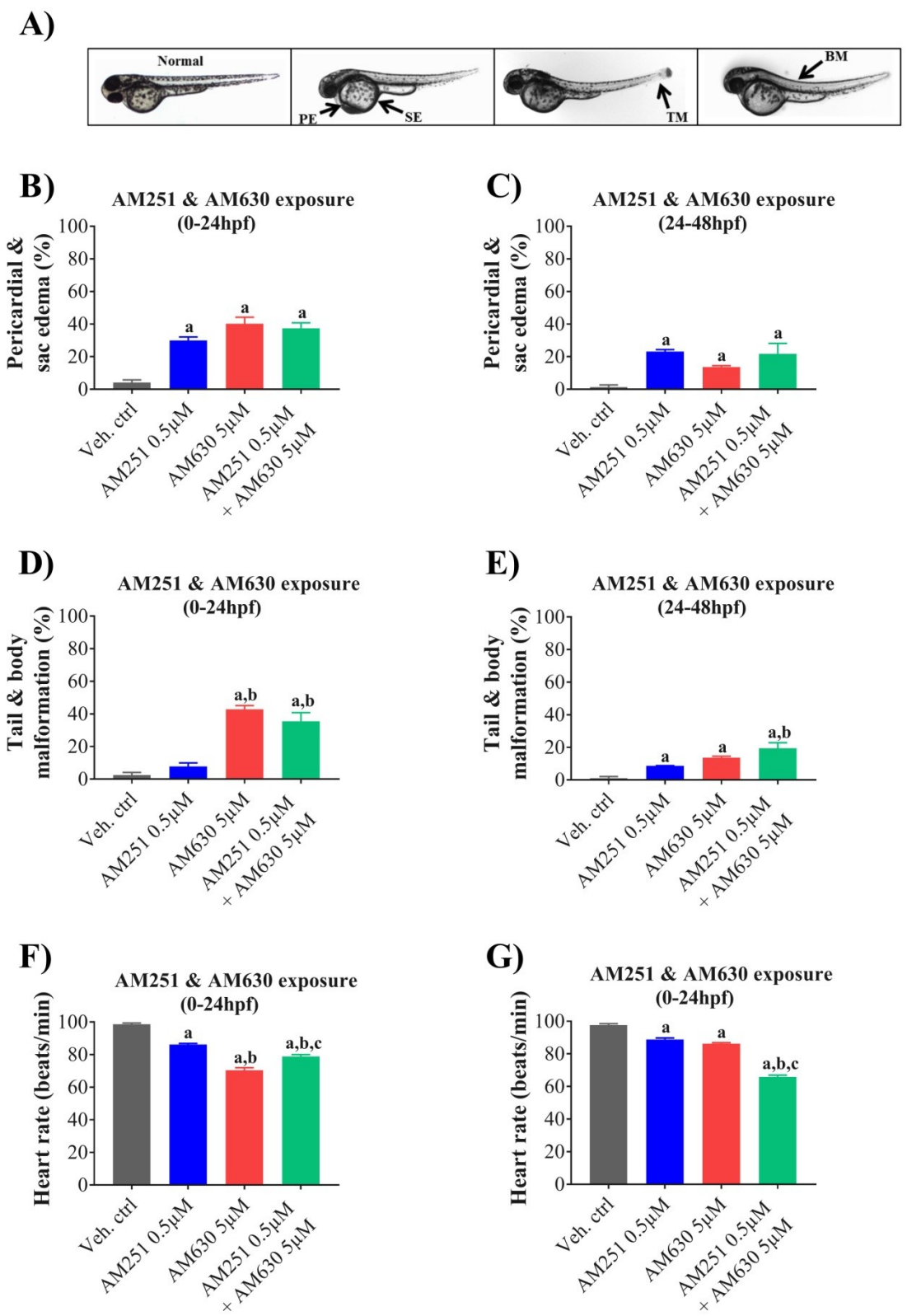
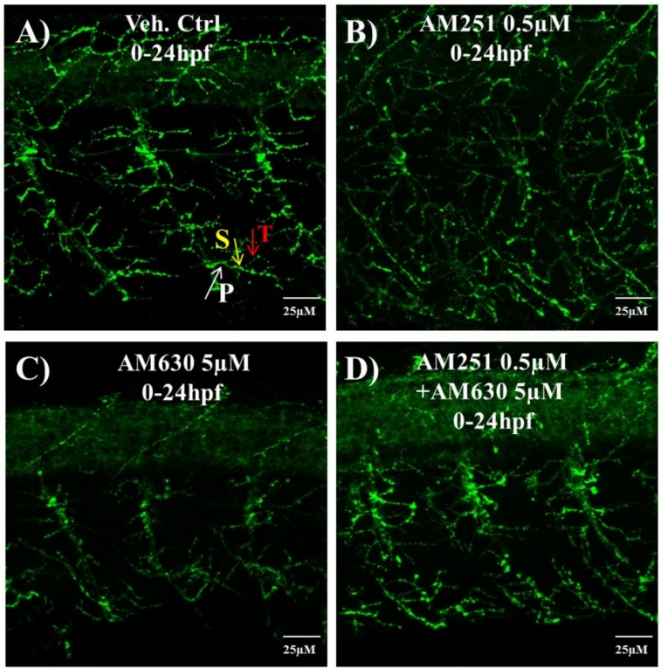
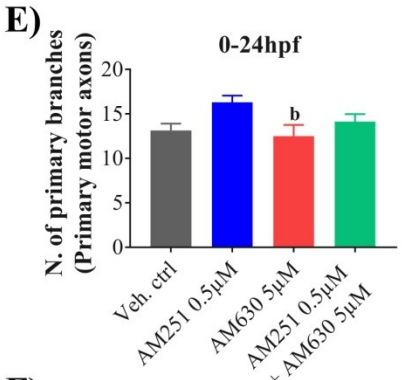
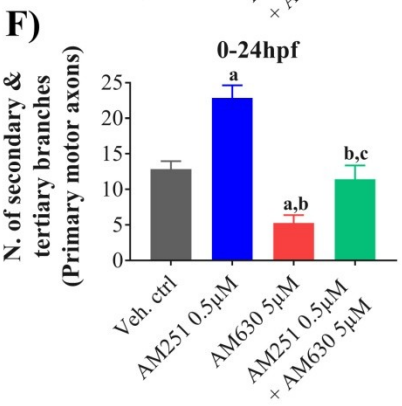
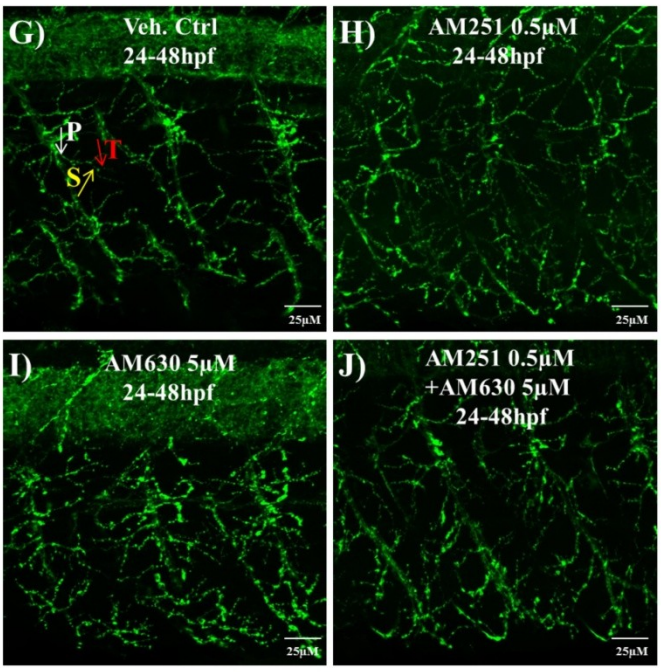

A)
Normal
PE
SE
TM
BM
B)
AM251 & AM630 exposure
(0-24hpf)
Pericardial & sac edema (%)
C)
AM251 & AM630 exposure
(24-48hpf)
Pericardial & sac edema (%)
D)
AM251 & AM630 exposure
(0-24hpf)
Tail & body malformation (%)
E)
AM251 & AM630 exposure
(24-48hpf)
Tail & body malformation (%)
F)
AM251 & AM630 exposure
(0-24hpf)
Heart rate (beats/min)
G)
AM251 & AM630 exposure
(0-24hpf)
Heart rate (beats/min)
Veh. ctrl
AM251 0.5μM
AM630 5μM
AM251 0.5μM + AM630 5μM

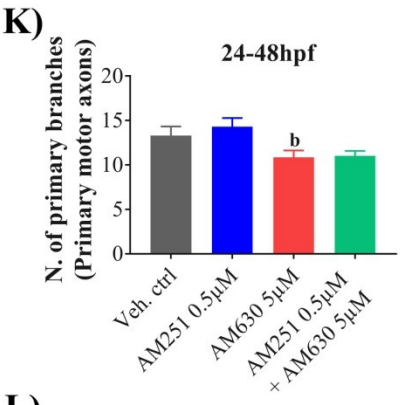
**Figure 4.2.Effect of the endocannabinoid receptor antagonists AM251 and AM630 on zebrafish morphological development and heart rate.** (A) Incidence of pericardial edema and yolk sac edema in embryos treated with AM251 and AM630 exhibit early morphological deformities such as pericardial edema (PE), yolk sac edema (SE), tail malformation (TM) and body malformation (BM) in zebrafish embryos at 2 dpf. (B,D) Rates of pericardial and yolk sac edema and tail and body malformation in embryos treated with AM251 and AM630 in the first 24 h of development (n=74, 56, 62 and 64 for vehicle control, AM251 0.5 μM, AM630 5 μM and AM251 0.5 μM +AM630 5 μM, respectively). (C,E) Rates of pericardial and yolk sac edema and tail and body malformation in embryos treated with AM251 and AM630 in the second 24 h of development. Data were obtained at 2 dpf (n=68, 62, 70 and 58 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+5 μM AM630, respectively). (F,G) Heart rate of embryos treated with AM251 and AM630 in the first and second 24 h of development (n=35, 35, 36 and 40 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+ 5 μM AM630 treatments, respectively, from 0 to 24 hpf, and n=35, 34, 35 and 49 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+5 μM AM630 treatments, respectively, from 24 to 48 hpf). Data were obtained at 2 dpf. Data are presented as means ±s.e.m. [a]Significantly different from vehicle control, $P<0.05$; [b] significantly different from 0.5 μM AM251, $P<0.05$;[c] significantly different from 5 μM AM630, $P<0.05$ (one-way ANOVA followed by Tukey's post hoc multiple comparisons test).


A)
Veh. Ctrl
0-24hpf
S
T
P
25μM
B)
AM251 0.5μM
0-24hpf
25μM
C)
AM630 5μM
0-24hpf
25μM
D)
AM251 0.5μM
+AM630 5μM
0-24hpf
25μM


E)
0-24hpf
N. of primary branches
(Primary motor axons)
20
15
10
5
0
b
Veh. ctrl
AM251 0.5μM
AM630 5μM
AM251 0.5μM
+ AM630 5μM


F)
0-24hpf
N. of secondary &
tertiary branches
(Primary motor axons)
25
20
15
10
5
0
a
a,b
b,c
Veh. ctrl
AM251 0.5μM
AM630 5μM
AM251 0.5μM
+ AM630 5μM


G)
Veh. Ctrl
24-48hpf
P
T
S
25μM
H)
AM251 0.5μM
24-48hpf
25μM
I)
AM630 5μM
24-48hpf
25μM
J)
AM251 0.5μM
+AM630 5μM
24-48hpf
25μM


K)
24-48hpf
N. of primary branches
(Primary motor axons)
20
15
10
5
0
b
Veh. ctrl
AM251 0.5μM
AM630 5μM
AM251 0.5μM
+ AM630 5μM

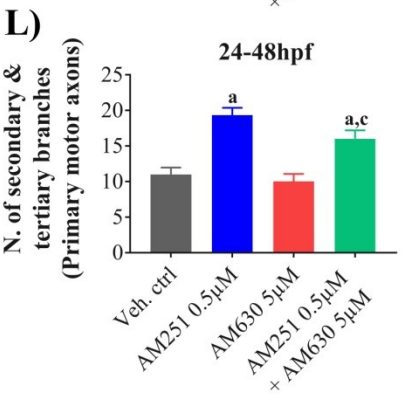

L)
24-48hpf
N. of secondary &
tertiary branches
(Primary motor axons)
25
20
15
10
5
0
a
a,c
Veh. ctrl
AM251 0.5μM
AM630 5μM
AM251 0.5μM
+ AM630 5μM

**Figure 4.3.Effect of the endocannabinoid receptor antagonists AM251 and AM630 on the branching patterns of primary motor axons in zebrafish embryos.** (A–D) Immunohistochemical staining of primary motor neurons using the Znp-1 antibody (green) in vehicle control preparations (n=7), embryos treated with AM251 (n=7), AM630 (n=6) or AM251+AM630 (n=7) in the first 24 h of development. Primary, secondary and tertiary branches in a primary motor axon are indicated with white (P), yellow (S) and red (T) arrows, respectively. (E,F) The number of primary, secondary and tertiary branches emanating from the main axon when treated in the first 24 h. (G–J) Immunohistochemical staining of primary motor neurons using the Znp-1 antibody (green) in vehicle control preparations (n=7), embryos treated with AM251 (n=7), AM630 (n=6) or AM251+AM630 (n=7) in the second 24 h of development. Primary, secondary and tertiary branches in a primary motor axon are indicated with white (P), yellow (S) and red (T) arrows, respectively. (K,L) The number of primary, secondary and tertiary branches emanating from the main axon when treated in the second 24 h. Scale bars: 25 μm. Data are presented as means±s.e.m. [a] Significantly different from vehicle control, $P<0.05$; [b] significantly different from 0.5 μM AM251, $P<0.05$; ;[c] significantly different from 5 μM AM630, $P<0.05$ (one-way ANOVA followed by Tukey's post hoc multiple comparisons test).

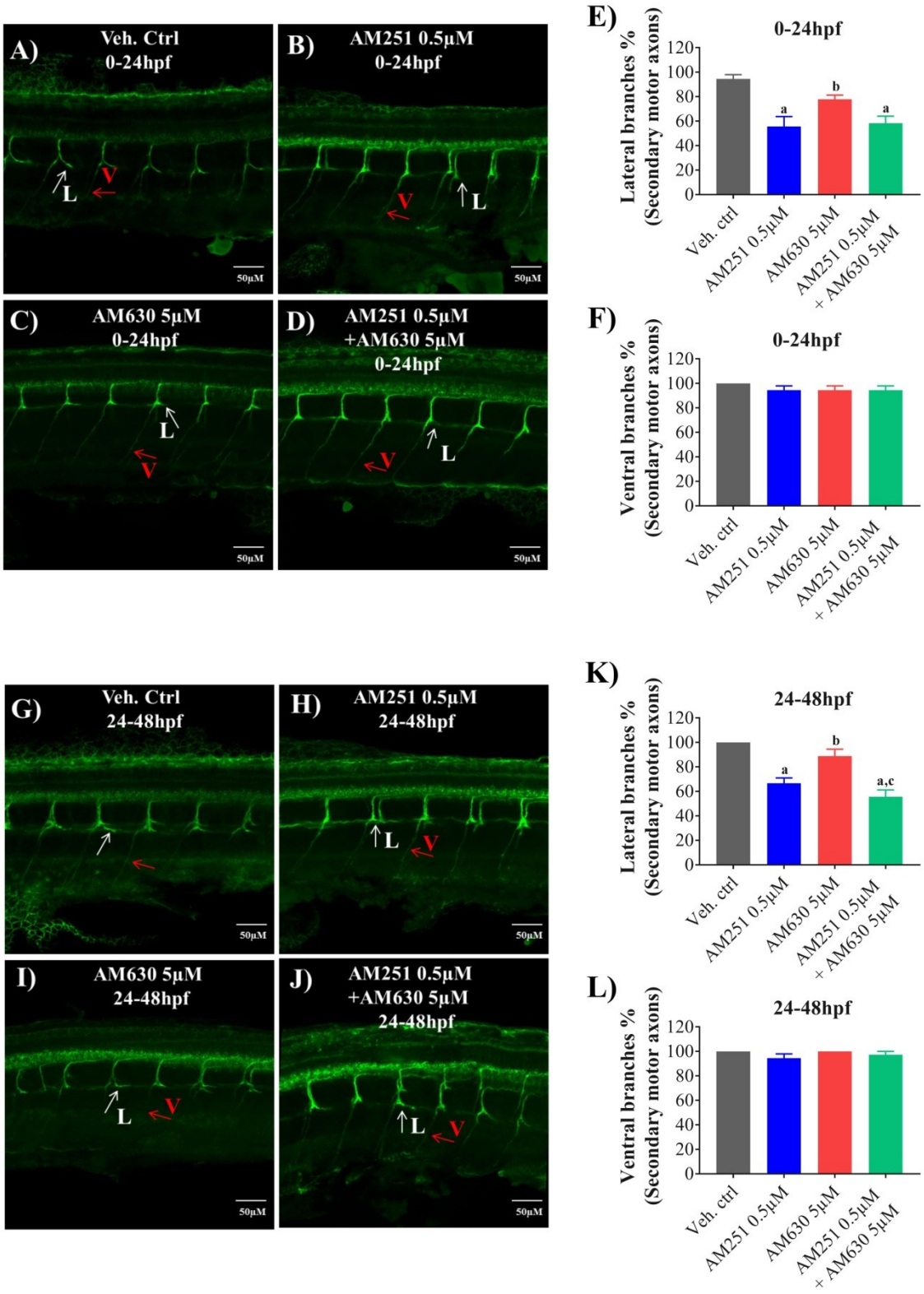

A)
Veh. Ctrl
0-24hpf
B)
AM251 0.5µM
0-24hpf
C)
AM630 5µM
0-24hpf
D)
AM251 0.5µM
+AM630 5µM
0-24hpf
E)
0-24hpf
Lateral branches %
(Secondary motor axons)
F)
0-24hpf
Ventral branches %
(Secondary motor axons)
G)
Veh. Ctrl
24-48hpf
H)
AM251 0.5µM
24-48hpf
I)
AM630 5µM
24-48hpf
J)
AM251 0.5µM
+AM630 5µM
24-48hpf
K)
24-48hpf
Lateral branches %
(Secondary motor axons)
L)
24-48hpf
Ventral branches %
(Secondary motor axons)
Veh. ctrl
AM251 0.5µM
AM630 5µM
AM251 0.5µM
+ AM630 5µM
L
V
50µM

**Figure 4.4.Effect of the endocannabinoid receptor antagonists AM251 and AM630 on the branching patterns of secondary motor axons in zebrafish embryos.** (A–D) Immunohistochemical staining of secondary motor neurons using the Zn8 antibody (green) in vehicle control preparations (n=7), embryos treated with AM251 (n=7), AM630 (n=6) or AM251+AM630 (n=7) in the first 24 h of development. Lateral and ventral branches projecting from secondary motor axons were counted from three different axons from each fish and were expressed as a percentage for each treatment. Lateral and ventral branches in a secondary motor axon are indicated with white (L) and red (V) arrows, respectively. (E,F) Lateral and ventral branches (%) emanating from the secondary motor axon when treated in the first 24 h. (G–J) Immunohistochemical staining of secondary motor neurons using the Zn8 antibody (green) in vehicle control preparations (n=7), embryos treated with AM251 (n=7), AM630 (n=6) or AM251+AM630 (n=7) in the second 24 h of development. Lateral and ventral branches in a secondary motor axon are indicated with white (L) and red (V) arrows, respectively. (K,L) Lateral and ventral branches (%) emanating from the secondary motor axon when treated in the second 24 h. Scale bars: 50 μm. Data are presented as means±s.e.m. [a] Significantly different from vehicle control, $P<0.05$; [b] significantly different from 0.5 μM AM251, $P<0.05$; ;[c] significantly different from 5 μM AM630, $P<0.05$ (one-way ANOVA followed by Tukey's post hoc multiple comparisons test).

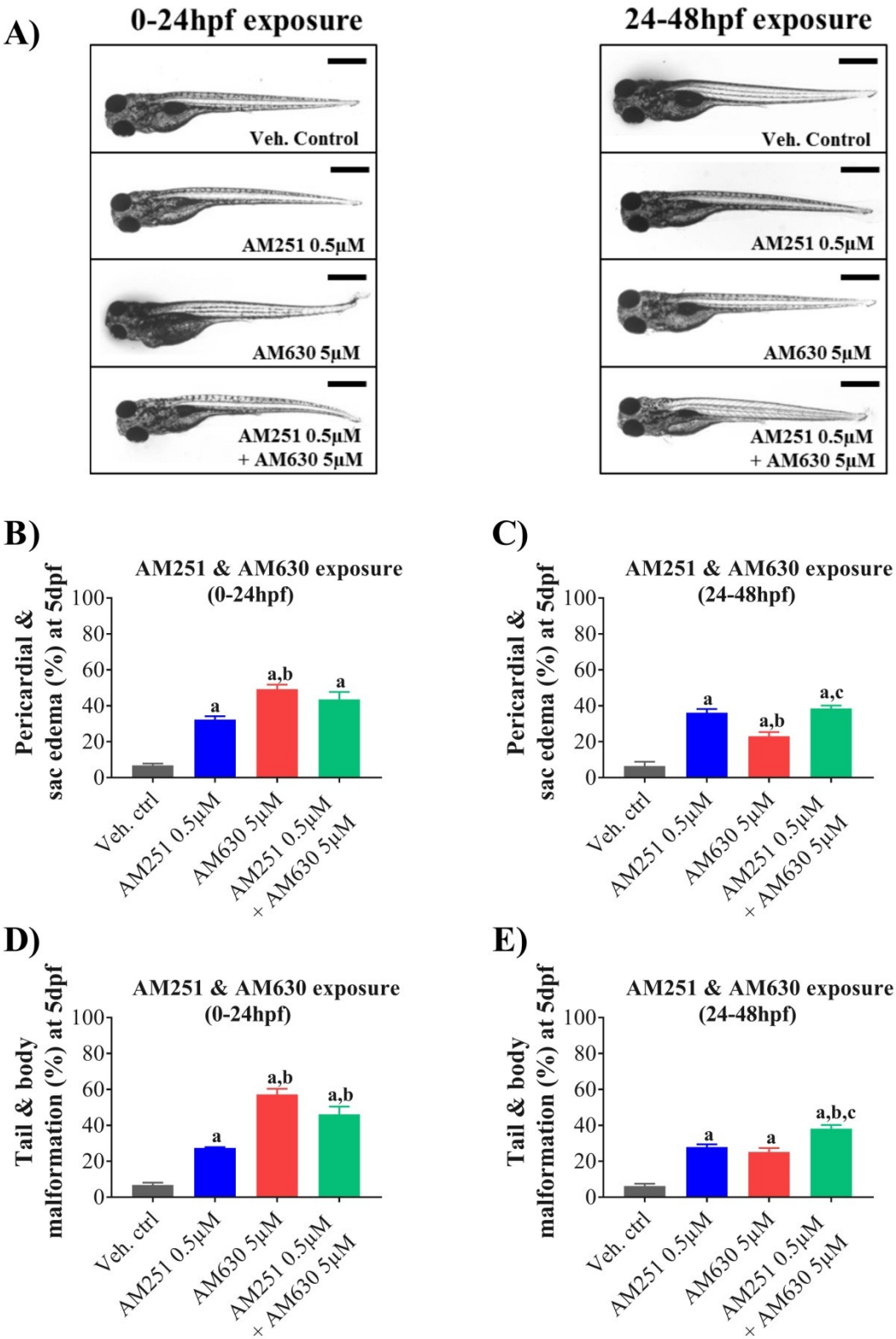

A)
0-24hpf exposure
24-48hpf exposure
Veh. Control
AM251 0.5µM
AM630 5µM
AM251 0.5µM
+ AM630 5µM
B)
AM251 & AM630 exposure
(0-24hpf)
Pericardial &
sac edema (%) at 5dpf
C)
AM251 & AM630 exposure
(24-48hpf)
D)
AM251 & AM630 exposure
(0-24hpf)
Tail & body
malformation (%) at 5dpf
E)
AM251 & AM630 exposure
(24-48hpf)
Veh. ctrl
AM251 0.5µM
AM630 5µM
AM251 0.5µM
+ AM630 5µM

**Figure 4.5.Effect of the endocannabinoid receptor antagonists, AM251 and AM630 on morphological development in 5 dpf zebrafish larva.** (A) Incidence of pericardial edema and yolk sac edema in 5 dpf larvae when treated with AM251 and AM630 in the first and second 24 h of development. Scale bars: 0.7 mm. (B,D) Rates of pericardial and yolk sac edema and tail and body malformation in 5 dpf larvae treated with AM251 and AM630 in the first 24 h of development (n=62, 56, 51 and 58 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+5 μM AM630, respectively). (C,E) Rates of pericardial and yolk sac edema and tail and body malformation in 5 dpf larvae treated with AM251 and AM630 in the second 24 h of development (24–48 hpf ) (n=74, 56, 62 and 64 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+5 μM AM630, respectively). Data are presented as means±s.e.m. [a] Significantly different from vehicle control, $P<0.05$; [b] significantly different from 0.5 μM AM251, $P<0.05$; [c] significantly different from 5 μM AM630, $P<0.05$ (one-way ANOVA followed by Tukey's post hoc multiple comparisons test).

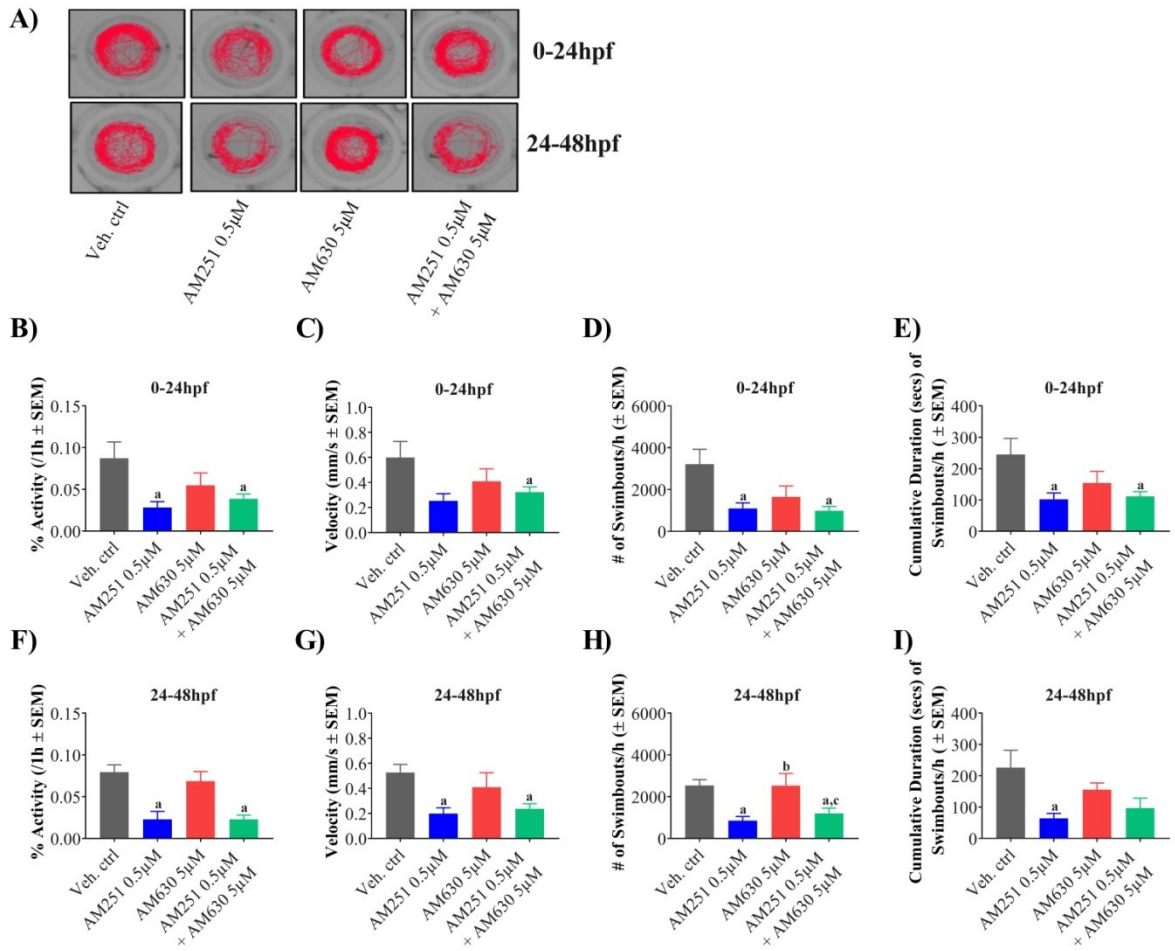

A)
0-24hpf
24-48hpf
Veh. ctrl
AM251 0.5µM
AM630 5µM
AM251 0.5µM + AM630 5µM
B)
0-24hpf
% Activity (/1h ± SEM)
C)
0-24hpf
Velocity (mm/s ± SEM)
D)
0-24hpf
# of Swimbouts/h (± SEM)
E)
0-24hpf
Cumulative Duration (secs) of Swimbouts/h ( ± SEM)
F)
24-48hpf
% Activity (/1h ± SEM)
G)
24-48hpf
Velocity (mm/s ± SEM)
H)
24-48hpf
# of Swimbouts/h (± SEM)
I)
24-48hpf
Cumulative Duration (secs) of Swimbouts/h ( ± SEM)

**Figure 4.6.Effect of the endocannabinoid receptor antagonists, AM251 and AM630 on locomotor activity in 5 dpf zebrafish larva.** (A) Representative image of a portion of a 96-well plate, where each well contains an individual larva. Eight larvae per row are displayed, each representing a replicate at the dose indicated. Red lines represent movement of the fish during 60 min. Larval tracing was recorded at 5 dpf after 48 h treatment with AM251 and AM630. (B–I) Changes in embryo mean activity (% rate for 1 h), mean velocity (mm s−1 for 1 h), frequency of swim bouts within 1 h and cumulative duration of swim bouts (s) for 1 h. The 0–24 hpf exposures (B–E) were vehicle control (n=24), 0.5 μM AM251 (n=20), 5 μM AM630 (n=19) and 0.5 μM AM251+5 μM AM630 (n=28), and 24–48 hpf exposures (F–I) were vehicle control (n=38), 0.5 μM AM251 (n=16), 5 μM AM630 (n=16) and 0.5 μM AM251+5 μM AM630 (n=24). Data are presented as means±s.e.m. [a] Significantly different from vehicle control, $P<0.05$; [b] significantly different from 0.5 μm AM251, $P<0.05$; ;[c] significantly different from 5 μM AM630, $P<0.05$ (one-way ANOVA followed by Tukey's post hoc multiple comparisons test).

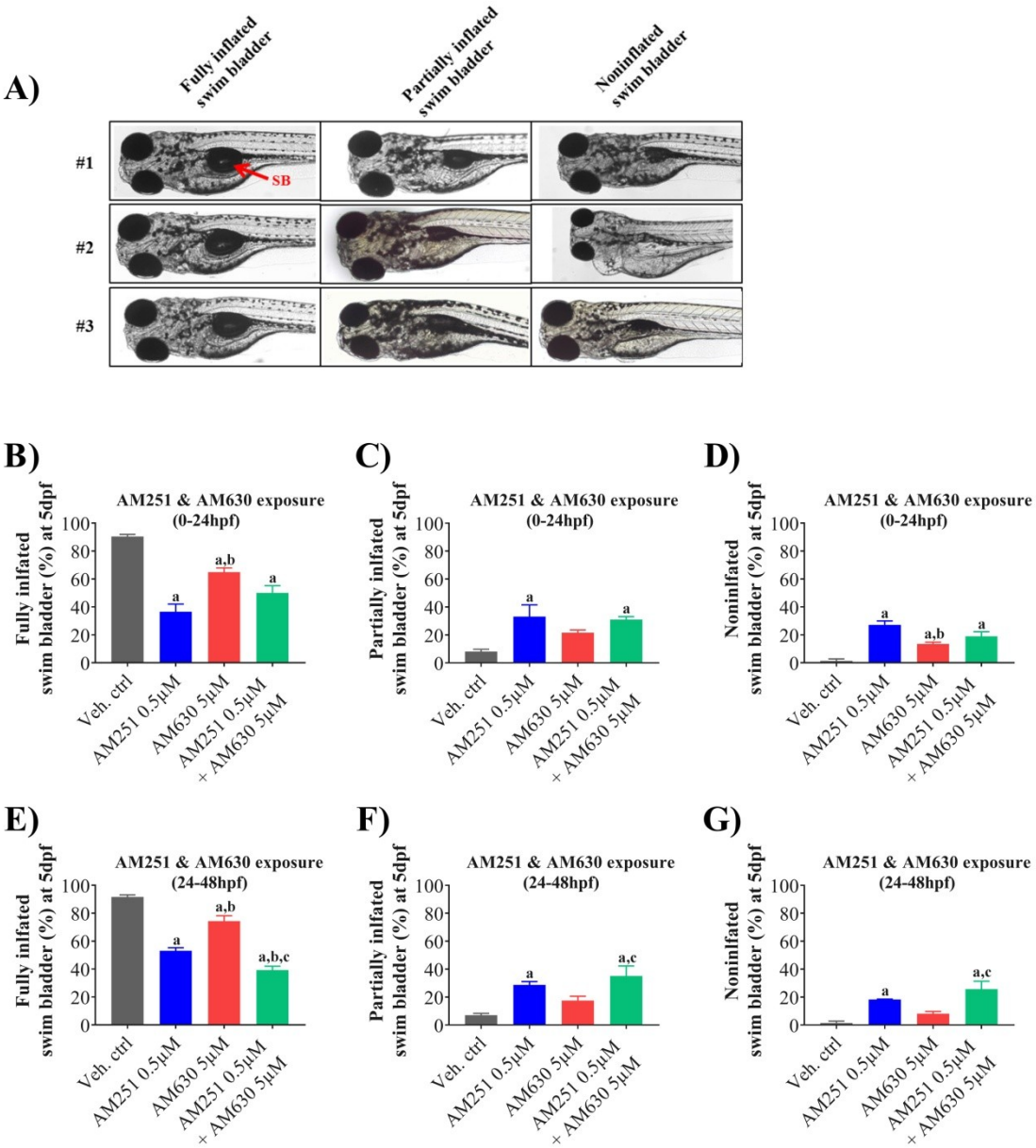

A)
Fully inflated swim bladder
Partially inflated swim bladder
Noninflated swim bladder
#1
#2
#3
SB
B)
AM251 & AM630 exposure (0-24hpf)
Fully inlfated swim bladder (%) at 5dpf
C)
AM251 & AM630 exposure (0-24hpf)
Partially inlfated swim bladder (%) at 5dpf
D)
AM251 & AM630 exposure (0-24hpf)
Noninlfated swim bladder (%) at 5dpf
E)
AM251 & AM630 exposure (24-48hpf)
Fully inlfated swim bladder (%) at 5dpf
F)
AM251 & AM630 exposure (24-48hpf)
Partially inlfated swim bladder (%) at 5dpf
G)
AM251 & AM630 exposure (24-48hpf)
Noninlfated swim bladder (%) at 5dpf
Veh. ctrl
AM251 0.5μM
AM630 5μM
AM251 0.5μM + AM630 5μM

**Figure 4.7.Effect of the endocannabinoid receptor antagonists AM251 and AM630 on development and inflation of 5 dpf larval zebrafish swim bladders.** (A) AM251 and AM630 treatments at the early stage either from 0 to 24 hpf or 24 to 48 hpf showed fully inflated to partial or non-inflated swim bladder (SB; red arrow) development in zebrafish larvae observed at 5 dpf. Representative images of three different fish, labelled 1, 2 and 3, show fully inflated, partially inflated or non-inflated swim bladders in larvae. (B–G) Rates of swim bladder inflation at 0–24 hpf (B–D: n=73, 59, 57 and 58 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+5 μM AM630 treatments, respectively) and at 24–48 hpf (E–G: n=78, 58, 61 and 60 for vehicle control, 0.5 μM AM251, 5 μM AM630 and 0.5 μM AM251+5 μM AM630 treatments, respectively). Data are presented as means±s.e.m. [a] Significantly different from vehicle control, $P<0.05$; [b] significantly different from 0.5 μM AM251, $P<0.05$; [c] significantly different from 5 μM AM630, $P<0.05$ (one-way ANOVA followed by Tukey's post hoc multiple comparisons test).

# Chapter 5. Results: Effects of FAAH and MAGL inhibition in early development of motor neurons and locomotion in zebrafish.

To further examine the role of the eCB system in zebrafish locomotor development, I used pharmacological tools to block the activity of the enzymes that degrade eCBs. FAAH and MAGL were both inhibited by JZL 195, while FAAH was selectively inhibited by URB 597 and MAGL was selectively inhibited by JZL 184 for the first 24 hrs of embryo fertilization, and then I specifically examined the developmental effects on motor neuron, neuromuscular junction (NMJ) and locomotor movement. My results discern the consequences of perturbing the CB pathway by increasing AEA and 2-AG bioavailability in zebrafish development.

## 5.1 Dose-dependent effects of FAAH and MAGL inhibition on embryo morphology at 2 dpf.

In these experiments, I attempted to investigate the effects of enhanced eCB signalling during early development in zebrafish embryos after an indirect manipulation of the eCB system. We exposed zebrafish embryos to egg water that contained inhibitors of eCB degrading enzymes, FAAH and MAGL, from 0 to 24 hpf, as shown in Fig 5.1A. The chemical compounds used in our study were: JZL 195 (dual inhibitor of FAAH and MAGL) [108], URB 597 (inhibitor of FAAH) [109] and JZL 184 (inhibitor of MAGL) [110]. The concentrations of the drugs used for the remainder of the studies were based on the preliminary results obtained from the dose-response studies in Fig 5.1C-H. The used concentrations of JZL 195, URB 597 and JZL 184 are also in a similar range reported in previous studies [91][92][89]. The representative images show embryo

morphology only at the selected doses, JZL 195 (2 μM), URB 597 (5 μM), and JZL 184 (5 μM) in Fig 5.1B.

Iused a range of FAAH and MAGL inhibitory drug concentrations, JZL 195 (0.5-10 μM), URB 597 (1-20μM), and JZL 184 (1-20μM). When examining hatching and malformation rates of embryos at 2 dpf, we observed a dose-dependent effect with increasing drug concentrations. Zebrafish eggs usually hatch between 2 dpf and 3 dpf, but I found that application of the FAAH and MAGL inhibitors resulted in delayed hatching rates (Fig 5.1C-E, $p<0.05$). For the malformation studies, I looked into deformities, such as curved bodies, pericardial edema and yolk sac edema. A dose-dependent trend in the rate of malformations was observed when embryos were exposed to JZL 195, URB 597, and JZL 184 (Fig 5.1F-H, $p<0.03$). These data clearly indicate that FAAH and MAGL inhibition (JZL 195 2 μM, URB 597 5 μM, and JZL 184 5 μM) can significantly lower the hatching rate while augmenting the rates of malformation.

## 5.2 Dual or selective inhibition of FAAH and MAGL enzyme activity by JZL 195, URB 597 and JZL 184 at 24 hpf.

Previous studies have demonstrated the actions and selectivity of JZL 195, URB 597, and JZL 184 toward FAAH and MAGL enzyme with a subsequent increase in AEA and 2-AG bioavailability in different animal models [108][109][110]. To further determine the efficacy and inhibitory activity imposed by the drugs in my animal model, I performed an *in vitro* enzyme inhibitory screening assay on zebrafish embryo protein extracts at 24 hpf (n=80-120 embryos, N=4 batches) (Fig 5.2). Prior to

performing the assay, eggs at 1-4 cell stage were treated with either vehicle control, JZL 195, URB 597 or JZL 184 and the total protein extracts were collected within 24 hours of the treatment.

I noted a significant reduction of FAAH activity in JZL 195 and URB 597 treated embryos (Fig 5.2). For example, embryos exposed to JZL 195 and URB 597 exhibited FAAH activity levels of 51 ± 7% ($p< 0.01$) and 55 ± 7% ($p<0.01$), respectively compared to 93 ± 8% activity of their vehicle control. Exposure to JZL 184 did not alter FAAH activity, which remained at a value of 84 ± 8% at 24 hpf (Fig 5.2A). With respect to the MAGL enzyme assay (Fig 5.2B), JZL 195 and JZL 184 showed a significant reduction in MAGL activity, 51 ± 5% ($p<0.01$) and 55 ± 7% ($p<0.01$), respectively. However, URB 597 did not show any significant change in MAGL activity when compared to vehicle control ($p<0.2$).

## 5.3 FAAH and MAGL inhibitory effects on motor neuron morphology and nAchRs expression at 2 dpf.

One of the main focuses of our research is to understand neurodevelopmental roles associated with locomotion in animal organisms. It is already established that eCB signalling can determine axonal growth in mice [53]. Therefore, in this part of the study, we aimed to examine the contributions of FAAH and MAGL enzymes toward axonal branching patterns emanating from primary and secondary motor neurons (Fig 5.3).

Znp1 immunostaining of primary motor neurons in zebrafish embryos (Fig 5.3B-E) revealed that exposure to JZL 195 significantly reduced both the number of primary (8

± 1.13, n=6, p<0.001) and secondary & tertiary branches (6.7 ± 1, n=6, p<0.001) extending from the main axon. A similar effect was observed on motor axonal branching patterns after URB 597 treatment whereby the number of primary branches was 9.8 ± 0.8 (n=6, p<0.004) and secondary & tertiary branches was 8.2 ± 0.9 (n=6, p<0.001). On the other hand, the selective MAGL inhibitor JZL 184 had no significant effect on neuronal branching (n=6, p< 0.6) compared to the control vehicle treatment (Fig 5.3F-G). The results indicate that either dual inhibition of FAAH and MAGL together or a selective inhibition of FAAH can interrupt primary motor neuron development.

Immunohistochemical analysis of secondary motor neuronal branches using the Zn8 antibody (Fig 5.3H-K) showed that only the dual inhibition of FAAH and MAGL enzymes resulted in abnormally shaped lateral and ventral branches growth. Embryos exposed to JZL 195 showed a drastic reduction in the proportion of normal lateral branches (39 ± 16%, n=6, p<0.01) and ventral branches (56 ± 11%, n=6, p< 0.001) emanating from secondary motor axon, compared to vehicle control (Lateral branches; 94 ± 5.6%, ventral branches; 100 ± 0%). Interestingly, embryos treated with JZL 195 exhibited a higher rate of misshapen or truncated secondary motor axonal branches. In contrast, embryos treated with URB 597 (n=6, p<0.5) and JZL 184(n=6, p<0.9) displayed no significant effects upon specific inhibition of FAAH or MAGL during secondary motor axonal growth (Fig 5.3L-M).

I then determined if FAAH and MAGL inhibition early in life could alter the expression of nAchRs at NMJs (Fig 5.4). To do that, I fluorescently labelled the postsynaptic membrane of the NMJs, using an α-BTX antibody (Fig 5.4A-D) that irreversibly binds nicotinic acetylcholine receptors. nAChRs puncta expressions was

then compared across different treatment groups to study FAAH and MAGL related effects here. The total number of α-BTX puncta per 2500 $\mu m^2$ counting showed again that only JZL 195 treated embryos express a lower number of nAChRs (65.94 ± 3.73, n=6, $p<0.001$). The total number of α-BTX puncta in URB 597 (93.2 ± 3.4, n=6, $p<0.7$) and JZL 184 (79.7 ± 4, n=6, $p<0.3$) exposed embryos were not different from vehicle controls (88 ± 3.5) (Fig 5.4E). We also further quantified the average size of the largest punctae present in the nAChR stained postsynaptic membrane at NMJs. The sizes of the nAChR puncta will likely be an indicative of developing or fully formed NMJ cluster. Consistent with previous observation, JZL 195 treatment sample expressed a largest cluster size of 14.9 ± 0.6μm (n=6, $p<0.01$), significantly different from the largest cluster size present in vehicle treated samples, 22.5 ± 2.3μm. Once again, URB 597 (n=6, $p<0.4$) and JZL 184 (n=6, $p<0.1$) treatments showed no difference here with the respect to vehicle when quantifying the largest nAChRs cluster size present at NMJs (Fig 5.4F). The outcome likely suggests that an unambiguous alteration in synaptic NMJs development might occur if FAAH and MAGL enzymes functions are altered at early stage.

## 5.4 JZL 195, URB 597 and JZL 184 induced FAAH and MAGL inhibition can affect locomotion at 5 dpf.

Since I observed a differential deficit in motor neuron morphology and nAChR expression at early life in zebrafish embryos, I was interested in determining if the observed physiological deformities can affect their locomotion behaviour at 5 dpf. Therefore to find out the FAAH and MAGL enzymes roles in locomotion development

in zebrafish, I recorded their swimming behaviour for an hour to measure movement distance (mm/hr), activity (%) and velocity (mm/s).

Larval locomotion studies demonstrated that an interruption of FAAH or MAGL enzyme can be detrimental toward movement perception (Fig 5.5A). Inhibition of FAAH by URB 597 ($n=24$, $p<0.001$) and MAGL inhibition by JZL 184 ($n=24$, $p<0.001$) tend to show a notable reduction in the swimming movement in zebrafish larvae compared to vehicle control. The obtained data also suggest that JZL 195 ($n=24$, $p<0.001$) administration can even have a more profound effect on locomotor development in zebrafish larvae (Fig 5.5B-D).

## 5.5 JZL 195 and URB 597 effects on primary motor neuron morphology are mediated through CB1R.

Considering the involvement of the eCB system in motor neuronal growth, and the effects of JZL 195 and URB 597 on motor neuron morphology (Fig 5.3B-G), I attempted to determine the mechanism by which these enzymes (FAAH and MAGL) were acting. To address this, I focused on the selective antagonism of CB1R and CB2R with AM251 and AM630, respectively. The concentrations of the CBR inhibitors used in this study are based on some preliminary results and also in the range of commonly used concentrations used previously (Fig 5.6A-M) [93][94][95][96].

Results showed that blockade of CB1R with AM 251 could prevent the JZL 195 mediated reduction in primary motor neuron branches (JZL 195, $7.8 \pm 0.8$; JZL 195 + AM 251, $11.8 \pm 0.8$, $n=6$, $p<0.04$). However, co-incubation of AM 630, a CB2R inhibitor, could not ameliorate JZL 195 induced reduction in primary branches

emanating from the primary motor axon (JZL 195, 7.8 ± 0.8; JZL 195 + AM 630, 9.5 ± 0.8, n=6, p<0.7) (Fig 5.6J-K). The CB1R blocker AM 251 also prevented the effects of JZL 195 on secondary & tertiary branches of the primary motor neuron axon (Fig 5.6L, n=6, p<0.04). Again, CB2R blocking by AM 630 could not prevent the branching deficit induced by JZL 195 (Fig 5.6M, n=6, p<0.8).

Interestingly, AM 251 also prevented the effects of URB 597 on motor neuron branching (Fig 5.6 J, L, n=6, p<0.04). But, AM 630 inhibition of CB2R was unable to prevent the URB 597 mediated effects (Fig 5.6K, M, n=6, p<0.9). Taken together, these results suggest that inhibition of the FAAH enzyme can perturb the development of primary motor neuronal morphology, most likely, due to the action of endogenous cannabinoids at CB1R.

## 5.6 JZL 195 can alter secondary motor neuronal branching patterns through CB1R.

To further investigate the eCB signalling roles on secondary motor neuron development, I co-administered either AM 251 (CB1R inhibitor) or AM 630 (CB2R inhibitor) with the dual FAAH and MAGL inhibitor, JZL 195 (Fig 5.7A-F, n=6). Importantly, AM 251 and AM 630 inhibitor treatment alone did not show any effect on their own (Fig 5.7G-J).Similarly, secondary motor axonal branching analysis revealed that only AM 251 co-treatment could inhibit JZL 195 induced branching anomalies (Fig 5.7G,I, n=6, p<0.03).

In contrast, AM 630 inhibition remained ineffective toward blocking JZL 195 actions at secondary motor axons (n=6, p<0.5). Overall, I have found that both the FAAH and

MAGL enzyme inhibition and CB1R activation are important for secondary motor neuronal development in zebrafish embryos.

## 5.7 The roles of CBRs on FAAH and MAGL inhibition mediated alteration in locomotion.

Since, a disturbance of eCB degradation enzyme (FAAH and MAGL) activity can alter locomotor development in zebrafish, I tried to determine if the effects were dependent upon CB receptors. Fig 5.8 shows that together with JZL 195, URB 597 or JZL 184, embryos were also co-treated with CB1R or CB2R inhibitors. All three different treatments JZL 195, URB 597 and JZL 184 showed a significant reduction in movement-related parameters, such as total distance moved, mean activity and mean velocity (Fig 5.8).

Interestingly, upon AM 251 co-administration, I observed that the effects of JZL 195 ($n=24$, $p<0.04$) and URB 597 ($n=24$, $p<0.03$) on locomotor activity could be rescued following CB1R blocking. Intriguingly, we could not relieve JZL 184 ($n=24$, $p<0.9$) induced effects in zebrafish larvae by inhibiting CB1Rs, thus suggesting a role of MAGL toward development of locomotion that is independent of eCB signalling pathway (Fig 5.8A,C,E). Likewise, the motor neurons, AM 630 co-treatment showed the locomotor development does not involve CB2Rs during JZL 195, URB 597 and JZL 184 treatment in zebrafish (Fig 5.8B,D,F, $n=24$, $p<0.9$).

Table 3. Effects of FAAH and MAGL inhibition during first 24 hpfin zebrafish embryos (Chapter 5).

| Figures | Findings |
|---|---|
| Fig 5.1 | 1. JZL 195, URB 597 and JZL 184 affected zebrafish gross morphology in a dose-dependent manner |
| Fig 5.3,5.6 | 2. Only dual inhibition of FAAH and MAGL by JZL 195 or selective inhibition of FAAH by URB 597 reduced primary motor neuronal branching through CB1R |
| Fig 5.3, 5.7 | 3. JZL 195 induced CB1R-mediated reduction of secondary motor neuron branching, while URB 597 and JZL 184 had no effects on secondary motor neuron |
| Fig 5.4 | 4. JZL 195 decreased nicotinic acetylcholine receptor (nAchRs) expression in neuromuscular junction (NMJs) |
| Fig 5.5 | 5. All the drugs, JZL 195, URB 597 and JZL 184 reduced zebrafish locomotor activity at 5 dpf |
| Fig 5.8 | 6. JZL 195 and URB 597 mediated effects on locomotor behaviour were mediated through CB1R, whereas JZL 184 showed a CBR-independent effects on locomotion |

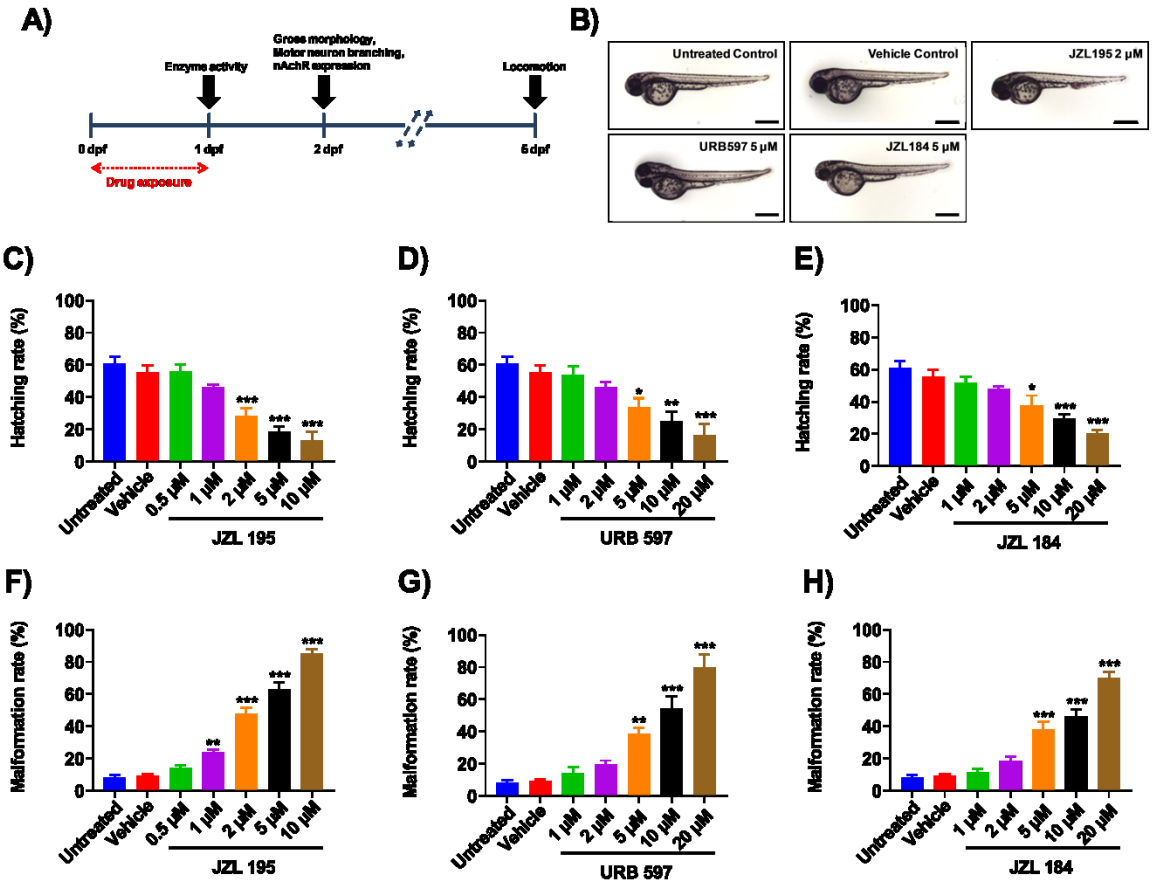
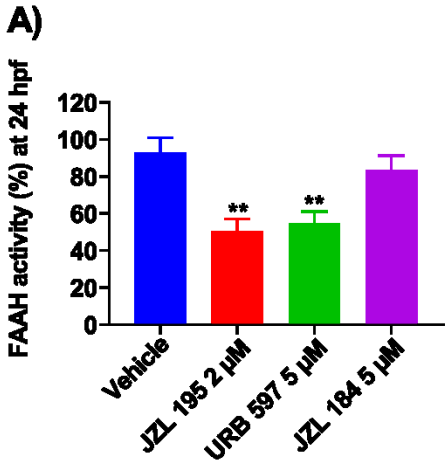

A)
Enzyme activity
Gross morphology,
Motor neuron branching,
nAchR expression
Locomotion
0 dpf
1 dpf
2 dpf
5 dpf
Drug exposure
B)
Untreated Control
Vehicle Control
JZL195 2 µM
URB597 5 µM
JZL184 5 µM
C)
Hatching rate (%)
Untreated
Vehicle
0.5 µM
1 µM
2 µM
5 µM
10 µM
JZL 195
D)
Hatching rate (%)
Untreated
Vehicle
1 µM
2 µM
5 µM
10 µM
20 µM
URB 597
E)
Hatching rate (%)
Untreated
Vehicle
1 µM
2 µM
5 µM
10 µM
20 µM
JZL 184
F)
Malformation rate (%)
Untreated
Vehicle
0.5 µM
1 µM
2 µM
5 µM
10 µM
JZL 195
G)
Malformation rate (%)
Untreated
Vehicle
1 µM
2 µM
5 µM
10 µM
20 µM
URB 597
H)
Malformation rate (%)
Untreated
Vehicle
1 µM
2 µM
5 µM
10 µM
20 µM
JZL 184

**Figure 5.1.FAAH and MAGL inhibition can affect zebrafish embryo morphology in a dose dependent manner at 2dpf.** (A) Schematic showing drug (JZL 195, URB 597 and JZL 184) exposure paradigm from 0-24 hpf on zebrafish embryo and measurement of different experimental parameters (enzymatic activity, primary or secondary motor neuron branching, nAChRs expression and locomotor activity) at 1, 2 or 5 dpf age. (B) Representative images show lateral views of control (untreated and vehicle), JZL 195 (2 μM), URB 597 (5 μM) and JZL 184 (5 μM) treated embryo morphology at 2 dpf; scale bars: 0.5 mm. (C-E) Hatching rates at different concentrations of JZL 195 (0.5 -10 μM), URB 597 (1-20 μM) and JZL 184 (1-20 μM); n=20, from four different batches(N=4). (F-H) Malformation (Pericardial & sac edema, curved tail & body) rates at different concentrations of JZL 195 (0.5 -10 μM), URB 597 (1-20 μM) and JZL 184 (1-20 μM); N=4 different experiment and n=6-20 embryos per experiment. *** Significantly different from vehicle control, $p<0.001$, ** $p<0.01$, * $p<0.05$. (One way ANOVA followed by Dunnett's multiple comparison tests).


A)
FAAH activity (%) at 24 hpf
120
100
80
60
40
20
0
**
**
Vehicle
JZL 195 2 µM
URB 597 5 µM
JZL 184 5 µM

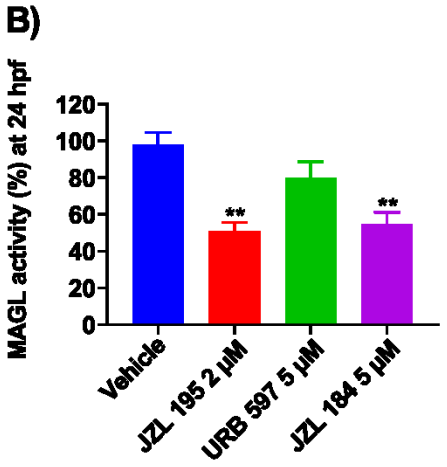

B)
MAGL activity (%) at 24 hpf
120
100
80
60
40
20
0
**
**
Vehicle
JZL 195 2 µM
URB 597 5 µM
JZL 184 5 µM

**Fig 5.2. JZL 195, URB 597 and JZL 184 mediated dual or selective inhibition of FAAH and MAGL enzyme activity at 24 hpf.** FAAH and MAGL enzyme activity (%) were measured on total protein extracts (30 µg per well) collected from treated zebrafish embryos at 24 hpf. (A) Bar charts showing dual or selected inhibition of FAAH enzyme activity (%) by JZL 195 (2 µM) and URB 597 (5 µM), respectively. (B) Bar charts showing dual or selected inhibition of MAGL enzyme activity (%) by JZL 195 (2 µM) and JZL 184 (5 µM), respectively. N=4 different experiments and n= 100-150 embryo extracts per experiment. ** Significantly different from their corresponding vehicle control, $p<0.01$. (One way ANOVA followed by Dunnett's multiple comparison tests).

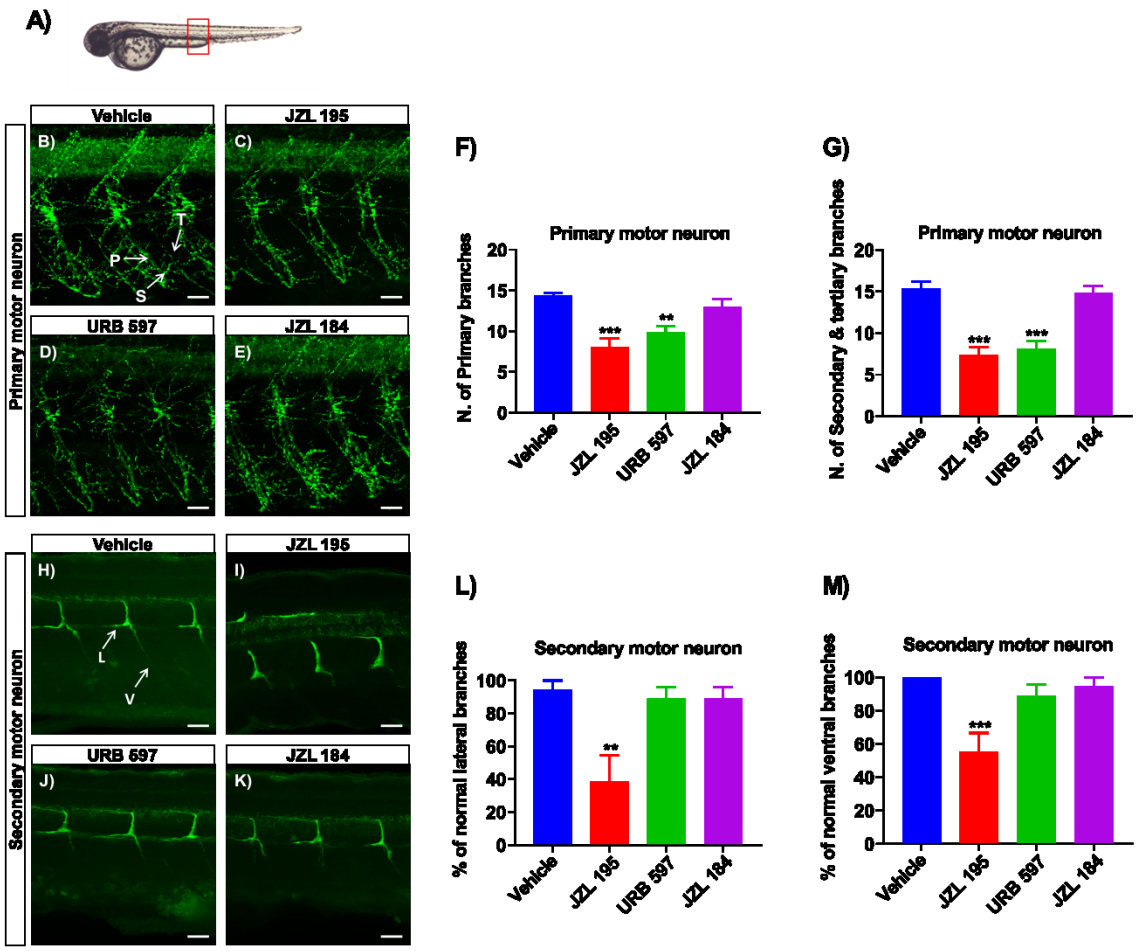
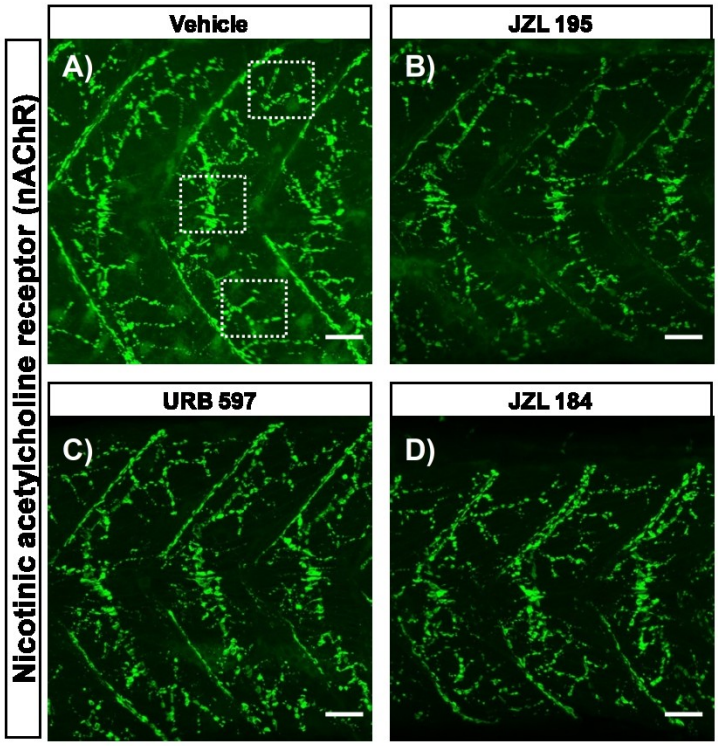

A)
Vehicle
JZL 195
URB 597
JZL 184
B)
C)
D)
E)
T
P
S
Primary motor neuron
Secondary motor neuron
H)
I)
J)
K)
L
V
F)
Primary motor neuron
N. of Primary branches
G)
N. of Secondary & tertiary branches
L)
Secondary motor neuron
% of normal lateral branches
M)
% of normal ventral branches
***
**

**Fig 5.3. JZL 195, URB 597 and JZL 184 mediated effects on branching patterns of primary and secondary motor neurons in 2 dpf embryos.** (A) Representative image shows lateral view of zebrafish embryo at 2 dpf and red box indicates the region from where the motor neuron images were acquired throughout the study. (B-E) Images show Znp1 immunostaining of primary motor neuron in zebrafish embryos upon FAAH and MAGL enzyme inhibition during first 24 hrs of development. White arrows indicate P (primary), S (secondary) and T (tertiary) branches emanating from primary motor axon. (F-G) Bar graphs show the quantification of primary, secondary & tertiary branches from primary motor axon. Branches from a single (middle) motor axon were quantified from each sample. (H-K) Images show Zn8 immunostaining of secondary motor neuron in zebrafish embryos upon FAAH and MAGL enzyme inhibition during first 24 hrs of development. White arrows indicate L (lateral) and V (ventral) branches emanating from secondary motor axon. (L-M) Bar graphs show the quantification of secondary motor axon (%) with normal lateral and ventral branches. Branches from three motor axons were quantified from each sample. The treatments were: vehicle control, JZL 195 (2 μM), URB 597 (5μM) and JZL 184 (5μM) and n=6, randomly collected from three different batches (N=3). Scale bars = 25μm. *** Significantly different from their corresponding vehicle control, $p<0.001$, ** $p<0.01$. (One way ANOVA followed by Dunnett's multiple comparison tests).


Vehicle
JZL 195
URB 597
JZL 184
A)
B)
C)
D)
Nicotinic acetylcholine receptor (nAChR)

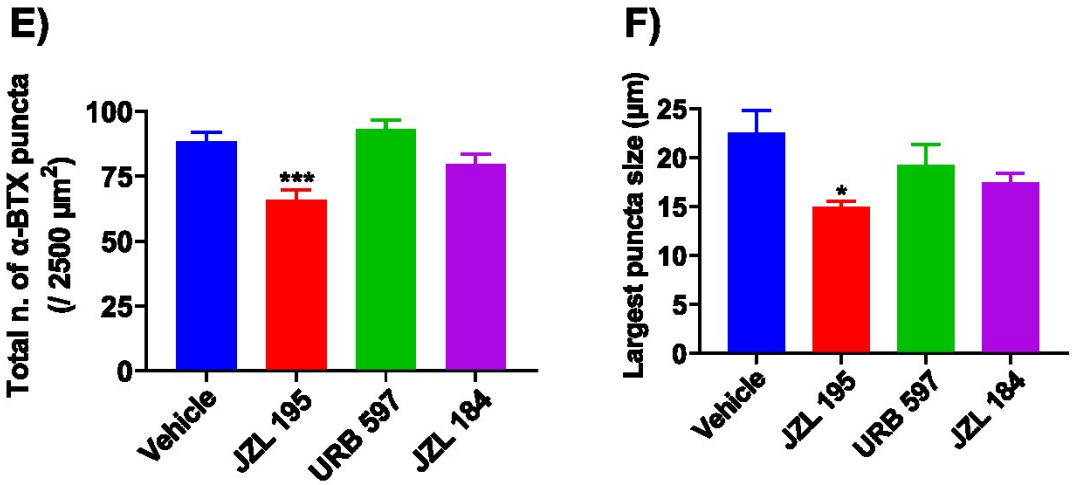

E)
Total n. of α-BTX puncta (/ 2500 μm²)
100
75
50
25
0
***
Vehicle
JZL 195
URB 597
JZL 184
F)
Largest puncta size (μm)
25
20
15
10
5
0
*
Vehicle
JZL 195
URB 597
JZL 184

**Fig 5.4. JZL 195, URB 597 and JZL 184 mediated effects on nicotinic acetylcholine receptors (nAChRs) expression in 2 dpf embryos.** (A-D) Representative images show α-BTX immunostaining of nicotinic acetylcholine receptors (nAChRs) in post synaptic NMJ in zebrafish trunk musculature upon FAAH and MAGL enzyme inhibition during first 24 hrs of development. White dashed boxes indicate the areas (1 in dorsal, 1 in middle and 1 in ventral; per 2500$\mu m^2$) from where the images were acquired. (E-F) Bar graphs show the quantification of total number of α-BTX puncta and the largest puncta size across different treatments. The treatments were: vehicle control, JZL 195 (2 μM), URB 597 (5μM) and JZL 184 (5μM) and n=6, randomly collected from three different batches (N=3). The total number and sizes were counted and averaged from three different areas of equal size (2500$\mu m^2$). Scale bars = 25μm. *** Significantly different from their corresponding vehicle control, $p<0.001$, * $p<0.05$. (One way ANOVA followed by Dunnett's multiple comparison tests).

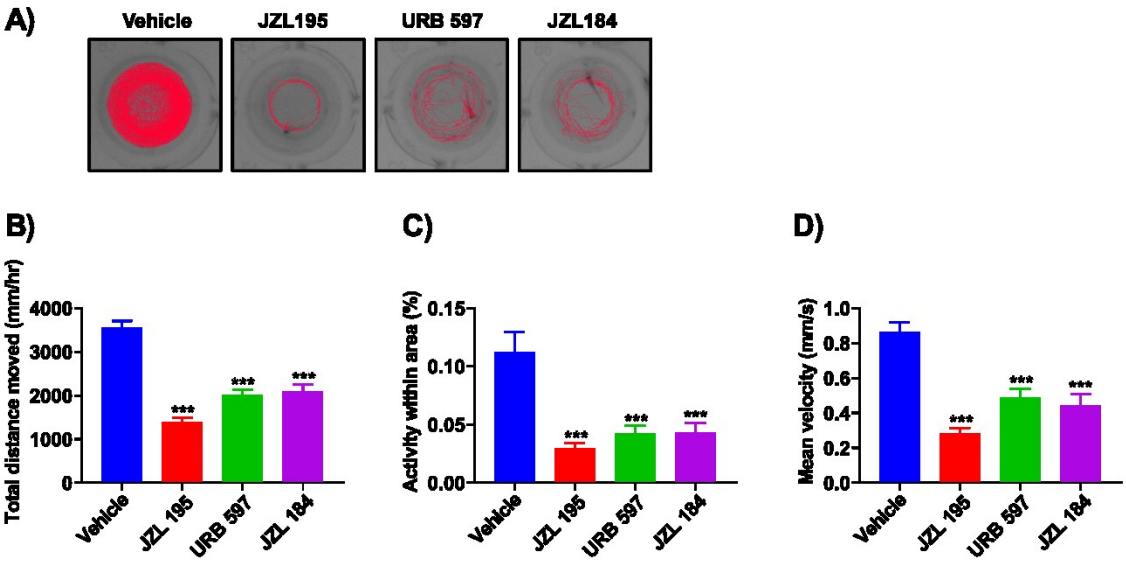
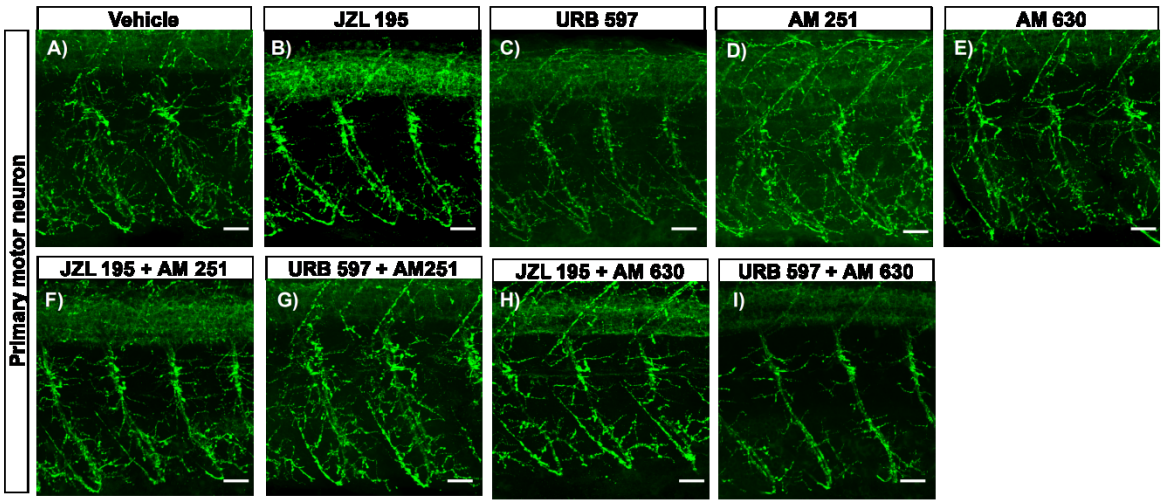
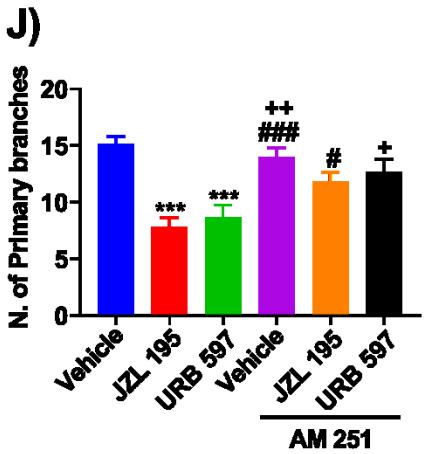
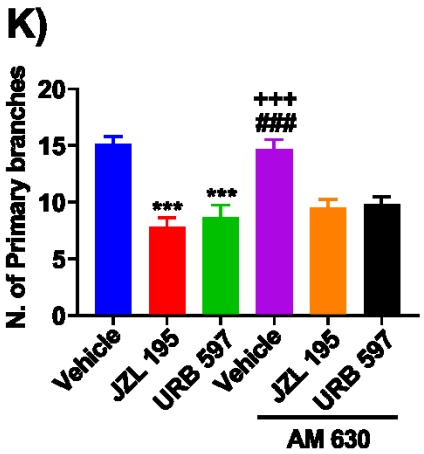
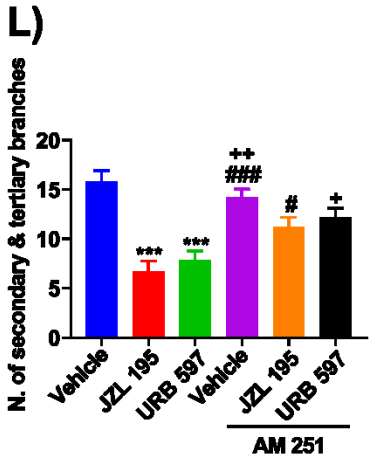

A)
Vehicle
JZL195
URB 597
JZL184
B)
Total distance moved (mm/hr)
4000
3000
2000
1000
0
***
***
***
Vehicle
JZL 195
URB 597
JZL 184
C)
Activity within area (%)
0.15
0.10
0.05
0.00
***
***
***
Vehicle
JZL 195
URB 597
JZL 184
D)
Mean velocity (mm/s)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
Vehicle
JZL 195
URB 597
JZL 184

**Fig 5.5. JZL 195, URB 597 and JZL 184 mediated effects on locomotor activity in 5 dpf embryos.** (A) Representative images show traces of larval movements at 5 dpf after vehicle control, JZL 195 (2 μM), URB 597 (5μM) and JZL 184 (5μM) treatments from 0- 24 hpf. Red line traces represent movement of larva in a single well for 60 mins time period. (B-D) Bar graphs show larval total distance moved (mm per hour), mean activity within area (%, per hour), and mean velocity (mm per second). n=24, randomly collected from three different batches (N=3). *** Significantly different from their corresponding vehicle control, $p<0.001$. (One way ANOVA followed by Dunnett's multiple comparison tests).


Primary motor neuron
Vehicle
JZL 195
URB 597
AM 251
AM 630
A)
B)
C)
D)
E)
JZL 195 + AM 251
URB 597 + AM251
JZL 195 + AM 630
URB 597 + AM 630
F)
G)
H)
I)


J)
N. of Primary branches
0
5
10
15
20
***
***
++
###
#
+
Vehicle
JZL 195
URB 597
Vehicle
JZL 195
URB 597
AM 251


K)
N. of Primary branches
0
5
10
15
20
***
***
+++
###
Vehicle
JZL 195
URB 597
Vehicle
JZL 195
URB 597
AM 630


L)
N. of secondary & tertiary branches
0
5
10
15
20
***
***
++
###
#
+
Vehicle
JZL 195
URB 597
Vehicle
JZL 195
URB 597
AM 251

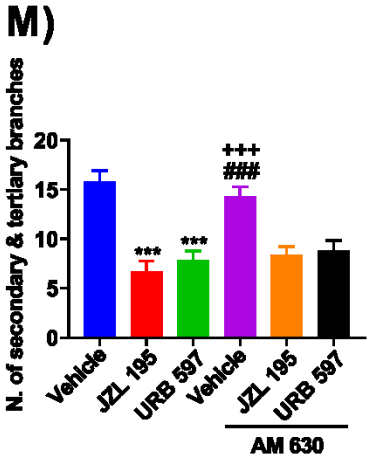
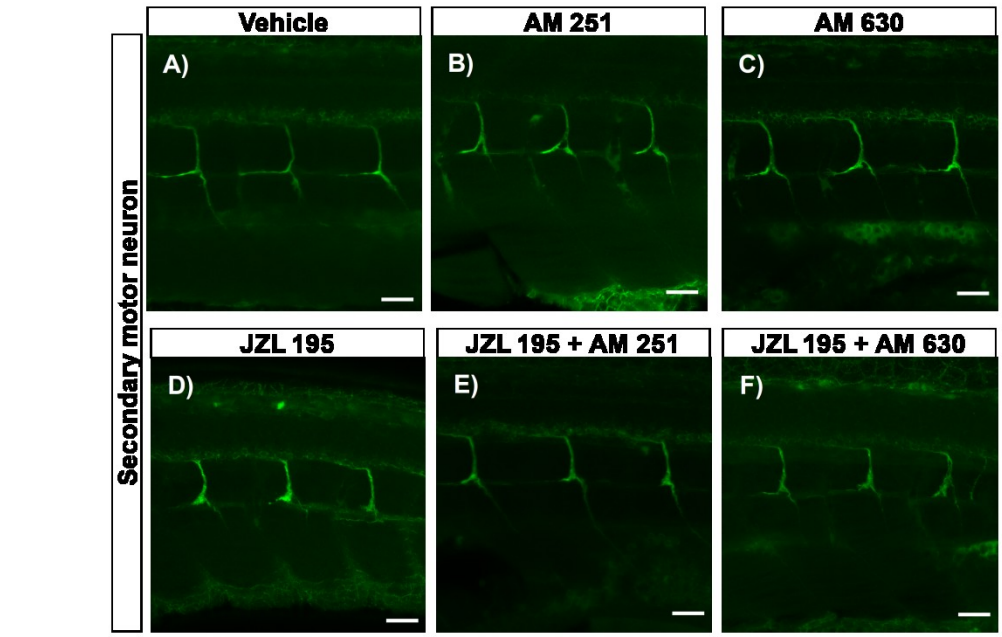

M)
N. of secondary & tertiary branches
0
5
10
15
20
***
***
+++
###
Vehicle
JZL 195
URB 597
Vehicle
JZL 195
URB 597
AM 630

**Fig 5.6. CB1 and CB2 receptor involvement in JZL 195 and URB 597 mediated effects on primary motor neuron branching in 2 dpf embryos.** (A-I) Representative images show Znp1 immunostaining of primary motor neuron in zebrafish embryos upon either JZL 195 (2 µM) and URB 597 (5 µM) treatments alone or in a combination with CB1 (AM 251; 50nM) and CB2 (AM630; 1µM) receptors inhibitor. (J,L) Bar graphs show the effects of JZL 195 and URB 597 on primary motor axonal branches during CB1 receptor inhibition by AM 251. (K,M) Bar graphs show the effects of JZL 195 and URB 597 on primary motor axonal branches during CB2 receptor inhibition by AM 630. The treatments were: vehicle control, JZL 195 (2 µM), URB 597 (5µM), AM 251 (50 nM), AM 630 (1 µM), JZL 195 (2 µM) + AM 251 (50 nM), JZL 195 (2 µM) + AM 630 (1µM), URB 597 (5 µM) + AM 251 (50 nM) and URB 597 (5 µM)+ AM 630 (1µM). n=6, randomly collected from three different batches (N=3). Scale bars = 25µm. $^{***}$ Significantly different from vehicle control, $p<0.001$. $^{###}$ Significantly different from JZL 195, $p<0.001$, $^{\#}p<0.05$. $^{+++}$ Significantly different from URB 597, $p<0.001$, $^{++}$ $p<0.01$, $^{+}$ $p<0.05$ (One way ANOVA followed by Tukey's post-hoc multiple comparison tests).


Secondary motor neuron
Vehicle
A)
AM 251
B)
AM 630
C)
JZL 195
D)
JZL 195 + AM 251
E)
JZL 195 + AM 630
F)

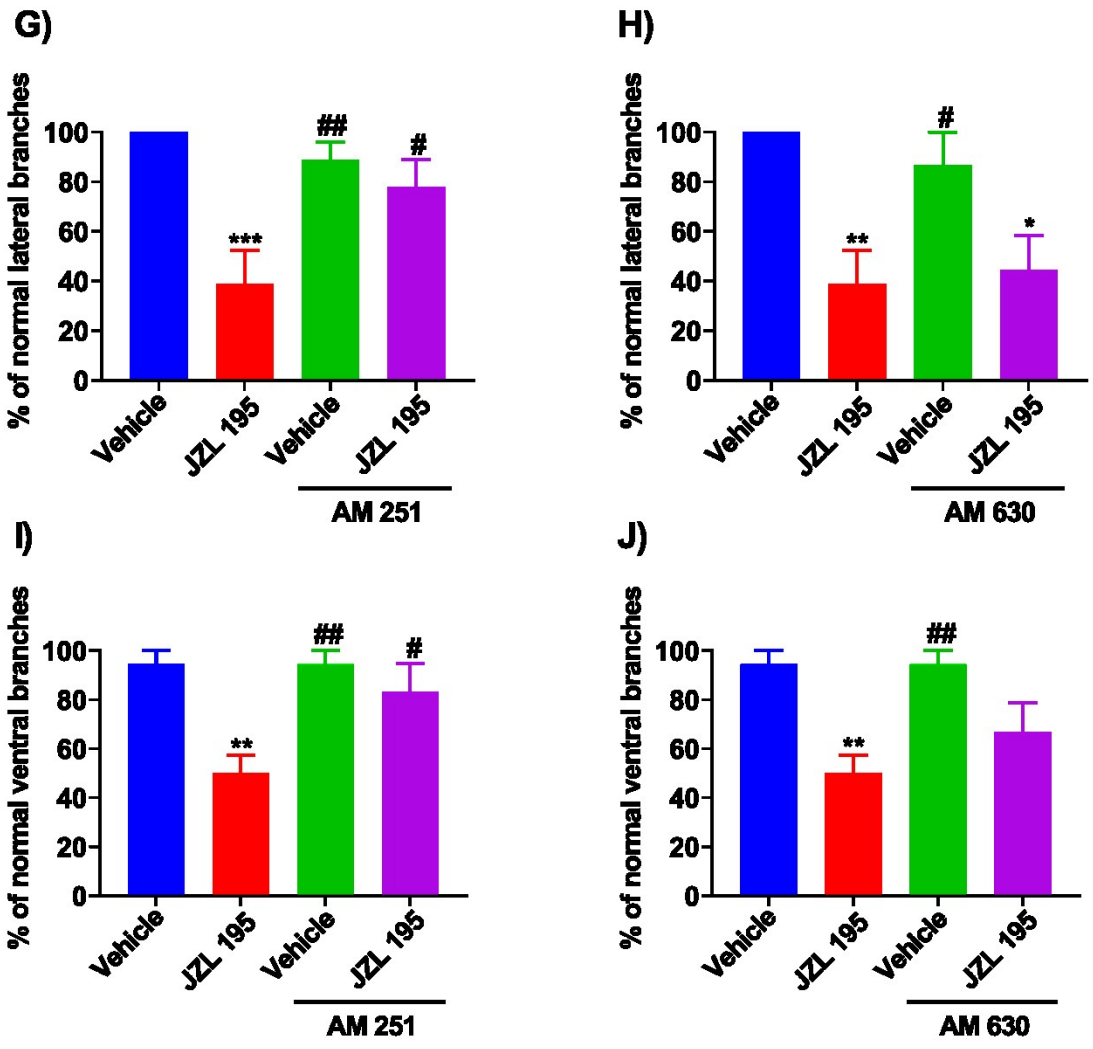

G)
% of normal lateral branches
100
80
60
40
20
0
***
##
#
Vehicle
JZL 195
Vehicle
JZL 195
AM 251
H)
% of normal lateral branches
**
#
*
Vehicle
JZL 195
Vehicle
JZL 195
AM 630
I)
% of normal ventral branches
**
##
#
Vehicle
JZL 195
Vehicle
JZL 195
AM 251
J)
% of normal ventral branches
**
##
Vehicle
JZL 195
Vehicle
JZL 195
AM 630

**Fig 5.7. CB1 and CB2 receptor involvement in JZL 195 mediated effects on secondary motor neurons branching in 2 dpf embryos.** (A-F) Representative images show Zn8 immunostaining of secondary motor neurons in zebrafish embryos upon either JZL 195 (2 μM) treatment alone or in combination with CB1 (AM 251; 50nM) and CB2 (AM630; 1μM) receptors inhibitor. (G,I) Bar graphs show the effects of JZL 195 on secondary motor axonal branches during CB1 receptor inhibition by AM 251. (H,J) Bar graphs show the effects of JZL 195 on secondary motor axonal branches during CB2 receptor inhibition by AM 630. The treatments were: vehicle control, JZL 195 (2 μM), AM 251 (50 nM), AM 630 (1 μM), JZL 195 (2 μM) + AM 251 (50 nM) and JZL 195 (2 μM) + AM 630 (1μM). n=6, randomly collected from three different batches (N=3). Scale bars = 25μm. $^{***}$ Significantly different from vehicle control, $p<0.001$, $^{**}$ $p<0.01$, $^{*}$ $p<0.05$. $^{\#\#}$ Significantly different from JZL 195, $p<0.01$, $^{\#}$ $p<0.05$. (One way ANOVA followed by Tukey's post-hoc multiple comparison tests).

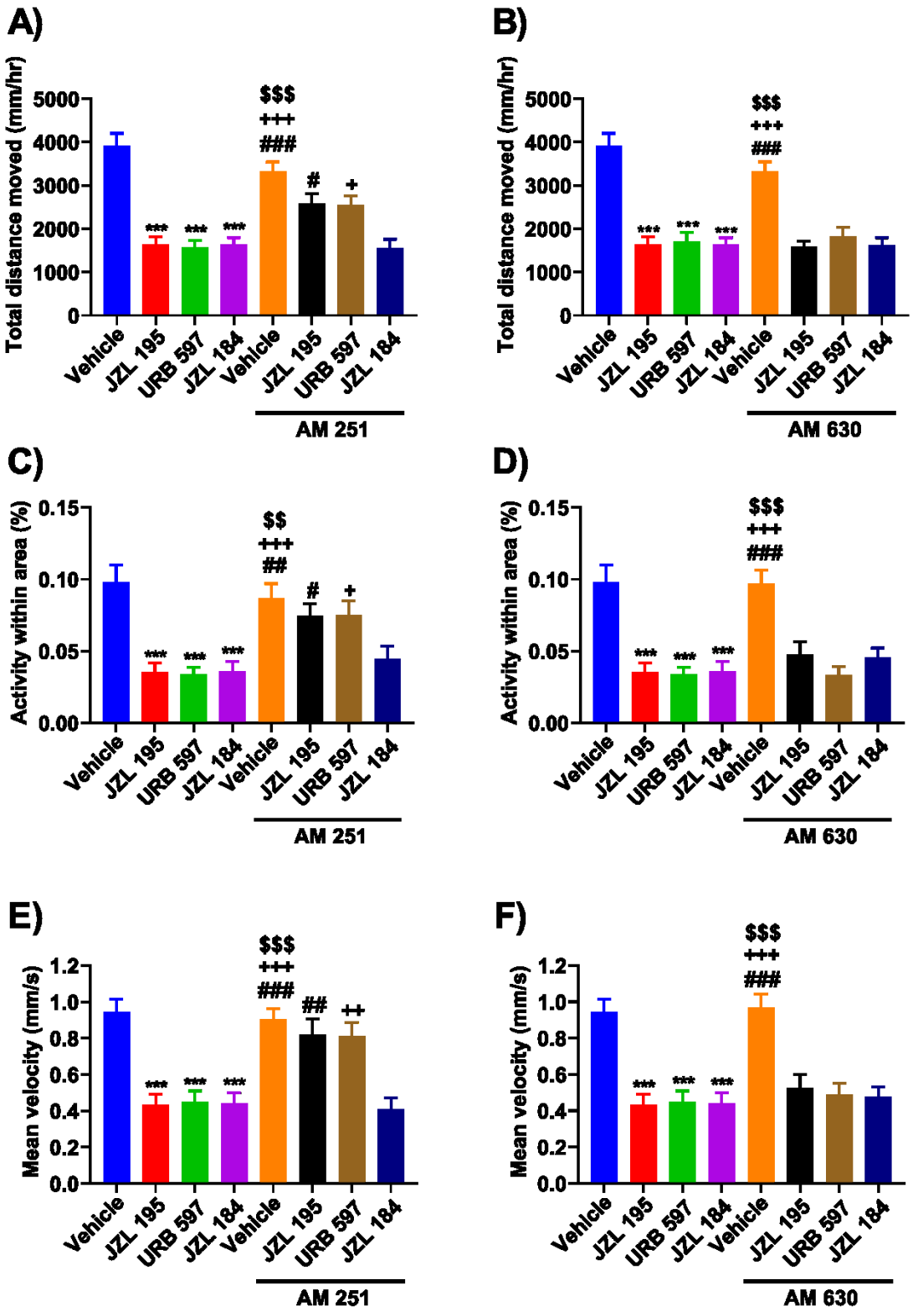

A)
Total distance moved (mm/hr)
5000
4000
3000
2000
1000
0
$$$
+++
###
#
+
***
Vehicle
JZL 195
URB 597
JZL 184
Vehicle
JZL 195
URB 597
JZL 184
AM 251
B)
Total distance moved (mm/hr)
AM 630
C)
Activity within area (%)
0.15
0.10
0.05
0.00
$$
+++
##
AM 251
D)
Activity within area (%)
AM 630
E)
Mean velocity (mm/s)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
AM 251
F)
Mean velocity (mm/s)
AM 630

**Fig 5.8. CB1 and CB2 receptor involvement in JZL 195, URB 597 and JZL 184 mediated effects on locomotor activity in 5 dpf embryos.** (A,C,E) Bar graphs show the effects of JZL 195, URB 597, and JZL 184 on locomotor activity movements during CB1 receptor inhibition by AM 251. (B,D,F) Bar graphs show the effects of JZL 195, URB 597, and JZL 184 on locomotor activity movements during CB2 receptor inhibition by AM 630. N=24, randomly collected from three different batches (N=3). $^{***}$ Significantly different from vehicle control, $p<0.001$. $^{###}$ Significantly different from JZL 195, $p<0.001$, $^{##}$ $p<0.01$, $^{#}$ $p<0.05$. $^{+++}$ Significantly different from URB 597, $p<0.001$, $^{++}$ $p<0.01$, $^{+}$ $p<0.05$. $^{\$\$\$}$ Significantly different from JZL 184, $p<0.001$ (One way ANOVA followed by Tukey's post-hoc multiple comparison tests).

# Chapter 6. Discussion

## 6.1 Brief overview

The goal of my study was to investigate the role of endocannabinoid (eCB) system pathway in embryonic and larval zebrafish locomotion and to determine how critically the pathway is involved in cellular and physiological development in an organism. The study particularly took into account the roles played by the key components of eCB into zebrafish embryogenesis, for the first 2 to 5 days of development. I examined the effects of blocking CB1R and CB2R activity during first 48 hours of post fertilization (hpf) and the subsequent effects on locomotor development. I also aimed to understand the differential actions of CB1R and CB2R in early life and to do that we perturbed CBR activity with pharmacological inhibitors selectively at either first or second day of development. I further explored the effects of inhibiting the eCB degrading enzymes, FAAH and MAGL, which augments endogenous AEA and 2-AG levels. This occurred through inhibition of FAAH and MAGL using JZL 195, URB 597 and JZL 184 and how they regulate early development in zebrafish embryos. My results support a conclusion that JZL 195 and URB 597 can affect motor neuron and locomotion development through CB1R activity. Although JZL 184 exposure did not affect motor neuron and nAChRs expression at NMJs, I observed a possible CBR- independent effects motor movements.

## 6.2 CB1R and CB2R are necessary to determine normal development and locomotion in zebrafish.

The goal of this portion of the study (chapter 3) was to investigate the role of the eCB system in early synaptic development by perturbing the system at select points. I focused on blocking the CB1 and CB2 receptors for first 48 hpf of development in zebrafish. To do this, I allowed embryos to develop in solutions containing a range of concentrations of the CB1 and CB2 receptor antagonist AM251 and AM630, respectively. My findings show that blocking cannabinoid receptors leads to alterations in hatching, survival, heart rate and locomotion and that this occurred in a dose-dependent manner. CB1R and CB2R are expressed in developing zebrafish embryos from as early as 1 h following egg fertilization [85] until adulthood. A previous research in our lab examined the effects of over-activation of the cannabinoid receptors by exposing embryos to THC and cannabidiol (CBD) for only 5 h during gastrulation [111], and found that brief activation of the CB receptors altered hatching, survival, heart rate, motor neuron development and responses to mechanical and sound stimuli. In this study, inhibition of the receptors resulted in a number of similar effects such as alterations in hatching, survival, heart rate and locomotion. CB1Rs are present from the earliest stages of neuronal life and in the developing chick they first appear in the CNS as early as the birth of the first neurons [112]. In embryonic organisms CB1 agonists and antagonists are capable of altering axonal growth [113]. The endocannabinoid system has the ability to control neuronal migration and differentiation by regulating growth factor activity. The endocannabinoid system has also been shown to modulate the expression of neurotransmitters in the basal ganglia that are involved in movement such as GABA and glutamate [114]. Interestingly the act of hatching occurs due to two main factors: 1) changes in the chemical composition of the chorion, and 2) a growing

embryo that exhibits strong movements. Thus, the locomotor deficits exhibited by the treated animals may play a significant role in hatching. My findings (suggest) that locomotion is altered following inhibition of cannabinoid receptors is consistent with a role in neuronal development. In this study we focused on locomotion at 5dpf. A surprising finding was the complete lack of activity when exposed to high concentrations of AM251 and AM630. My results suggest that heart rate is acutely sensitive to perturbations of the eCB system. When embryos were exposed to AM251 and AM630 at concentrations that were low enough to have no effect on survival, hatching or locomotion, there was still a significant effect on heart rate. Several studies have reported on the inconsistent effects of the endocannabinoid system on heart activity of adult organisms. For instance, acute activation of the eCB system has been shown to increase heart rate, decrease heart rate or have no effect [115][116][117]. Although the activation of peripherally localized CB1 receptors is thought to be responsible for depression of cardiovascular activity [118], a possible role of cannabinoid receptors on heart rate cannot be ruled out. CB1 and CB2 receptors are differentially expressed during development. In embryonic rat the CB1 receptor is expressed throughout the nervous system, eyes, digestive tract, endocrine organs and lungs before gestational stage E8 to the end of gestation around E22 [34], whereas CB2 receptors were only found in the liver from embryonic day 13 (E13), but continued to be present after birth [34]. CB2 receptor expression is present from very early in development, around the 1 hpf time point at relatively higher levels than CB1 [85]. Throughout the first 2 days, CB2 mRNA levels are much greater than CB1 mRNA, implying a greater functional role for CB2 in the early stages of development. However, by day 5 CB2 expression drops considerably and CB1 mRNA levels increase. These findings suggest that CB2 receptors may play a more prominent role

during the early stages of development compared with CB1 receptors. My results show that inhibition of either receptor in the first 2 days of life leads to similar developmental effects, but our exposure paradigm was broad and it will be of interest to narrow the exposure timeframes to shorter and more precise developmental stages. While I did not attempt to block both receptor types simultaneously, this type of experiment may offer insights into the combined roles of the cannabinoid receptors versus other potential modes of action of eCBs. For example, it is not known to what extent eCBs act via gpr18, gpr55, 5-HT2 or TRPv1/ TRPA1 during development. Additional studies will be required to parse out the nature/role of these various receptors. My results suggest that there might be some overlap in the functions of these receptors in early development. That inhibition of either CB1 or CB2Rs leads to similar effects suggests that they may have overlapping roles/functions in development. Knockout or knockdown of CB receptors may help to determine this. Future studies using morpholinos to knockdown CB1 and CB2R separately, or full knockout of these receptors using CRISPR-Cas9 will be critical to fully ascertain their roles during development. In zebrafish the eCB system has been shown to be involved in normal axonal growth and fasciculation [88]. Morpholino knockdown of the CB1 receptor resulted in aberrant patterns of axonal growth of neurons known to express cannabinoid receptors such as the reticulospinal neurons in the hindbrain. Interestingly, the axonal growth of neurons that are not thought to express CB1 receptors was unaffected [88]. Zebrafish have 2 cb2 genes but only 1 cb1 gene [119]. The expression profile of cb1 indicates that there are low levels of expression in the first 24 h of development [85][84] followed by increasing levels while cb2 gene expression indicates relatively high but fluctuating levels in the first 5 days of development, but decreasing levels thereafter [85]. My results are consistent with a previous finding that investigated the developmental effects of cannabinoids on

zebrafish embryos and larvae where 24 hpf embryos were exposed to AM251 for various time intervals ranging from 1 hour to 96 h [94]. In that study it was found that AM251exposure inhibited locomotion at high concentrations. In my study we incubated embryos immediately after egg fertilization until 48 hpf, and so even though AM 251 was present in the first 24 hours of development, because cb1 levels are relatively very low during this period [85], the effects of AM251 are likely very minimal. In larval zebrafish, anandamide has been shown to modulate lipid metabolism [120][121]. The overall results suggest that general perturbation of the endocannabinoid system, either by overactivation or inhibition of the CB receptors, alters organismal and neuronal growth. This underscores a homeostatic role for the eCB system in development.

## 6.3 CB1R and CB2R play differential roles in zebrafish motor neuron and locomotor development.

Further in my study at chapter 4, I showed that preferentially blocking the cannabinoid receptors CB1R or CB2R in zebrafish embryos during either the first or second 24 h of development can result into differential developmental effects. My findings here were crucial to understand CB1R and CB2R roles at a time dependent manner. The studies again show that blocking cannabinoid receptors leads to alterations in hatching, survival, heart rate and locomotion and that this occurred in a dose-dependent manner. My results in this section are also consistent with other studies and suggest that CB receptors play multiple roles during early development in events such as hatching, as well as in survival, heart rate, motor neuron development, responses to mechanical and sound stimuli, and ability to locomote [122][123][120]. In the present study, I again focused on motor neurons and locomotion, and show that inhibition of the CB1R or

CB2R resulted in different effects depending on the time of exposure. Both antagonists had stronger effects on hatching, survival, edema and body malformations when used at 0–24 hpf compared with the 24–48 hpf exposure. However, blocking the CB2Rs with AM630 was significantly more effective when applied in the first 24 h. This was evident when comparing the morphology at 2 dpf. In fact, the morphological deficits persisted throughout development, and by 5 dpf, animals exposed to the CB receptor blockers showed significantly more abnormalities than at 2 dpf. A previous study examined the expression of CB1 and CB2 receptors in developing zebrafish and found that CB1Rs are expressed at a very low level in the first day of development, while CB2Rs are more highly expressed [85]. Zebrafish express a single form of the CB1R [84], but two forms of the CB2R owing to gene duplication [86]. Thus, the expression of CB1R and CB2R mRNA was such that CB2 levels are greater in the early stages but CB1 levels rise dramatically by the time of hatching, implying a greater role for CB2Rs in early development, and CB1Rs in later development, when the nervous system is rapidly maturing. CB1 receptor activity has been linked to motor neuron development through a number of factors. For instance, transgenic studies using CB1R knockout mice (CB1 −/− mice) show that CB1R tune the balance between deep- and upper-layer cortical projection neurons [124]. The CB1 receptors are also coupled to the regulation of the Ctip2-Satb2 regulatory code by altering transcription, and are linked to the development of corticospinal tracts [124]. In embryonic organisms, CB1 agonists and antagonists are capable of altering axonal growth [125], and signalling through the eCB system has been shown to play chemo-attractive and chemorepulsive roles in developing cortex [51]. A number of other studies provide solid evidence for an interaction between the eCB system and several different growth factors during early development. For example, neurite outgrowth of cerebellar neurons is impacted by

CB1R activation coupled to fibroblast growth factor (FGF) receptor activity [51]. Moreover, CB1R interaction with tyrosine kinase B (TrKB) receptors in cortical interneurons is necessary for interneuron migration and specification [51]. Finally, activation of CB1R by agonists such as methanandamide increased self-renewal of neuronal-like cells in the subventricular zone via a Notch-related pathway [126]. Importantly, activation of CB1R also led to an increase in neurite growth and extension. Thus, the eCB system has the ability to control neuronal migration and differentiation by regulating growth factor activity. My finding showed that locomotion is altered following inhibition of CB receptors is in line with a role in neuromuscular development. Blocking CB1Rs in either the first or second 24 hpf results in an increase in the number of secondary and tertiary branches emanating from the main axons of primary motor neurons. This result was consistent and robust, but differed dramatically from the effects of blocking the CB2Rs, which resulted in a decrease in the number of branches. Our results suggest that there may be an interplay between the actions of these receptors on neuronal growth. With regard to the secondary motor neurons, we found that treatment with the CB1R blocker altered lateral branching in approximately 50% of the cases. Surprisingly, the ventral branches were completely unaffected by any of the treatments. Our findings compare well with other studies where the eCB has been shown to impact neuronal growth, axonal branching and pathfinding [127]. CB1 and CB2 receptors are differentially expressed during development in embryonic rats. Zebrafish express a single form of the cb1 gene and two forms of the cb2 receptor gene [128], with mRNA transcripts appearing as early as 1 hpf [85]. CB2 receptor expression is present from very early in development, around the 1 hpf time point at relatively higher levels than CB1 [85]. The time course for the expression of CB1R and CB2R show opposite patterns. Throughout the first stages of development until the end of

gastrulation, CB2 mRNA levels are much greater than CB1 mRNA, implying a greater functional role for CB2Rs in the early stages of development. This is consistent with our data, which generally show a greater effect of CB2R than CB1R in the first 24 h of development. However, by day 5, CB2R expression drops considerably and CB1 mRNA levels increase [85]. In all of our experiments, we used a combination of AM251 and AM630 to effectively inhibit both CB1R and CB2R simultaneously during the first or second 24 h of development. Only in a few instances did we find that co-application of both antagonists resulted in an augmented effect, and often co-application resulted in an effect that was intermediate compared with application of either antagonist alone. Overall, these findings suggest that CB1Rs and CB2Rs may play opposite roles during development. It is important to recognize that although we performed complete dose–responses for the effects of both antagonists on hatching, survival, morphology and cardiac activity, we chose a single dose to use throughout the remainder of the study for logistical purposes. Knockout or knockdown of the CB receptors may help to determine their roles. Future studies using morpholinos to knockdown CB1R and CB2R separately, and full knockouts using CRISPR-Cas9, will be critical to fully ascertain their roles during development. In fact, morpholino knockdown of the CB1 receptor has already been performed and shows that morphants exhibit abnormal patterns in the growth of hindbrain reticulospinal neurons that are known to express CB receptors [88]. Interestingly, the axonal growth of neurons that do not express CB1Rs was unaffected [88]. My findings are consistent with this earlier study. Moreover, our findings that inhibition of CB1R or CB2R suppressed locomotor activity is also consistent with aberrant motor neuron branching in our treated animals, and with a previous finding in which researchers exposed 1 dpf animals to AM251 for time frames ranging from 1 to 96 h [106]. In that study, the researchers found that

exposure to high concentrations of AM251 reduced locomotion. Taken together, my results suggest that alterations in the eCB system, either by an upregulation or a reduction in activity of the CB receptors, impacts growth and development. These findings highlight the differential roles played byCB1R and CB2R at various time points in the early stage of life in zebrafish.

## 6.4 FAAH and MAGL suppression affect the early development of motor neuron and locomotion via CB receptors.

The studies performed in chapter 5 shows that eCB accumulation at early life can lead to improper development of motor neurons (PMN, SMN) and alteration in locomotion in embryonic and larval zebrafish. I particularly targeted the eCB degrading enzymes, FAAH and MAGL to determine their physiological roles during developmental stages and to do that, I preferentially blocked FAAH and MAGL enzyme activity by using JZL 195 (dual FAAH/MAGL inhibitor), URB 597 (FAAH) or JZL 184 (MAGL) for the first 24 hours of embryo fertilization.

My findings indicate that either dual inhibition of FAAH/MAGL or inhibition of FAAH can reduce axonal branching associated with PMNs at 2 dpf through a CB1R-related mechanism. Furthermore, only the dual inhibitor of FAAH/MAGL (JZL 195) exhibited CB1R-mediated reduction in SMN branching and post-synaptic nAChRs expression at NMJs. Interestingly, MAGL inhibition had no effects on motor neuron development and nAChR expression at 2 dpf. Notably, all three of the enzyme inhibitors reduced larval swimming at 5 dpf. However, only the CB1R selective

inhibitor (AM 251) was able to rescue JZL 195 and URB 597 induced locomotor changes.

The eCB pathway and its key components are expressed over many developmental stages across different animal models [129][130]. In zebrafish embryos, FAAH and MAGL, the two main catabolic enzymes of eCBs can be expressed as early as 1 hour following egg fertilization. In zebrafish, FAAH appears to be present at high levels in brain, and a moderate expression has been identified in skin and testis. MAGL is highly expressed in the brain, kidney, eye and spleen [85]. It is well known that the key actions of FAAH and MAGL are to control the eCB breakdown, thus indirectly modulating eCB signalling via regulation of AEA and 2-AG availability [131].

First, I confirmed the efficacy of the drugs in my study, by testing an in-vitro 96 well-plate based FAAH and MAGL enzyme activity assay. My tests suggested that the concentrations I used for JZL 195, URB 597 and JZL 184 were potent enough to preferentially block the activity of FAAH and MAGL enzyme. The dose that I finally chose to use was based on the efficacy of FAAH and MAGL, and resulted in minimal embryo malformation rates at 2 dpf. It is reported that a reduction in MAGL activity, induced by either genetic deletion or pharmacological inhibitors can lower 2-AG hydrolysis activity by more than 80% [132][133]. Similarly, mice treated with FAAH inhibitors or FAAH (-/-) mice, experience an accumulation of 10 fold higher levels of AEA in brains with no change in 2-AG content[134][135][110]. Therefore, it is very likely that a perturbation of FAAH and MAGL will result in an upregulation of AEA and 2-AG, respectively.

Other studies have suggested that agonists and antagonists that act on CB1 receptors can impair motor neuron axon growth during development [113].eCB signalling itself may also play both chemo-attractive and chemo-repulsive roles during cortical development[51][136]. Further, hippocampal slices from rat embryos show that DAGLα (2-AG synthesizing enzyme) and CB1 can be localized to the growth cones of developing neurons, indicating a possible role of 2-AG in early development [137]. In contrast, my study found no effects of MAGL interruption on motor neuron development when axonal branching numbers and patterns were counted from PMNs. Surprisingly, pharmacological inhibition of FAAH in my study showed that it can direct axonal growth from PMNs after first two days of development in zebrafish embryos. Although not much is known about the role of FAAH in neurogenesis during early life, FAAH activity does impact both embryo development and fetal viability. For example, reduced AEA hydrolysis activity in peripheral lymphocyte can induce higher abortion rate in women, suggesting an adverse effect of AEA accumulation during pregnancy [138].

Even though there were variable effects of JZL 195, URB 597 and JZL 184 during PMN, SMN and NMJ development, all three drugs reduced zebrafish larval motor activity at 5 dpf. The locomotor deficits were more pronounced in JZL 195 treated larvae compared with the other compounds. Comparative profiling between these three inhibitors in this study allowed me to address single versus dual augmentation of AEA and 2-AG activity during locomotor development. A previous report of a study performed on mice showed that dual inhibition of FAAH/MAGL (JZL 195) or specific inhibition of MAGL (JZL 184) can induce hypomotility, but there were no effects when FAAH was inhibited (PF-3845). The authors of that study claimed that locomotion can

be modulated exclusively by a 2-AG/MAGL mechanism [108]. In contrast, my study revealed that not only MAGL but also an inhibition of FAAH can affect motor movement, thus confirming a role of both AEA/FAAH and 2-AG/MAGL in locomotor development. The difference between these two findings might be organism specific, or could be attributed to the different FAAH inhibitors in the two different studies. Additionally, my experimental model focused particularly at very early developmental phases whereas those in the rodent study did not. Exposure to JZL 195 and URB 597 resulted in aberrant branching in primary motor neurons at 2 dpf, and was followed by a deficit in larval locomotion at 5 dpf. Both the motor neuron morphological and locomotor abnormalities were prevented when CB1R was inhibited. Thus, it is highly likely that the JZL 195 and URB 597-mediated motor movement changes were due to their aberrant motor neuron development occurring through CB1R overactivation. However, no effects on axonal branching and postsynaptic development of NMJ were observed during JZL 184 induced reduction in locomotion. Therefore, it can be suggested that the JZL 184 associated changes in locomotion are not related to motor neuron or NMJ synaptic development. In addition to that, neither CB1R nor CB2R inhibition prevented the JZL 184 linked motor movement deficits. It is also possible that the motor movement changes upon 2-AG elevation are mediated via a non CB1R/CB2R mechanism.

FAAH and MAGL inhibition can also cause a significant reduction in the arachidonic acid (AA) levels in brain [139]. On the other hand, postnatal neurogenesis can also be driven by AA [140]. As a result, any contribution that may arise from depleted AA levels should be taken into account here. In addition to AEA hydrolysis, FAAH enzyme can also target N-palmitoyl ethanolamine (PEA), which is an anti-inflammatory and

analgesic compound [141]. Besides MAGL, there are other serine hydrolase enzymes, α-β-hydrolase domain 6 (ABHD6) and α-β-hydrolase domain 12 (ABHD12) that can degrade 2-AG too [132]. However, the inhibitors of the main enzymes FAAH and MAGL impact the eCB pathway such that the endogenous cannabinoid signalling is enhanced. Since the final targets of eCBs are the CB receptors (CB1 and CB2), GPR55, peroxisome proliferator-activated receptors (PPARs) and vanilloid receptors (TRPV1), FAAH and MAGL enzymes are becoming a very promising target to treat a wide range of pathological conditions[142][143][144]. Although some exogenous cannabinoids have already received market approval to be used as a therapeutic option for anorexia, neuropathic pain and multiple sclerosis treatment, they can also impose some major neurological side effects, such as cognitive and motor dysfunctions. Therefore, exogenously administered cannabinoids are not recommended for long-term treatment[145][146]. Rather, increasing endogenous cannabinoids (eCBs) via inhibition of their catabolic enzymes, FAAH and MAGL are suggested to minimize cannabinoid-like side effects [147][148]. For instance, the FAAH inhibitor, URB 597 can mitigate THC-like side effects, such as catalepsy, hyperthermia[134].

Even though recent research findings discuss in length the potential benefits of FAAH and MAGL inhibitors, chronic ablation of FAAH and MAGL have unwanted consequences [149]. For instance, one of the FAAH inhibitors, BIA 10-2474 showed serious adverse events during its phase I clinical trial in Rennes, France. Following the treatment, one participant was brain dead while two other participants exhibited major neurological side effects [149]. Another study demonstrated that a permanent elevation of AEA combined with early life stress can have a severe negative impact on the stress

response pathway through CB1R down-regulation during brain neurodevelopment [131].

On the other hand, chronic inhibition of MAGL may also antagonize the eCB system, thereby inducing a mild physical dependence and an impaired eCB mediated synaptic plasticity [150]. The studies conducted here also prove that FAAH and MAGL enzyme inhibition in early life can have serious consequences ranging from an impaired motor neuron development to motor movement abnormalities. Despite considerable efforts being carried out to understand the impacts of the eCB pathway in early development, a clear and comprehensive view of FAAH and MAGL role has not been achieved yet. Therefore, before further examining FAAH and MAGL directed therapeutic options, it would be prudent to focus more on investigating their physiological and functional roles.

## 6.5 Conclusion and future directions

Together, my studies clearly indicate a perturbation of eCB system can be critical for the embryonic development. The results suggest that CB1R and CB2R inhibition can cause aberrant innervations, motor neuron axonal deformities and would likely result into weaker and uncoordinated movement. Therefore, it is clear that both CB1R and CB2R are crucial toward determining motor neuron and locomotor development in zebrafish. Similar developmental defects were also observed when FAAH and MAGL activities were suppressed to increase AEA and 2-AG availability. I also observed an association of CB1Rs, which was confirmed when AM 251 was able to rescue the JZL 195 and URB 597 mediated effects. Therefore, my studies also suggest that AEA

accumulation possibly working through CB1R during embryogenesis can affect the development of motor neuron and locomotion.

To further extend our understanding on eCB pathway in early development, the following areas of research can be addressed:

1. My study included the application of pharmacological inhibitors to suppress CB1R and CB2R activity during evaluation of their physiological relevance in zebrafish. However, to minimize off-target effects and increased specificity toward the target receptor genes, I will design morpholinos to knockdown CB1 and CB2 receptor in zebrafish embryos, particularly at 1-4 cell stage. It would be interesting to observe if the suppression of CB1R and CB2R protein synthesis using splice-blocking morpholinos can translate a result similar to what was observed with their pharmacological inhibitors. However, the use morpholinos can also exhibit disadvantages, such as incomplete knockdown and occasional off-target effects. Notwithstanding, these studies will help us to clearly identify a profound role of CB receptors in eCB signalling pathway during early development.

2. Since, free larval activity is altered at 5 dpf in zebrafish due to a perturbation of eCB signalling; it is highly possible that the fast escape swimming behaviour at early stage might be affected too. Hence, faster C-escape response properties in zebrafish can be measured to determine any eCB mediated effect. This experiment can be easily set up upon application of a gentle stimulus at head or tail of zebrafish embryos as described previously [151]. Since C-escape responses are triggered by Mauthner cell and other reticulospinal neurons, the morphology of these neurons can also be investigated.

3. Although several studies reported that JZL 195, URB 597 and JZL 184 can preferentially block FAAH and MAGL enzymes to increase AEA and 2-AG bioavailability, my studies does not include measurement of eCB upregulation. The FAAH and MAGL enzyme assay used in our study only measures the efficiency and selectivity of the drugs. Therefore, it would be interesting to use a quantification technique, such as LC-MS to measure endogenous AEA and 2-AG levels.

4. eCB signalling pathway is a complex system but highly organized at pre and post-synaptic levels. The study in chapter 5 mainly investigates on the catabolic enzymes of the pathway, while the synthetic enzymes, NAPE-PLD and DAGL are equally important during embryogenesis. Therefore, further experimentation on eCB components by solely targeting synthetic enzymes can be exploited to understand more about this pathway.

5. The study in chapter 5 showed aggravated effects on locomotion upon FAAH and MAGL inhibition. Interestingly, neither CB1R nor CB2R could rescue the JZL 184 induced reduction in locomotion. However, not only motor neuron and NMJ development, but also other factors might play a role here to affect locomotion. Therefore it might be important to look into the muscle morphology of the embryos if it is deformed after FAAH and MAGL inhibition. Deformities at the level of red and white muscle fiber can also be involved during the deficit in locomotion.

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