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Chapter 2

Zebrafish as a High-Throughput In Vivo Model for Testing the Bioactivity of Cannabinoids

Lee Ellis

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Abstract

Zebrafish represent an established vertebrate model system that helps to bridge the research gap between cell line/invertebrate studies and mammalian systems. While the initial testing of tetrahydrocannabinol (THC) using Zebrafish occurred in 1975, zebrafish are currently a burgeoning model for testing the bioactivity of cannabinoids. Zebrafish express both CB1 and CB2 receptors along with all of the other major endocannabinoid-related genes. Zebrafish endocannabinoid gene function has been associated with addiction, anxiety, development, energy homeostasis and food intake, immune system function, learning and memory. Both adult and larval zebrafish have been used to test the therapeutic potential of THC and cannabidiol (CBD) against various disease models such as models of nociception, epilepsy, stress/anxiety and addiction. This chapter will review recent studies that have used zebrafish as a model for testing the bioactivity of cannabinoids and provide insight on potential future work in this area.

Keywords: zebrafish, cannabinoid, pain, stress, addiction, epilepsy

1. Introduction

The use of zebrafish as a vertebrate model for biological research began in the late 1960s in the lab of George Streisinger at the University of Oregon. However, it was not until the middle of the 1980s that a community of researchers working on zebrafish began to emerge. Since that time the use of zebrafish as a model organism has continued to increase. Over the past 3 decades the use of zebrafish as a model species has contributed to our understanding of developmental biology, toxicology, drug efficacy and disease.

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As a vertebrate, the zebrafish model provides more information than can be obtained from cell lines and invertebrate studies, while at the same time remaining low-cost and high-throughput compared with mammalian models. It has been estimated that screening 1 drug with rodent models costs approximately 50× more than through zebrafish assays and zebrafish testing can be done in days versus weeks to months for analogous rodent assays [1].

Another major advantage to using zebrafish as a model species is that they show high genetic homology to mammals. The sequencing of the zebrafish genome was begun in 2001 and the reference genome was published in 2013 [2]. This revealed that ~70% of human genes have at least 1 zebrafish ortholog and ~84% of genes known to be associated with human disease have a zebrafish counterpart. This then provides an important platform with which to begin to study genes linked to human disease. The initial studies that made use of zebrafish were largely entrenched in forward genetic screening, which revealed their genetic tractability and helped to lead the way to the generation of clonal lines [3]. While these original studies were begun nearly 40 years ago, since then an ever increasing number of genetic tools have been developed and used to alter the zebrafish genome such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) [4, 5]. These tools along with a fully sequenced genome provide a stage for the creation of any number of informative transgenic and knockdown/knockout lines. Along with this, zebrafish reach maturity by 90 days post fertilization and produce hundreds of eggs per clutch on a weekly basis. As screening for germline transmission is the general bottle neck in the generation of transgenic lines, the high fecundity of zebrafish allows for more rapid screening and development of transgenic lines compared with mammalian models. The use of transgenic models has a broad applicability and can potentially contribute to all facets of zebrafish research.

One of the major advantages of zebrafish as a model species is that their embryos are fertilized and develop externally providing easy access to embryos and larvae. Importantly, all major organs are formed by 1 day post fertilization (dpf) and larvae hatch from their chorion and become free swimming by 3 dpf. Larvae can live off of the nutrition provided by their yolk sac until 5 dpf, at which time they begin to feed. During this period, larvae are largely transparent making the development of organs and body patterning visible. Their rapid development and transparency provides an ideal setting for testing the effects of various compounds on normal development along with their potential acute toxicity. Standard toxicity testing models exist, including the OECD recognized fish embryo toxicity assay (FET) that tests the effects of compound exposure on normal development from 6 to 72 hpf (OECD guideline 236, adopted July 2013). The general and behavioral toxicity (GBT) assay tests the effects of compound exposure on larvae from 72 to 120 hpf [6]. Additionally, the effect of compounds on the larval heart rate has been shown to be a predictive indicator of potential bradycardia related cardiotoxicity [7]. This is important when screening neuroactive compounds as the blockage of numerous ion channels, often the target of neuroactives, can lead to arrhythmias [8]. The toxicity profiling of potential therapeutics at early stages of development allows for the identification of off-target side effects as well as the potential to calculate a therapeutic window when the toxicity profile is compared with the level of compound required to have a positive effect on disease models.

The use of zebrafish in the field of neuroscience continues to increase and a number of recent reviews have highlighted both the strengths and weaknesses of using zebrafish to study neuroactive compounds and brain disorders [9–15]. The zebrafish brain has many analogous regions to those of higher vertebrates and the complexity of both juvenile and adult zebrafish brains has been well documented [16]. In addition to brain morphology, the neurochemistry and endocrine responses linked to zebrafish neuroactivity is highly homologous to other vertebrates including the same neurotransmitters, receptors, synthetic/metabolic enzymes and hypothalamo-pituitary hormones [9–11, 15, 17–19].

It has been demonstrated that zebrafish are sensitive to a large number of neurotropic drugs including: antipsychotics, mood stabilizers, anxiolytics, antidepressants, ethanol, hypnotics, stimulants, hallucinogens, antiepileptics, analgesics and cognitive enhancers [15, 16]. In addition, both adult and larval zebrafish can be used to model numerous neural disorders including pain/nociception, anxiety, stress, PTSD, ADHD, Autism, epilepsy, learning & memory deficits, psychiatric disorders, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, schizophrenia, bi-polar disorder, addiction and brain cancer [6, 9, 11, 14–16, 20–25]. This provides *in vivo* models with which to test not only the bioactivity of various neuroactive compounds, but also allows for the testing of their potential efficacy against numerous models of disease. Use of these models can provide an indication of the level of compound required to oppose a disease phenotype, which is required for the calculation of a therapeutic window for new drugs. The disease models also provide a platform for the testing and potential re-purposing of neuroactive compounds currently on the market. Finding an effective treatment for the disease models may help to provide clues to the etiology of human disease and insights into additional therapeutic targets.

Many of the neuronal disease models developed using zebrafish are centered on the assessment of aberrant behavior in both larvae and adults, which each provide their own distinct advantages [9, 11, 15, 16]. One of the major advantages of using larvae over adults stems from their reproducible patterns of behavior and potential to be screened in a high throughput fashion. Activity patterns can be assessed in multi-well plates allowing for up to 96 larvae to be tested simultaneously using benchtop tracking systems. As mentioned, larvae become free swimming between 3 and 5 dpf and develop stereotypical behavioral and stimulus response patterns. These include their response to startling stimuli such as noise, light–dark transitions and touch. Importantly the behavioral activity patterns are highly quantifiable and can be altered by neuroactive compounds with various targets. The assessment of adult behavior, while much lower throughput, does have some advantages over larval testing as it can often provide more intricate behavioral paradigms than can be obtained with larvae. Specifically, adult behavior can be tracked in 3 dimensions and various models of learning and memory, conspecific interactions and place preference exist that are not found for larvae. Many of these models are analogous to rodent behavioral models [16].

In addition to models of behavior, as previously mentioned, larval zebrafish are nearly transparent for their first week of development and a number of transgenic lines exist that completely lack pigment. This provides unparalleled access to an intact vertebrate brain. Numerous studies have used *in situ* hybridization and immunohistochemistry to map and

profile neural activity using indicators such as *c-Fos*. Recent technical advances have allowed for a more in depth assessment of neuronal activity than is possible in mammalian systems. The assessment of neural activity has been accomplished using genetically encoded calcium indicators and whole brain imaging in immobilized larvae [26]. More recently the assessment of neural activity in freely behaving larvae at near cellular resolution has become possible [27]. The development of this new technology now allows for links to be made between localized brain activity and various behaviors and stimulus response patterns that is currently not possible with mammalian models.

While the original use of zebrafish as a model species was focused on genetics, they are currently contributing ever evolving models to the fields of developmental biology, neuroscience, molecular biology and pharmacology research.

2. Functions of the zebrafish endocannabinoid system

The initial use of zebrafish for testing the toxicity of THC occurred in 1975 [28]. However, it has only been the last 10–15 years that interest in the study of the zebrafish endocannabinoid system (ECS) has begun to grow. As outlined below, the zebrafish ECS shows genetic homology to mammalian systems and is involved in many of the same physiological processes. Importantly, the route of administration for cannabinoids to zebrafish is relatively straight forward as they can be added to the bath solution with either methanol or dimethyl sulfoxide (DMSO) as a solvent.

2.1. Gene expression patterns

The initial sequencing and mapping of the expression pattern of the CB1 receptor (CB1R) in both larvae and adults found that the zebrafish CB1R showed a 69% nucleotide identity and a 73.6% amino acid identity with the human CB1R [29]. Larvae begin to express the CB1R by the 3 somite stage of development [30] and, as expected, show a widespread and distinct expression pattern throughout the CNS (preoptic area, dorsal telencephalon, periventricular hypothalamus, tegmentum and anterior hindbrain) by 48 hpf that continues into adulthood [29, 31]. The general pattern of expression for the CB1R in the adult zebrafish brain appears to be homologous to that of mammals.

Shortly after the cloning of the CB1R zebrafish were found to express two CB2 receptor (CB2R) orthologs that showed 98% genetic identity with each other and a 39% amino acid identity with the human CB2R [32]. Importantly, similar to the CB1R, the expression patterns of the CB2R were homologous to those found in mammals with low levels in the brain and higher levels in the intestine, retina, gills, heart, muscle, pituitary and spleen.

Zebrafish also express the transient receptor potential vanilloid type 1 cation channel (TrpV1) and the G-protein coupled receptor 55 (Gpr55) early in development. Both receptors are known to bind endocannabinoids [33]. The cannabinoid receptor interacting protein (CRIP1A) is also expressed early in development [31].

In addition to the cannabinoid receptor genes, the genes responsible for the synthesis and catabolism of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) begin to

become expressed between 1 and 12 hpf and their expression levels continue to increase throughout development. These include the AEA biosynthetic enzymes *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-pld) and αβ-hydrolase domain containing 4 (Abhd4), the AEA catabolic enzyme fatty acid amide hydrolase (faah), the 2-AG biosynthetic enzyme diacylglycerol lipase α (DAGL α) and the 2-AG catabolic enzymes monoglyceride lipase (mgll) and prostaglandin-endoperoxide synthase 2 (ptgs2b) [31, 33]. Importantly the expression of these biosynthetic enzymes is accompanied by an increase in the protein levels of AEA and 2-AG [33]. The tissue distribution pattern of these enzymes is vast with high levels found in the brain, muscle, heart, intestine, eyes and reproductive organs (ovary and testis) of adults (**Table 1**) [31]. In larvae mgll, dagla and cnr1 are expressed in different regions of the brain and all 3 show some overlap with the expression pattern of CB1R [31, 33].

2.2. Growth and development

The CB1 receptor is present at early developmental stages in mammals and it has been suggested that the ECS may contribute to CNS development, such as axonal elongation, myelination, migration, cell proliferation and synaptogenesis [34].

In zebrafish it has been shown that the developmental expression of the CB1R and Dagl2α occurs at the same time during larval development, suggesting that larvae are able to both synthesize and respond to 2-AG during development [35]. The same study demonstrated that morpholino knockdown of the CB1R expression lead to aberrant patterns of axonal growth. It was subsequently shown that knockdown of Dagl2 alters axon formation in the midbrain-hindbrain region and alters different patterns of behavior which suggests that 2-AG plays a role in axon formation which subsequently affects the control of vision and movement in larvae [33]. Additionally, the highest level of CB1R expression in the developing larvae occurs at the time of hatching, which may suggest that the proper expression of the CB1R is necessary for the increase in movement that is required for the hatching process [30].

In addition to neuronal development, it has also been shown that endocannabinoid signaling is required for normal embryonic liver development and function [36]. Alteration of this normal development appears to impact the structure and function of the adult liver and may impact metabolic homeostasis. It has also been shown that the CB2R plays a role in the production, expansion and migration of hematopoietic stem cells suggesting that it may play a role hematopoiesis during development [37].

2.3. Feeding and lipid metabolism

The consumption of *cannabis* is well known to stimulate appetite and numerous animal and human studies have detailed the role that the endocannabinoid system plays in appetite regulation, weight gain, energy balance, and lipid metabolism. Rodent models have shown that both the endocannabinoids as well as THC stimulate appetite and can produce hyperphagia [38–41], while CB1R antagonists can suppress appetite [42, 43]. In humans genetic variations in the CB1 receptor and a dysregulation of the endocannabinoid system have been linked to obesity [44, 45]. The first therapeutic targeting this system that was brought to market was the inverse agonist for the CB1 receptor rimonabant. It was shown to lead to weight loss in

Distribution			
Protein name	**Abbreviation**	**High levels**	**Low levels**
Cannabinoid receptor 1	CB1R	Brain	Eyes, testis
Cannabinoid receptor 2	CB2R	Intestine, eyes, gills, heart, muscle, pituitary, kidney, spleen	Brain, testis
Transient receptor potential vanilloid type 1 cation channel	TrpV1	Sensory neurons	
G protein-coupled receptor 55A	GPR55A	Brain, spleen, testis	
Cannabinoid receptor interacting protein	(CRIP1A)	Brain, eyes, testis	
N-acylphosphatidylethanolamine-selective phospholipase D	NAPE-pld	All organs	
αβ-Hydrolase domain containing 4	Abhd4	Spleen, testis	All organs
Fatty acid amide hydrolase	faah	Brain	Skin, testis
Fatty acid amide hydrolase 2a	faah2a	Brain	Intestine, eyes, testis
Diacylglycerol lipase α	DAGL α	Brain, muscle, kidney, eyes, testis, spleen	
Diacylglycerol lipase β	DAGL β	Brain, muscle, kidney, eyes, testis	Spleen
Monoglyceride lipase	mgll	Brain, kidney, spleen, eyes	
Prostaglandin-endoperoxide synthase 2	ptgs2a	Skin, spleen, eyes	
αβ-Hydrolase domain containing 6b	Abhd6b	Not detectable	
αβ-Hydrolase domain containing 6a	Abhd6a	Intestine, liver, testis	
αβ-Hydrolase domain containing 12	abhd12	Brain, muscles, eyes, reproductive organs	Kidney, heart, intestine
Glycerophosphodiester phosphodiesterase1	gde1	All organs	
N-acylspihingosine amidohydrolase 1a	naaa1a	Reproductive organs	
Peroxisome proliferator-activated receptor αβ	pparab	Muscles, spleen, brain, heart, eyes	
Peroxisome proliferator-activated receptor γ	pparg	Muscles, spleen, testis	

Adapted from Oltrabella et al. [31].

Table 1. Organ distribution patterns of cannabinoid related proteins in adult zebrafish

overweight subjects and was marketed as a therapeutic for the treatment of obesity [46]. In line with this, it has been shown that CB1 receptor knockout animals are thinner than controls and have less adipose tissue, which is thought to relate to both decreased caloric intake as well as changes in metabolic factors [47]. Less adipose tissue may also be linked to the therapeutic potential of targeting the CB1 receptor in the treatment of obesity, as obesity in humans is linked to hepatic stenosis, which was shown to be reduced by treatment with rimonabant [44]. Since the initial development of rimonabant, the endocannabinoid system has been of interest

for the potential role its dysregulation may play in obesity [44]. Unfortunately, the side effect profile of rimonabant resulted in its withdrawal from the marketplace.

Zebrafish provide a model with which to study the role of the endocannabinoid system in appetite regulation and lipid bioaccumulation. Similar to what was found for mammals, rimonabant led to the suppression of feeding in juvenile fish [48]. In larvae it was found that rimonabant exposure led to larger yolk sacs during development, suggesting a decrease in the use of fat stores, which may be related to a decreased appetite. Exposure of adult zebrafish to melatonin, a known regulator of energy homeostasis, suppressed appetite through the downregulation of the CB1R gene expression [49]. It then appears that similar to rodents, modulation of the zebrafish endocannabinoid system can regulate appetite.

Zebrafish are also an established model for the study of lipid biology [50–52]. With respect to the endocannabinoid system, it has been shown that overexpression of the CB1R in liver leads to hepatic lipid accumulation, while suppression leads to a loss of lipid accumulation during hepatogenesis [53]. It has also been found that bisphenol A exposure produces hepatostenosis in adult zebrafish liver by increasing the liver levels of 2-AG and AEA [33]. Stimulation of the endocannabinoid system through CB1 and CB2 receptor activation can influence lipid deposition during embryogenesis through an up-regulation of the lipoprotein lipase gene [54]. Additionally, exposure to two non-psychoactive cannabinoids, namely CBD and tetrahydrocannabivarin (THCV), can lead to a decrease in intracellular lipid levels in zebrafish yolk along with human hepatocytes and adipocytes [55]. This activity does not appear to be linked to CB1R or TRPV1-R activation, but it may suggest a use for both cannabinoids in the treatment of obesity.

2.4. Learning and memory

The effects of cannabinoids on learning and memory in mammalian models is complex and often depends on the model employed and the neural pathways that are activated. However, cannabinoid exposure has been shown to lead to memory impairments for numerous rodent learning paradigms [56].

Zebrafish also have a number of different learning paradigms that include habituation learning, conditioned place preference, avoidance learning, associative learning and spatial memory tests. These learning paradigms are largely based on appetitive and/or fear conditioning [57]. Importantly, a number of these training models have been used to test the cognitive effects of various psychoactive drugs [58]. As many of these models involve the activation of different neuronal pathways, only some of which express cannabinoid receptors, the role of cannabinoid exposure on the development, retention and recall of memories can vary. One such example is a model of fear learning where adult fish were taught to associate the presentation of the alarm pheromone known as the Schreckstoff substance [59] with the presentation of a red light [60]. The response to Schreckstoff substance typically resulted in an increase in bottom dwelling and an increase in erratic movements, both of which are linked to stressful stimuli. Following training, the fish then respond to the red light stimulus, a previously inert stimulus, by showing a similar pattern of behavior. Pre-exposure to THC reduced, but did not eliminate the bottom dwelling and had only a minor effect on erratic movements

[60]. A previous study from the same group evaluating spatial memory and found that THC exposure did not affect associative memory but did impair spatial cognition and memory retrieval [60]. In addition to THC, high levels of CBD also appear to reduce memory retention in a spatial memory test [61]. While the number of studies testing the role of cannabinoids on learning and memory using zebrafish is currently limited, it appears the model has great potential in assessing the role of the endocannabinoid system in multiple aspects of learning and how this can be influenced by various cannabinoids.

3. Developing models

3.1. Pain

The treatment of chronic pain is the largest indication for medical cannabis [62–67]. This is not surprising given that endocannabinoids are known to act as retrograde transmitters blocking the transmission of pain signals at both GABAergic and glutamatergic synapses [68]. Unfortunately the etiology of pain is vast and thus there is not an all-encompassing treatment for pain. Often, an analgesic is only effective for a subset of patients or can only partially reduce pain, but cannot eliminate it [69, 70]. This often leads to multiple drugs being used in combination, which opens the door to various drug interactions that can lead to a number of potential adverse effects and an increased side effect profile.

It is now widely accepted that zebrafish have similar somatosensory systems to higher vertebrates and they can detect painful stimuli (nociception) [71–83]. The models that have been developed vary and include thermal and chemical stimuli that is either bath applied or focally by injection. The models also make use of both acute and chronic nociceptive stimuli and have been developed using larvae and adults. This then provides a number of platforms with which to test potential analgesics that may have links to different disease etiologies.

Recently, a novel model of nociception has been developed and used to test and compare a number of known therapeutics with THC and CBD. The model made use of a short-term exposure to acetic acid which led to tissue damage on the surface of zebrafish larvae and a distinct, reproducible, activity pattern that appears to indicate a multifaceted nociceptive response [83]. The study revealed that THC and CBD had different effects on the behavioral response pattern that varied from those of the known analgesics. Interestingly, of the compounds tested CBD had the most unique effect increasing the rate at which the larval activity pattern returned to that of controls. This would seem to suggest that CBD shortened the recovery from the nociceptive stimulus. This is consistent with literature that has suggested CBD is a strong candidate for pain management [84–86]. Importantly, this activity was found at a concentration of CBD that had no effect on baseline activity for controls, suggesting that there would be a low potential for side effects. One of the major issues surrounding the therapeutics currently used for pain management lies in their side effect profile, which is often vast and can range from relatively minor (constipation) to severe (addiction). This is especially evident for opioids, which are the most commonly used therapeutic for chronic pain, but have one of the highest addictive potentials [87]. While more work is required to test the effect of other cannabinoids and extracts

on the various zebrafish models of nociception, the initial indications are that zebrafish will be valuable for assessing the efficacy of potential therapeutics for pain management.

3.2. Addiction

Recent data suggests that approximately 9% of individuals that use cannabis show symptoms associated with addiction, including tolerance and withdrawal [88]. Comparatively the rate of dependence for tobacco is 67.5% and for alcohol is 22.7% [89]. Zebrafish represent an underutilized model with which to study the addictive properties of cannabinoids. While it has been demonstrated that zebrafish can be used to study the pathology of addiction to numerous drugs of abuse, including, alcohol, cocaine, morphine, nicotine, amphetamine, diazepam and salvinorin A [90–95], thus far their use to study the addictive properties of cannabinoids has been minimal. Changes in both larval and adult zebrafish behavior can be linked to numerous phenotypes associated with addiction that include conditioned place preference for drugs of abuse, relapse, changes in social behavior, along with symptoms indicating the development of tolerance and withdrawal [90, 93, 96–99]. It has also been found that the genetic pathways linked to addiction are highly conserved in zebrafish [100]. Currently, with respect to cannabinoids, only one study has shown that zebrafish larvae develop tolerance to the effects of cannabinoids after chronic exposure [101]. As the levels of THC in cannabis plant strains is varied and the refinement and extraction processes allow for other cannabinoids to be used at higher levels both medicinally and recreationally, there is a need to develop models with which to test the addictive properties of both pure cannabinoids on their own, in combination and as part of a complex mixture or extract. Zebrafish have the potential to be such a model.

3.3. Stress and anxiety

One of the known difficulties in using cannabinoids as therapeutics lies in their effect on stress and anxiety. A sought after symptom of cannabis use is the euphoric feeling that often leads to it being considered an anxiolytic. However, it has been broadly shown that as the levels of cannabinoids (specifically THC) are elevated there can be an increase in anxiety-related effects [102]. This is important not only from a side effect perspective, but also becomes an issue when cannabinoids are used to treat anxiety related disorders such as PTSD.

Zebrafish provide numerous models with which to assess stress responses in both larvae and adults. Measurements such as scototaxis (light-dark preference), thigmotaxis (wall hugging), shoaling and the amount of time spent in the bottom of a tank are used as standard measures of stress. Induction of stress can occur by chemical means such as neuro-hyperactive compounds or exposure to the alarm substance. Stress can also be induced physically by touch or following the placement of a fish in a novel setting (novel tank response). Various visible stimuli can also lead to stress responses such as changes in back ground light/dark levels or the appearance of an image of prey. All of these models seem to activate both unique and overlapping neural pathways and thus could provide insight into the mechanism of action of any potential anxiolytic effect [103–105]. An example of the use of zebrafish stress models for testing cannabinoids was outlined in a recent paper that evaluated the acute effects of both THC and CBD on larval behavior [106]. Zebrafish larvae show a preference for light and a transition from a light to a dark setting results in an increase in activity in the form of darting type movements which are

thought to be a stress response. It was found that while THC reduced the baseline activity in the light, the response to a light-dark transition was still evident. Exposure to CBD had a much different response with almost no effect on the baseline activity in the light accompanied by a concentration-dependant reduction in the light-dark transition until it was eliminated. This may suggest that CBD is showing anxiolytic effects at the levels tested [107].

It is currently felt that there is insufficient evidence to support the use of cannabis for the treatment more complex stress disorders such as PTSD [108]. Recently work has begun to establish zebrafish models of complex disorders such as PTSD [109, 110]. The development of these models will provide additional systems with which to test the efficacy of various cannabinoids and combinations thereof in the treatment of anxiety related disorders and may help provide insight into their etiology.

3.4. Uptake and metabolism

The adsorption and bioavailability of cannabinoids provides a challenge for their use as therapeutics. This is particularly true for orally ingested cannabinoids, which show low and, at times, unpredictable bioavailability [111]. The interaction between various cannabinoids along with their interaction with other therapeutics can affect their bioavailability. This is important since the effects of various cannabinoids can be bimodal (hyperactivity at low concentrations and sedation at higher concentrations). Having the ability to measure their uptake, bioaccumulation and excretion will provide insights into the exact levels found within the fish. Knowing the true concentration response profile based on the amount of compound found within zebrafish may also allow for comparisons to be made to the dose-response patterns found for mammals.

Previous work has shown that testing the uptake, metabolism and secretion of cannabinoids is possible using zebrafish larvae [106]. A number of important findings came from this study. First it was found that common pharmacokinetic cannabinoid metabolites are produced by the zebrafish larvae including the phase 1 and phase 2 metabolites hydroxylated THC (11-hyrdoxy-THC, 8-hydroxy-THC), 11-nor-9-carboxy THC, THC-glucuronide, hydroxyl-CBD and CBD-glucuronide. Both the cannabinoids and their metabolites were found to accumulate in the larvae with the metabolites eventually excreted into the bath. It was also shown that there appeared to be bioaccumulation of the cannabinoids in the larvae and a non-linear increase in the amount found in the larvae compared with the bath levels. The same study also revealed that when THC and CBD were co-administered the levels of metabolites that were produced was altered compared to when they were administered alone. This suggests that the complex chemical composition of various cannabis plant strains will also affect the normal metabolism of the individual cannabinoids. It then appears that it will be important to evaluate the uptake kinetics and metabolism of various cannabis derived compounds both alone and in combination.

3.5. Seizures

Approximately 1% of the world's population is purported to have epilepsy with 30% of those affected having multi-drug resistant epilepsy. This often leads to the requirement for strong

anti-seizure medications or drug cocktails. In general this leads to an ever increasing side effect profile that is often debilitating in and of itself. The treatment of seizures is one of the oldest reported uses of cannabis and it has recently garnered attention along with the use of pure cannabinoids (CBD) for their ability to treat severe forms of refractory childhood epilepsy (i.e. Dravet syndrome [112]). However, to date there still remains some controversy regarding its efficacy, with some groups suggesting there is no concrete evidence that it is effective [113]. While it has been purported that cannabinoids, in particular CBD, can mitigate, to some degree, epileptic seizures, unfortunately, with the exception of the childhood epilepsy study [112], the numerous human studies that have evaluated the effect of cannabinoids on seizures have either been from small sample groups, had insufficient controls or were not blinded, which confounds any potential outcomes of the studies [114]. The study was able to show that there was a reduction in seizure frequency in patients with Dravet syndrome following the addition of CBD to their current prescription regime. The one question that does remain is whether the reduction in seizures was due to the direct effect of CBD or if the effect was due to the effect of CBD on the patient's current medication.

The lack of high-quality human trials for testing anti-epileptics stem from the difficulty in properly designing and/or interpreting the results of human studies. This is partially due to the fact that most study participants are already on another anti-epileptic drug, which often varies between participants in either the drug target or the dosage. During the course of the clinical trials often the levels of either the cannabinoid or the existing therapeutic have to be modified for an individual in order to resolve issues relating to side effects. This makes the proper grouping of different treatment regimens difficult.

There are currently a number of zebrafish models of epilepsy that have been generated to provide a platform for identifying new seizure medications and potentially to understand the etiology of the disease [115]. For instance, a number of small molecules that target different receptors or ion channels can be used to induce seizures or neural hyperactivity in larvae [103, 116, 117]. These platforms provide high throughput testing models with multiple etiologies. While CBD has been shown to be effective in the treatment of some forms of epilepsy, the mechanism of action is still largely unknown. The existing zebrafish seizure models provide multiple platforms with which to evaluate both the efficacy and potentially the mechanism of action for cannabinoids in the treatment of epilepsy. The further development of these models will be of great benefit for discerning the true therapeutic potential of various cannabinoids for the treatment of epilepsy.

3.6. Smoke toxicity

Currently the main delivery method for cannabinoids both medicinally and recreationally is by the inhalation of smoke from marijuana cigarettes. This is generally because of the rapid onset of effects compared with other delivery methods, which is beneficial from the perspective of symptom relief and also allows for a level of self-regulated dose control that is not possible with other delivery methods such as edibles, which can often take up to 90 min to reach peak effect [111]. Unfortunately, some of the major caveats to an inhaled product are that dosing is often inconsistent and difficult to titrate and they have the potential to have similar health risks as are found for smoked tobacco [118]. It has been suggested that high

doses of THC containing products are associated with an increased risk of developing respiratory infections [119]. However, it has been difficult to establish a clear relationship between smoked cannabis and more severe lung disorders, such as cancer, since tobacco use is often co-morbid with cannabis. While in general the number of smoked cannabis cigarettes is lower on a per day basis, this risk cannot be overlooked. Additionally, the inhalation delivery methods for cannabis smoke are somewhat more diverse than for tobacco and include pipes, water pipes, burning on metal and vaporizers. All of these delivery methods produce smoke with its own set of chemical characteristics that depend on the temperature at which the smoke was created and any filtering that occurred before the smoke was inhaled. Processing of the plant material into oils or resins before combustion adds another level of complexity to the potential chemical diversity of the smoke that is inhaled.

Zebrafish larvae are an established model for testing vertebrate toxicity, teratogenicity and environmental risk assessment [120–122]. The use of these models has proven to be a valuable resource for testing the toxicity of various extracts and condensates obtained from tobacco cigarette smoke [6, 123, 124]. It was found that smoke from tobacco cigarettes was more toxic and produced different phenotypes than that of nicotine alone, suggesting, that the other toxic components found in tobacco smoke are having an effect. The use of the previously validated smoke testing models for testing cannabis smoke has the potential to provide information on developmental, cardiac, behavioral/neural and acute toxicity.

3.7. Multi-drug interactions and polypharmacology

One of the major complexities of working with cannabis is the fact that it is currently known to be comprised of 500+ constituents and more than 100 cannabinoid molecules [125]. The potential of interactions between many of these compounds is high and has been widely demonstrated for THC and CBD. It has been shown that the zebrafish can be used to assess the interaction of THC and CBD with respect to their effects on locomotor activity along with the uptake and metabolism of each compound [106]. Many of the aforementioned zebrafish models have the potential to be used to assess the potential interaction of THC and CBD and likely other cannabinoids. This is important as one of the next steps in the use of the zebrafish models for testing cannabinoids is to begin to test various extracts and isolates from cannabis for their bioactivity. Having an understanding of how the pure compounds interact in an *in vivo* system and how this relates to their activity in complex mixtures derived from plant material will be extremely valuable. In addition to the interaction of the various compounds found within cannabis, the use of cannabis or cannabinoids as therapeutics is also complicated by the fact that many patients are already taking a prescription drug for their particular indication. The zebrafish testing platforms appear to have the potential to characterize some of these interactions as well.

Understanding the pharmacokinetics and pharmacodynamics of cannabis is complicated by the fact that CBD (and potentially other cannabinoids) has numerous targets and mechanisms of action that contribute to its various biological effects. Similar to CBD a high percentage of neuroactive compounds have multiple targets and act on them within similar concentration ranges. This polypharmacology has both advantages and disadvantages. As many disease etiologies are not entirely known and may be multifactorial, there may be a substantial benefit of having activity on multiple targets. However, this may also increase the side effect potential and the potential

of interacting with other therapeutics. It has been suggested that large-scale zebrafish behavioral testing models can be used to help discern the polypharmacological mechanisms of neuroactive compounds [126]. This provides an ideal platform with which to test cannabis derivatives.

4. Discussion

One of the unique characteristics of researching the effects of cannabis and cannabinoids using various models and for various disease indications is that often there is already clinical data on the effects in humans. While much of the data is often anecdotal in nature, it does allow for animal model testing to be used to back validate the findings of the clinical trials. By designing top-down translational research studies we can begin to elucidate the biological basis of the clinical findings and potentially provide information on the mechanism of action of therapeutic compounds. This is particularly true for cannabis uses where the cannabinoid mechanism of action is often difficult to discern. The use of animal models of disease may help to elucidate these mechanisms and further define the etiology of the disease.

As outlined in this chapter, zebrafish have an established endocannabinoid system that is highly analogous to that of humans. Additionally, as a model system both adults and larval zebrafish provide numerous models of disease that have been shown to be efficacious for testing the therapeutic potential of novel compounds. Importantly the response patterns in these various disease models following exposure to different cannabinoids reveal unique characteristics for each cannabinoid. Thus far, only a limited number of these models have been used to test the efficacy of cannabinoids. However, the framework is in place for an expansion of the use of zebrafish in this field.

Author details

Lee Ellis

Address all correspondence to: lee.ellis@nrc-cnrc.gc.ca

National Research Council of Canada, Halifax, Nova Scotia, Canada

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