Published in final edited form as:

Toxicol Appl Pharmacol. 2019 February 01; 364: 133–143. doi:10.1016/j.taap.2018.12.021.

Multigenerational consequences of early-life cannabinoid exposure in zebrafish

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Abstract

While Δ^9 -tetrahydrocannabinol (THC) has been widely studied in the realm of developmental and reproductive toxicology, few studies have investigated potential toxicities from a second widely used cannabis constituent, cannabidiol (CBD). CBD is popularized for its therapeutic potential for reducing seizure frequencies in epilepsy. This study investigated developmental origins of health and disease (DOHaD) via multigenerational gene expression patterns, behavior phenotypes, and reproductive fitness of a subsequent F1 following an F0 developmental exposure of zebrafish (*Danio rerio*) to THC (0.024, 0.12, 0.6 mg/L; 0.08, 0.4, 2 μ M) or CBD (0.006, 0.03, 0.15 mg/L; 0.02, 0.1, 0.5 μ M). Embryonic exposure at these concentrations did not cause notable morphological abnormalities in either F0 or F1 generations. However, during key developmental stages (14, 24, 48, 72, and 96 hours post fertilization) THC and CBD caused differential expression of *c-fos*, brain-derived neurotrophic factor (*bdnf*), and deleted-in-azoospermia like (*dazl*), while in F1 larvae only CBD differentially expressed *dazl*. Larval photomotor behavior was reduced (F0) or increased (F1) by THC exposure, while CBD had no effect on F0 larvae, but decreased activity in the unexposed F1 larvae. These results support our hypothesis of cannabinoid-related developmental neurotoxicity. As adults, F0 fecundity was reduced, but this was not in F1 adults. Conversely, in the adult open field test there were no significant effects in F0 fish, but a significant reduction in the time in periphery was seen in F1s from the highest THC exposure group. The results highlight the need to consider long-term ramifications of early-life exposure to cannabinoids.

Keywords

Cannabidiol; tetrahydrocannabinol; multigenerational; development; behavior; zebrafish

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1. Introduction

As cannabinoid exposure increases among the North American population, whether for recreational or medicinal purposes, it is important to delineate potential hazards associated with cannabis use. Currently, the majority of the states have passed some form of medical marijuana legislation within the United States and a number of additional states, including Mississippi, possess laws specific for CBD drug trials and regulate the way in which patients obtain medical cannabis. While a previous study revealed roughly fifty percent of medical cannabis users were below the age 35, strikingly few demographic statistics are available concerning physician-related prescriptions to young patients (Nunberg et al., 2011). Moreover, in a recent trial roughly sixty drug-resistant epilepsy patients with a mean age of 9.7 years old and as young as 2.5 years old were exhibited a significant decrease in seizures following a newly approved CBD formulation, Epidiolex (Devinsky et al., 2017, US FDA, 2018). Therefore, it is of great importance to patients, doctors, and the toxicology community to understand the potential developmental origins of health and disease (DOHaD) following cannabis exposure during early-life stages and through critical developmental periods.

⁹-Tetrahydrocannabinol (THC) and CBD have been used for several millennia as a traditional medicine to relieve indications including insomnia, pain, nausea, satiety, and recently seizure frequency/severity (reviewed in Bostwick, 2012). THC has previously been implicated in developmental and reproductive toxicities. For example, early research in rodents and humans following chronic use, resulted in adverse effects on neurodevelopment, cognitive ability, and reproduction such as spermatogenic apoptosis (Costa et al., 2015; Nahas et al., 2002; Rodríguez et al., 2017; Schönhofen et al., 2015; Tortoriello et al., 2014; Wu et al., 2011). Very few studies have focused on CBD's safety profile; however, our recent work has depicted similar related toxicities between THC and CBD in zebrafish (*Danio rerio*) (Carty et al., 2018). These related toxicities include: morphologically deformed phenotypes, differential regulation of key developmental genes, and abnormal behavioral attributes. In view of this, we present additional evidence of toxicities resulting from THC and CBD developmental exposure as well as multigenerational consequences of an F0 only exposure.

Key developmental stages (e.g., toddler through adolescence) are sensitive to cannabinoid toxicity due to important periods of neurodevelopment and neurotransmitter maturation (Pitsilis et al., 2017). Zebrafish are useful in characterizing the molecular mechanisms of cannabinoid toxicity due to their highly conserved endogenous cannabinoid ligands and receptors to humans (Reviewed by Oltrabella et al., 2017). Developmental cannabinoid receptor 1 (CB1) knockdown studies in zebrafish, which have 72% sequence similarity to humans, have been conducted to assess its functional characteristics (Watson et al., 2008). In these studies, CB1 morphants exhibited a distinct phenotype of disorganized medial longitudinal fasciculi (MLF) and disturbed axonal growth (Watson et al., 2008). In chick and rodent studies, cannabinoid receptors, which are among a class of G-protein coupled receptors (GPCRs) that transduce their signaling via adenylyl cyclase/cAMP and additional downstream mechanisms, are very active during early development time periods (Psychoyos et al., 2012). Therefore, exogenous ligands such as THC or CBD have the ability to alter

these endogenous systems leading to adverse developmental outcomes. Adverse outcomes have been reported in zebrafish following THC exposure, resulting in cognitive impairments, including anxiety, learning, and memory (Ruhl et al., 2014; Stewart and Kalueff, 2014); however, no previous study offers a comparative analysis of THC versus CBD.

Similarly, a few studies offer insight into the reproductive and multigenerational consequences of THC exposure (Dalterio et al., 1984; Lewis et al., 2012; Thomas, 1975), but existing literature has not focused on CBD as a generational toxicant until recently (Achenbach et al., 2018; Carty et al., 2018). Evidence of multigenerational THC-mediated toxicities in rodents following an adolescent exposure to THC were reported by (Watson et al., 2015). The reduced pregnancy rates were thought to be correlated with reduced male infertility following THC exposure which is well established (Agirregoitia et al., 2010; Costa et al., 2015; Lewis et al., 2012; Nahas et al., 2002). The molecular mechanism of male infertility following THC exposure is less studied; however, CB1 and cannabinoid receptor 2 (CB2) are both expressed in human sperm cells and are implicated in the regulation of sperm motility (Agirregoitia et al., 2010). Epigenetics may play a role in the adverse multigenerational effects following cannabinoid exposure, but more attention is needed in this area of research (Watson et al., 2015).

In this study, we use zebrafish, a well-established model for assessing teratology and developmental neurotoxicity (Dach et al., 2018; Hagstrom et al., 2018; Noyes et al., 2015, Levin and Cerutti 2009). Embryos/larvae were exposed to 0.024, 0.12, 0.6 mg/L (0.08, 0.4, 2 μ M) THC, 0.006, 0.03, 0.15 mg/L (0.02, 0.1, 0.5 μ M) CBD, or 0.05% DMSO control. These concentrations were chosen to overlap with therapeutic doses for THC and CBD in humans, which vary drastically depending on the patient and application but are generally below 100 mg/kg per dose (Klotz et al., 2018, Mersiades et al., 2018). A recent publication focusing on the uptake, metabolism, and behavioral effects of THC/CBD on zebrafish larvae observed a maximal detected concentration of 3.6, 11.4, and 70.6 mg/kg for 0.1, 0.5, and 1.5 μ M THC, respectively, and 21.2 mg/kg for 0.5 μ M CBD (Achenbach et al., 2018). Embryos/larvae were collected across a concentration-response and time gradient (14, 24, 48, 72 and 96 hours post-fertilization (hpf)). At these developmental stages, RT-qPCR analyses of select genes were used to inform the mechanistic role of CBD or THC developmental toxicity. Each time point represents a key developmental stage in morphogenesis and neurogenesis signaling that could be disrupted by cannabinoid exposure.

We hypothesized that altered expression of *bdnf* (Collo et al., 2014) and *c-fos* (Stranahan et al., 2013) were indicative of neurotoxicity while altered *dazl* (Li et al., 2016) would be a reproductive biomarker gene. In addition to gene expression, we studied the effects of a F0 generation developmental exposure to THC or CBD on both F0 development and their subsequent offspring (F1; second generation) in the context of behavior and reproductive success. This project is novel due to the lack of empirical data comparing the toxicities of the two most exploited phytocannabinoids, THC and CBD. In addition, evidence of CBD's therapeutic potential (Devinsky et al., 2017) in children diagnosed with drug-resistant forms of epilepsy will inherently increase human exposure during critical developmental stages.

2. Methods and Materials

2.1. Zebrafish Care and Exposure

Tg(fli1:egfp) zebrafish were obtained through the Zebrafish International Resource Center (ZFIN, Eugene, Oregon). In brief, three breeding tanks of 60 adult zebrafish were spawned overnight containing water from an Aquatic Habitats zebrafish flow-through system (Aquatic Habitats, Apopka, Florida) within the following parameters: pH 7.5–8.0, dissolved oxygen 7.2–7.8 mg/L, conductivity 730–770 μ S, temperature 27°–29°C. The next morning, newly fertilized eggs were randomly sorted into scintillation vials, $n = 5$ vials per exposure group per time point; 15–30 fish per vial depending on exposure sampling time period (14 hpf $n \approx 30$, 24 hpf $n \approx 30$, 48 hpf $n \approx 25$, 72 hpf $n \approx 20$, 96 hpf $n \approx 15$ – at each of these time points, all embryos/larvae in a vial were collected as one replicate), containing embryo water with a salinity of 60 ppm Instant Ocean (Instant Ocean, Cincinnati, Ohio) at pH 7.4–7.7. Beginning at ~6 hpf, embryos were exposed to concentrations below the reported lethal effect levels (Carty et al., 2018) namely, 0.024, 0.12, 0.6 mg/L (0.08, 0.4, 2 μ M) THC, 0.006, 0.03, 0.15 mg/L (0.02, 0.1, 0.5 μ M) CBD, or 0.05% DMSO control water at a 0.6:1 (mL water:fish) ratio in static conditions without water changes. Separate exposures were conducted for the time point qPCR, larval behavior, reproductive and open field studies. For all endpoints measured, embryos and fish were collected from two to three separate spawning events maximized by using at least three individual spawning tanks per cohort. Embryos were then randomized in exposure vials to ensure genetic diversity. For fish raised past 96 hpf, embryos from two to three separate spawning events were kept until adulthood; these fish were randomly sub-selected for open field testing, as well as reproduction. Culture and exposure protocols were IACUC approved. The NIDA Drug Supply Program supplied all THC and CBD used throughout the developmental exposures.

2.2. GC-MS Water Analysis

Exposure concentrations of THC and CBD in water and tissue bioaccumulation were measured at 0, 4, 96 hours post-treatment (hpt), as described in (Carty et al., 2018; Kudo et al., 1995). In brief, we confirmed THC and CBD concentrations in our highest water and tissue samples (0.6 mg/L THC and 0.15 mg/L CBD (0.05% DMSO)). Concentrations were calculated based on a 5-point standard curve (0.016 – 10 mg/L). Percent recovery was $70.7 \pm 0.1\%$ for water samples and $12.4 \pm 0.02\%$ for tissue samples. Exposure water concentrations at 0 hpt were verified via GC-MS as follows: 0.6 mg/L THC (nominal) = 0.67 ± 0.05 mg/L THC and 0.15 mg/L CBD (nominal) = 0.07 ± 0.05 mg/L CBD. Water and tissue concentrations are listed in Table 1.

2.3. RT-qPCR

Samples from all exposure concentrations were assessed in the F0 generation, while only 0.05% DMSO control and the highest concentrations (0.6 mg/L THC and 0.15 mg/L CBD) were assessed in F1 larvae. Zebrafish (F0 and F1) were collected and pooled at 14, 24, 48, 72, or 96 hpf ($n = 5$ vials; 15–30 fish per vial) and placed in RNAlater (Invitrogen, Waltham, Massachusetts) and then stored at -80°C . Whole embryo/larval tissue was homogenized in TRIzol (Invitrogen #A33251) followed by RNA extraction using an RNeasy mini-kit (Qiagen #74104) in conjunction with gDNA removal via an RNase-Free DNase set (Qiagen

#79254) following manufacturer's recommended protocol. RNA was quantified and evaluated for purity (260/280 ratio = 1.9–2.1) on a NanoDrop 2000 (Thermo Fisher, Waltham, Massachusetts). RNA (250 ng) was then reverse transcribed to 10 ng/μL cDNA following manufacturer's protocol (Invitrogen kit #4304134). RT-qPCR was performed using an Applied Biosystems 7200 real-time cycler with SYBR Green detection chemistry (Applied Biosystems #4309155) following manufacturer's protocol (25 μL reaction volume). Parameters for RT-qPCR were as follows: 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by 95°C for 15 sec–60°C for 1 min–95°C for 15 sec dissociation curve. Primers were optimized as in (Corrales et al., 2014) and detailed in Table 2. Samples were screened in duplicate followed by 2⁻ C_T method evaluation (Livak and Schmittgen, 2001).

2.4. Larval Behavior

F0 generation zebrafish exposed to CBD, THC, or control (n = 24) for 96 hpf and unexposed F1 generation zebrafish (96 hpf; n = 47–48) were transferred to a 96-well plate (one fish per well) followed by a 5-minute acclimation period in full light. A single cohort from at least three separate tanks was utilized for both F0 and F1 behavioral analysis. Larvae exhibiting a lack of touch response or deleterious morphology were excluded from the behavioral assessment. Larval zebrafish were assessed in a ZebraBox utilizing ZebraLab 3.3 software (ViewPoint, Montreal, Canada). Behavior was observed via a 30 min period of light:dark cycling (0–10 min, 100% light; 10–20 min, dark, 0% light; and 20–30 min, 100% light) (Kirla et al., 2016).

2.5. Reproductive Assessment

F0 fish were developmentally exposed until 96 hpf at which time they were transferred to clean system water for rearing until adulthood. The F1 offspring were not exposed other than via the F0 germline and were raised under standard culture conditions to adulthood. At 6–9 months of age, two spawning pairs (two males and two females) for the F0 (exposed) or F1 (parents exposed) were placed into static breeding tanks (n = 3–7 spawning tanks per concentration) with 750 mL system water from our primary zebrafish culture unit (Aquatic Biosystems) and placed in a 28°C temperature controlled room. Water changes were conducted twice a week and during spawning events. Mating pairs were allowed a one-week acclimation period for which one spawning event took place. In week two we collected eggs for three consecutive days to obtain eggs from all tanks and to ensure both females per tank spawn (Reed and Jennings, 2011). Following each egg collection, debris and water were removed and replaced with clean embryo medium (60 ppm Instant Ocean; pH 7.5–7.8). Egg production was calculated as the average number of eggs per breeding tank. Collected eggs were subsequently counted and screened for fertilization, mortality, malformations, and hatching every 24 hours until 96 hpf. There was no gross incidence of malformations in any treatment relative to control. Following reproductive assessment fish were returned to their respective tanks until use for the Open Field Test.

2.6. F0 and F1 Adult Open Field Test

F0 fish were developmentally exposed and reared until adulthood, as described above. All behavioral testing was performed using apparatuses with system water from our primary

zebrafish culture unit (Aquatic Biosystems) adjusted to the holding room temperature on adult fish aged 18 months for the F0 and 12 months for the F1 fish. To circumvent the test battery effect, each test was performed on a separate cohort of naïve fish. Zebrafish behavior was recorded by trained observers that manually scored different behavioral endpoints with subsequent movement analysis by Ethovision XT13 (Noldus Information Technology, Netherlands). The zebrafish open field test is conceptually similar to the rodent open field test (Christmas and Maxwell, 1970). Open field testing is used in animal models to assess locomotive function and anxiety (Stewart et al., 2014). We used this test to quantitate locomotion, thigmotaxis, and freezing activity. In brief, an opaque white bucket of 21 cm diameter and 24 cm height was filled with water to a height of 12 cm. The tank was divided into two zones: center (13 cm diameter circle), and periphery (4 cm between the wall and the center). None of the tanks housing zebrafish treated with the same concentration (3–4 tanks per concentration) were recorded consecutively to minimize diurnal variation. Zebrafish were placed in the center of the bucket and swimming activity was immediately recorded and analyzed for 6 min using Ethovision XT13 to calculate total distance traveled, time in center, freezing frequency, and freezing duration. The bucket was recorded from above with a color GigE camera. The F0 groups tested include: 0.024, 0.12, and 0.6 mg/L THC and 0.006, 0.03, and 0.15 mg/L CBD as well as a control (0.05% DMSO) group $n = 7$ –10 per sex per treatment. The F1 groups tested include: 0.6 mg/L THC, 0.006 and 0.03 mg/L CBD, and a control group (0.05% DMSO) with $n = 8$ –10 per sex per treatment.

2.7. Statistical Analysis

RT-qPCR was assessed using one-way analysis of variance (ANOVA) on the CT followed by Dunnett's post-hoc test ($p < 0.05$; $n = 3$ –5). Each time-point was analyzed separately against their corresponding control. Reproductive success and F1 survival was also analyzed using one-way ANOVA followed by Dunnett's post-hoc test ($p < 0.05$). Error was identified as standard error of the mean (SEM) for all data points.

For larval zebrafish locomotor activity, we collected movement with a velocity > 5 mm/sec continuously throughout the 30-min period and the duration of activity every 2 minutes was averaged for each light or dark phase for statistical analysis ($n=24$ (F0); $n = 47$ –48 (F1)) using one-way ANOVA with repeated measures followed by Dunnett's post-hoc test ($p < 0.05$). Analysis of adult behavior was assessed by two-way ANOVA followed by Bonferroni post-hoc test ($p < 0.05$). A one-way ANOVA was performed for significant treatment main effects when there was no sex or interaction effects. All statistical analysis was conducted using GraphPad Prism 5.0 software.

3. Results

3.1. F0 and F1 Gene Expression

In order to assess aberrant gene expression during crucial developmental time periods, zebrafish were collected at 14, 24, 48, 72, and 96 hpf and analyzed for differential gene expression of biomarkers for neurodevelopment (*c-fos* and brain-neurotrophic factor, *bdnf*) as well as germline fitness (deleted-in-azoospermia-like, *dazl*). The expression relative to the 14 hpf control of both *c-fos* and *bdnf* increased during development for all treatments and

controls, while the expression of *dazl* changed less over time (Figure 1, A, E, and H). Time point analysis of the F0 generation revealed *c-fos* to be differentially expressed, with upregulation seen at earlier time points (14 hpf THC) and downregulation observed at later time points (48 and 72 hpf) for the 0.6 mg/L THC exposure (Figure 1). *bdnf* displayed aberrant up-regulated patterns of expression at multiple time points (48 & 96 hpf) at 0.6 mg/L THC. *dazl* also showed significant upregulation compared to control at 72 hpf following exposure to 0.6 mg/L THC (Figure 1).

CBD developmental F0 exposure led to fewer significant changes in gene expression. *c-fos* expression at 24 hpf (0.15 mg/L CBD) was significantly higher than control (Figure 2). Similar to THC, the expression relative to the 14 hpf control of both *c-fos* and *bdnf* increased across development for all treatments and controls, while the expression of *dazl* changed less over time (Figure 2, A, C, and F). Like with THC, *bdnf* was consistently up-regulated during development at 24 and 72 hpf, at the highest concentrations of CBD (0.15 mg/L; Figure 2). Conversely, at a lower concentration of 0.03 mg/L CBD a significant decrease in expression of *bdnf* at 24 hpf was observed. While *dazl* was differentially expressed throughout development following THC exposure, CBD significantly decreased *dazl* expression only at 14 hpf with 0.15 mg/L (Figure 2). As expected, F1 developmental expression patterns of *c-fos*, *bdnf* and *dazl* relative to the 14 hpf control were consistent with those in F0 (Figure 3, A, B, and C). Fewer adverse changes in gene expression were observed in the F1 generation. No significant changes in *c-fos* or *bdnf* expression in the F1s were detected. Only *dazl* expression was significantly up-regulated in the F1 generation at 48 and 96 hpf following developmental exposure to F0 parents at 0.15 mg/L CBD (Figure 3).

3.2. F0 and F1 Locomotor Larval Activity

Locomotor activity was conducted at 96 hpf in both F0 and F1 larvae. In F0 larvae, statistically significant locomotor hypoactivity in the dark phase was only observed in larvae exposed to the lowest concentration of THC (0.024 mg/L) (Figure 4). No other statistically significant changes in locomotor activity relative to controls were detected in exposed F0s. A more complicated picture with respect to parental cannabinoid exposure dose and subsequent F1 behavioral phenotypes was observed. For example, F1 larvae from parents exposed to the lowest concentration of THC (0.024 mg/L) displayed a non-significant reduction in locomotor activity during the dark phase, mirroring their parents' locomotor hypoactivity (Figure 4). However, F1 of parents exposed to 0.12 mg/L THC exhibited significantly increased locomotor activity during the dark phase, while the highest and lowest concentrations (0.024, 0.6 mg/L THC) proved to be unchanged compared to control (Figure 4). F1 larvae from 0.03 mg/L CBD exposed parents displayed a reduction in average locomotor activity during the dark phase while their parents, at the larval stage, were unaffected.

3.3. F0 Reproductive Success and F1 Survival

Following three consecutive days of egg collection from 6–9 month old F0 (developmentally exposed) or F1 (unexposed) zebrafish, we examined total offspring, survival, fertilization, and hatching rate (Table 3). When comparing total eggs produced, there was significantly reduced fecundity in F0s exposed to CBD (0.15 mg/L) and THC (0.024 and 0.12 mg/L).

Survival and fertilization percentages were not affected in the F1 generation. While we have previously observed delayed hatching rates in zebrafish larvae exposed to high concentrations of CBD (Carty et al., 2018), at these lower concentrations there were no significant changes at 48 or 72 hpf (Table 3).

3.4. F0 and F1 Adult Open Field Test

Developmentally exposed adult F0 or unexposed F1 zebrafish were observed in an open field chamber and evaluated for distance traveled, velocity, time in periphery, center visits, freezing duration, and freezing frequency. The F1 fish (parents THC treated) spent significantly less time in the periphery than the control fish (t-test, Figure 5), while no significant effect was observed for CBD-treated (F0 & F1) or THC-treated (F0) fish. An initial two-way ANOVA analysis showed no significant sex differences but did indicate a significant interaction in the CBD F1 subjects for distance moved, velocity, and freezing frequency (Supplemental Figure 1). A significant main effect for concentration was found in the CBD-treated F0 fish for the following parameters: increase in freezing duration, decrease in center visits, decrease in distance moved and velocity in the THC-treated F0 fish, but no effect for sex or interaction was detected. Therefore, subsequent analysis was done utilizing one-way ANOVA after collapsing sex that showed a non-significant dose-dependent trend of increasing freezing duration for both THC- and CBD-treated F0 fish (Supplemental Figure 2).

4. Discussion

Previously, we assessed the morphological, behavioral, and differential expression of imperative developmental genes (Carty et al., 2018) at higher concentrations bracketing the published THC LC₅₀ of 3.37 mg/L (Akhtar et al., 2018). While, the THC LC₅₀ was confirmed by our previous exposure in the Fli1:eGFP zebrafish line (3.65 mg/L), we did not expect the CBD LC₅₀ to be upwards of seven times lower. Several observations were made while characterizing the differences between THC and CBD exposure: 1) CBD caused dysmorphologies at concentrations lower than that of THC, 2) at concentrations 0.6 mg/L CBD, we observed a non-hatched phenotype, 3) larvae behavior was consistent with a predicted non-monotonic concentration response in both THC or CBD exposed fish, and 4) genes important for neurodevelopment and reproduction were differentially expressed (Carty et al., 2018). To further investigate lower dose effects following developmental cannabinoid exposure, three additional aspects were investigated here, namely: 1) what are the consequences of sub-lethal exposures of CBD and THC, 2) are genes that are critical for neuronal growth and reproductive fitness differentially regulated at critical stages of development, and 3) are there multigenerational effects on gene expression, behavior or reproduction. In order to answer these questions, we used concentrations of THC and CBD which allowed survival through adulthood. We also assessed gene expression at several time points (14, 24, 48, 72, and 96 hpf) throughout development of both F0 and F1 generations.

Our laboratory has studied *dazl* and how its expression is sensitive to xenobiotic exposure via hypermethylation patterns. Specifically, parental exposure to benzo[a]pyrene (BaP) caused hypermethylation of the *dazl* promoter and decreased *dazl* gene expression and

reduction of F1 offspring (Corrales et al., 2014). Herein, we assessed *dazl* expression as early as 14 hpf due to primordial germ cells (PGCs) completing their migration to gonadal tissue at roughly the 15-somite stage (16.5 hpf) (Dosch, 2015). Following CBD exposure in F0 larvae, *dazl* was differentially expressed in both F0 and F1 generations. In the F0s, CBD exposure caused differential expression of *dazl* during the segmentation stage (14 hpf) which can affect germline development and PGC maturation. In our reproductive success assay, we also observed a significant decrease in egg production in the F0 fish developmentally exposed to either THC or CBD. However, more studies are required to determine the correlative response between differential *dazl* expression and egg production. A future experiment determining the effect of THC or CBD on sperm concentration and motility could help clarify the reduction in fertilized egg production. It should be noted that decreased egg production was observed in CBD exposed fish at concentrations much lower than THC. In the F1 generation, *dazl* was differentially expressed; however, no significant decrease in fecundity was observed. In mice, *Dazl* mutations lead to a loss of oocyte formation in females and a complete loss of germ cells regardless of sex (Ruggiu et al., 1997). In humans, THC is an established reproductive toxicant (Lewis et al., 2012). Cannabis use in men caused a reduction in endocannabinoids (e.g. AEA and 2-arachidonoylglycerol) responsible for THC signal transduction in seminal plasma. Moreover, infertility is a direct consequence of THC exposure via the transient receptor potential cation channels receptor (Lewis et al., 2012). Studies also suggest low birth weights (< 2,500 g) and preterm deliveries in children after regular maternal cannabis use (Gunn et al., 2016). More research is required to understand the role of DAZL in human infertility following THC exposure.

In order to assess neurodevelopment and potential neuronal activity in exposed larvae, we measured *c-fos* and *bdnf* at multiple developmental time points. F0 larvae exposed to THC or CBD were sensitive to cannabinoid exposure resulting in differentially expressed *c-fos* and *bdnf* throughout development. However, unexposed F1 generation larvae expressed *c-fos* and *bdnf* similar to controls and were not sensitive to parental exposure to THC or CBD. The rationale for using *c-fos* in this study to represent neuronal activity is well established (Baraban et al., 2005; Buenafe et al., 2013; Ellis et al., 2012; Kalueff et al., 2014; Rahn et al., 2015). The expression of *c-fos* has also been directly correlated with seizure generation as well as anxiety-like behavior (Baraban et al., 2005; Jesuthasan, 2012). In this study, THC reduced the expression of *c-fos* while CBD increased expression.

To further investigate the potential for developmental neurotoxicity, we assessed *bdnf* expression throughout development. The BDNF protein is ubiquitously expressed throughout human, mammalian, and zebrafish brains and is critical for CNS function and development (Cacialli et al., 2016; Cunha et al., 2009; Murer et al., 1999). The primary pathway for BDNF protein signal transduction is through the TrkB pathway, and they together affect numerous functions including neuronal plasticity, neuronal survival, learning and memory and neurodegenerative diseases (Bekinschtein et al., 2008; Cacialli et al., 2016; Cunha et al., 2009). In zebrafish, maternally inherited *bdnf* is observed through 24 hpf followed by steady endogenous increase in expression past eight days post fertilization (De Felice et al., 2014). Similarly, we observed significant up-regulation of *bdnf* at very early stages of development (14 hpf) through stages post-embryogenesis (96 hpf). In transgenic

mice, BDNF overexpression has been directly correlated with learning and memory impairment (Cunha et al., 2009). Therefore, it is imperative to uncover the possible ramifications of increased *bdnf* expression in developing zebrafish. While the F1 generation of THC and CBD exposed zebrafish did not recapitulate a differential expression of *bdnf*, we did observe aberrant behavior in larval and adult zebrafish. The expression of *bdnf* in exposed parents may lead to an abnormal behavioral phenotype especially regarding photomotor and anxiety-like responses (Cunha et al., 2009).

Several recent studies outline the therapeutic and potential adverse effects of cannabinoids and their influence on *bdnf* expression (Butovsky et al., 2005; Campos et al., 2015; D'Souza et al., 2009; ElBatsh et al., 2012; Fishbein et al., 2012; Mori et al., 2017; Winsauer et al., 2015). Consistent with our results, moderate (1.5 mg/kg) and low (0.002 mg/kg) doses of THC upregulated *bdnf* expression in various regions of brain tissue in mice (Butovsky et al., 2005; Fishbein et al., 2012). Regulation of cannabinoid signaling via CB1 can also be modulated by BDNF, which presents broad implications in cannabinoid mediated toxicities relevant to the endocannabinoids (De Chiara et al., 2010). More research is needed to identify the exact mechanisms for the detrimental versus beneficial roles of THC and CBD.

Prior work investigating zebrafish behavior following cannabinoid exposure reported a non-monotonic (inverted U-shaped) concentration-response to CBD exposure (Nazario et al., 2015). Our goal was to reproduce these behavior abnormalities in larval zebrafish following exposures to sub-lethal THC or CBD. We also wanted to study the likelihood of F0 behavioral phenotypes carrying-over to the F1 larvae that lacked direct cannabinoid exposure. In our previous study, THC and CBD caused a concentration-dependent reduction in locomotor behavior in 96 hpf zebrafish leading to significant locomotor hypoactivity at the highest concentrations of THC and CBD (Carty et al., 2018). In the current study, we observed significant locomotor hypoactivity, but at very low concentrations of THC in the F0 generation which supports the non-monotonic concentration response prediction. Additionally, the F1 generation displayed a locomotor hyperactivity (THC) and locomotor hypoactivity (CBD) phenotype at concentrations not typical of a monotonic dose response. Reduced swimming distance and velocity in a novel tank test were also seen in adult zebrafish exposed to 40 mg/L CBD (Jensen et al., 2018). Brigante et al., (2018) also explored the ramifications of CBD on behavior and development. In their study, motor activity of zebrafish embryos was significantly increased at 24 hpf following exposure to 20–300 µg/L CBD, but no changes were observed at 48 hpf at concentrations as high as 300 µg/L.

Open field adult behavior was measured in order to better understand the potential anxiogenic or anxiolytic effects of cannabinoids across generations. A CBD dose-dependent anxiolytic response has been reported in numerous studies (reviewed by: Crippa et al., 2018). Thigmotaxis, swimming in close proximity to tank walls, is an established behavioral characteristic of anxiety in fish (Baiaamonte et al., 2016; Nielsen et al., 2018) and is increased in high-anxiety states (Stewart et al., 2014). Time in periphery (thigmotaxis), defined here as the area within 4 cm of the walls, was significantly decreased in the F1 fish whose parents were exposed to 0.6 mg/L THC. This result suggests generational effects observed in offspring that were not apparent in the F0 generation. Freezing behavior in

zebrafish is implicated as a fear response mechanism and is a measure of anxiety (Egan et al., 2009). We observed a trend of dose-dependent increase in freezing duration following CBD or THC exposure in F0 adults. Our results support the hypothesis that abnormal expression of *c-fos* or *bdnf* in the F0 generation may lead to cognitive impairments in the F1 generation. However, measuring cortisol levels in zebrafish in future experiments would help elucidate if the results in behavior are due to cognitive impairment or a reduction in anxiety.

Herein, we established for the first time, a multigenerational zebrafish model for investigating the potential developmental toxicities following THC or CBD exposure. This study is relevant due to the increased therapeutic treatment opportunities of cannabinoids in young children for the treatment of devastating forms of epilepsy. Our initial hypothesis was that THC would pose a significantly greater risk to reproduction and neurodevelopment than CBD. While we did observe reproductive deficiencies and molecular clues for neurotoxicity following THC exposure, we also observed more pronounced adult behavioral and gene expression abnormalities following CBD exposure. These results indicate a need for additional cannabis-based studies in order to better understand the mechanistic properties of cannabinoids and to realize the potential risks of its therapeutic application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We owe gratitude to The University of Mississippi – School of Pharmacy for their continued support and to The University of Mississippi – Center of Research Excellence in Natural Products Neuroscience for providing initial support for this project. We would also like to thank James Gledhill, Patricia Ward, Alex Fan, and Kayla Frost for their technical assistance in collecting and counting offspring as well as helping with the timed exposures.

Funding sources

Research reported in this publication was supported by the National Institute on Drug Abuse and the National Institute of General Medical Sciences under Award Numbers R21DA044473–01 and P20GM104932, respectively. This content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Highlights

- CBD caused adverse developmental outcomes at much lower concentrations than THC
- THC and CBD exposure led to expression changes in developmentally imperative genes
- Sub-lethal concentrations of THC and CBD affected larval and adult zebrafish behavior
- THC and CBD decreased zebrafish offspring in the F0 but not the F1 generation

Figure 1.

THC time point gene expression (F0). Developmental stage specific differential expression of *c-fos* (A-D), *bdnf* (E-G), and *dazl* (H-I) following exposure to THC or DMSO. Line graphs depict the developmental expression patterns normalized to their corresponding 14 hpf. Bar charts represent time points that were statistically significant throughout development normalized to control (note all bar graphs have the same y-axis with the exception of *bdnf* 48 hpf). Exposure groups were normalized to 18s and the CT values were assessed for significance by one-way ANOVA with Dunnett's post-hoc test ($p < 0.05$) (*) ($n = 5$). Bars represent mean fold change ($2^{-C_T} \pm \text{SEM}$).

Figure 2. CBD time point gene expression (F0). Developmental stage specific differential expression of *c-fos* (A,B), *bdnf* (C-E), and *dazl* (F,G) following exposure to CBD or DMSO. Line graphs depict the developmental expression patterns normalized to their corresponding 14 hpf. Bar charts represent time points that were statistically significant throughout development normalized to control. Exposure groups were normalized to 18s and the CT values were assessed for significance by one-way ANOVA with Dunnett's post-hoc test ($p < 0.05$) (*). ($n = 5$). Bars represent mean fold change ($2^{-C_T} \pm \text{SEM}$).

Figure 3.

THC and CBD developmental gene expression (F1). F1 developmental stage specific differential expression of *c-fos*, *bdnf*, and *dazl* following exposure to THC, CBD, or DMSO via RT-qPCR. Exposure groups were normalized to 18s and the CT values were assessed for significance by a student's t-test ($p < 0.05$) ($n = 5$). For reference, line graphs depict the developmental expression patterns normalized to their corresponding 14 hpf, while the bar charts represent time points that were statistically significant. THC (0.6 mg/L) did not provide any multigenerational differential expression (F1) following a development exposure to F0 parents. Bars represent mean fold change ($2^{-C_T} \pm \text{SEM}$).

Figure 4.

THC and CBD larval zebrafish behavior in F0 (A,B) and F1 (C,D). F0 and F1 larval zebrafish behavior were assessed at 96 hpf for locomotor activity in a ViewPoint ZebraBox for a total of 30 minutes (0 – 10 min, light: 10 – 20 min, dark (grey box): 20 – 30 min, light) as described in (Carty et al., 2018). Locomotor durations greater than a velocity of 5 mm/sec were averaged over the duration of the dark phase F0 (E,F), F1 (G,H), and analyzed for statistical significance using one-way ANOVA with repeated measures followed by Dunnett's post-hoc test ($n = 24$ (F0), $n = 47-48$ (F1), * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). Concentrations along with (n) are provided in the graphs above. Bars represent mean $\pm$ SEM.

Figure 5.

CBD open field test. Summary of behavioral data collected from a 6 minute open field test ($n = 12-18$) from adult, 18-month old F0 (developmentally exposed) or 12-month old F1 (unexposed) zebrafish treated with increasing concentrations of CBD. Statistical analysis was performed using a one-way ANOVA followed by Dunnett's post-hoc test for every group other than the F1 THC where a t-test was utilized ($p < 0.05$). Each bar represents the mean $\pm$ SEM.

Table 1.

Cannabinoid Water and Tissue Concentrations. Data presented as mean measured concentration $\pm$ SD of 2–3 replicates.

Compound	Time (hpt)	Measured Water Concentration mg/L	Measured Tissue Concentration $\mu\text{g/g} \pm \text{SD}$
Control	0	N.D.	N.D.
	4	N.D.	N.D.
	96	N.D.	N.D.
0.6 mg/L THC	0	0.67 ± 0.05	-
	4	0.59 ± 0.05	3.84 ± 0.31
	96	0.12 ± 0.01	5.57 ± 1.41
0.15 mg/L CBD	0	0.07 ± 0.05	-
	4	0.06 ± 0.04	2.40 ± 0.17
	96	0.01 ± 0.002	8.61 ± 4.69

N.D. = not detected

hpt = hours post-treatment

Table 2.

RT-qPCR Primers

Gene	Accession	Primer sequence	r ²	Efficiency %
c-fos	BC065466	F: 5'-CAC CGA TAC ACT GCA AGC TGA A-3' R: 5'-CAG GTT GGC GAT GTC GTT CT-3'	0.997	106.2
dazl	BC076423	F: 5'-AAT GCC CAG CTT GCC TAA TG-3' R: 5'-CAC GCG GCC GAA ACC-3'	0.974	84.06
bdnf	BC058301	F: 5'-GAC TCG AAG GAC GTT GAC CTG TA-3' R: 5'-CGG CTC CAAAGG CACTTG-3'	0.977	97.95
18s	FJ915075	F: 5'-TGG TTA ATT CCG ATA ACG AACGA-3' R: 5'-CGC CAC TTG TCC CTC TAA GAA-3'	0.999	75.61

Table 3.

F0 fecundity and F1 survival

Group	Nominal water concentration (mg/L) at 6 hpf (tanks)	Average # eggs per tank	% Fertilized	% Survival at 24 hpf	% Hatched at 48 hpf	% Hatched at 72 hpf	% Survival at 96 hpf
F0 Control	0.05% DMSO (5)	198 ± 24	98.2 ± 1.4	78.9 ± 7.5	20.9 ± 8.9	96.5 ± 2.4	74.1 ± 8.9
F0 THC	0.024 (3)	64 ± 19*	100.0 ± 0.0	90.4 ± 4.9	18.4 ± 15.4	95.7 ± 2.3	89.8 ± 4.8
	0.12 (3)	61 ± 34*	99.2 ± 0.6	61.3 ± 24.0	40.7 ± 2.0	100.0 ± 0.0	60.4 ± 26.0
	0.6 (5)	110 ± 26	99.3 ± 0.3	91.7 ± 1.6	25.3 ± 8.3	99.2 ± 0.8	89.4 ± 3.2
F0 CBD	0.006 (3)	192 ± 33	95.8 ± 2.2	85.3 ± 5.9	24.4 ± 3.9	99.0 ± 0.3	77.6 ± 5.7
	0.03 (3)	113 ± 19	98.9 ± 0.5	87.5 ± 5.4	14.1 ± 2.5	98.1 ± 1.2	84.0 ± 5.7
	0.15 (3)	66 ± 14*	92.8 ± 6.0	55.3 ± 7.6	23.6 ± 10.5	97.5 ± 2.5	49.5 ± 9.3
F1 Control	0.05% DMSO (6)	71 ± 18	97.9 ± 2.1	79.0 ± 9.2	63.2 ± 18.0	98.6 ± 1.4	74.3 ± 10.6
F1 THC	0.6 (5)	55 ± 23	99.5 ± 0.5	79.1 ± 6.3	50.3 ± 14.4	95.7 ± 2.6	76.1 ± 8.4
F1 CBD	0.006 (7)	82 ± 19	96.5 ± 2.9	76.6 ± 7.3	21.0 ± 7.5	87.7 ± 4.9	66.2 ± 7.2
	0.03 (6)	46 ± 13	98.1 ± 1.9	94.7 ± 2.7	30.9 ± 10.4	96.7 ± 2.1	89.1 ± 5.5