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Research Article

Transcriptomic Changes and the Roles of Cannabinoid Receptors and PPARγ in Developmental Toxicities Following Exposure to Δ^9-Tetrahydrocannabinol and Cannabidiol

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ABSTRACT

Human consumption of cannabinoid-containing products during early life or pregnancy is rising. However, information about the molecular mechanisms involved in early life stage Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) toxicities is critically lacking. Here, larval zebrafish (*Danio rerio*) were used to measure THC- and CBD-mediated changes on transcriptome and the roles of cannabinoid receptors (Cnr) 1 and 2 and peroxisome proliferator activator receptor γ (PPARγ) in developmental toxicities. Transcriptomic profiling of 96-h postfertilization (hpf) $cnr^{+/+}$ embryos exposed (6 – 96 hpf) to 4 μM THC or 0.5 μM CBD showed differential expression of 904 and 1095 genes for THC and CBD, respectively, with 360 in common. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched in the THC and CBD datasets included those related to drug, retinol, and steroid metabolism and PPAR signaling. The THC exposure caused increased mortality and deformities (pericardial and yolk sac edemas, reduction in length) in $cnr1^{-/-}$ and $cnr2^{-/-}$ fish compared with $cnr^{+/+}$ suggesting Cnr receptors are involved in protective pathways. Conversely, the $cnr1^{-/-}$ larvae were more resistant to CBD-induced malformations, mortality, and behavioral alteration implicating Cnr1 in CBD-mediated toxicity. Behavior (decreased distance travelled) was the most sensitive endpoint to THC and CBD exposure. Coexposure to the PPARγ inhibitor GW9662 and CBD in $cnr^{+/+}$ and $cnr2^{-/-}$ strains caused more adverse outcomes compared with CBD alone, but not in the $cnr1^{-/-}$ fish, suggesting that PPARγ plays a role in CBD metabolism downstream of Cnr1. Collectively, PPARγ, Cnr1, and Cnr2 play important roles in the developmental toxicity of cannabinoids with Cnr1 being the most critical.

Key words: cannabidiol; tetrahydrocannabinol; PPARγ; RNAseq; development; behavior.

Marijuana laws are changing at a rapid pace across the world. As of January 2021, consumer access to cannabis is high, with countries such as Canada, Georgia, South Africa, Uruguay, as well as 15 states, 2 territories, and the District of Columbia in the United States of America (USA) having legalized recreational use. Medicinal cannabis use is legal in 43 countries and almost all states in the USA. Roughly 22%–30% of young adults in the USA between the ages of 18 – 30 admit to using marijuana in the past month (Schulenberg *et al.*, 2020), whereas 11%–36% of teenagers aged 13 – 17 admit using in the last year (Miech *et al.*,

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2020). The incidence of cannabis usage in pregnant women (in the first trimester) has more than doubled in the past decade (Volkow *et al.*, 2019). In fact, marijuana use among pregnant women is higher than any other illicit drug (Volkow *et al.*, 2019) and 70% of both pregnant and nonpregnant women believe there is slight or no risk of marijuana use (Ko *et al.*, 2015). Therefore, there is an ongoing need to understand the potential developmental effects of exposure including possible long-term effects of early life exposure (Bobst *et al.*, 2020).

Cannabis contains more than 545 known chemical compounds including the psychoactive Δ9-tetrahydrocannabinol (THC) and nonpsychoactive cannabidiol (CBD) (Gonçalves *et al.*, 2019). The endocannabinoid system, on which cannabinoids interact, consists of 2 cannabinoid receptors 1 and 2 (CB1 and CB2 in humans and rodents, Cnr1 and Cnr2 in fish), 2 endocannabinoids—anandamide and 2-arachidonoylglycerol, and anabolic/catabolic enzymes (Lu and MacKie, 2016). The endocannabinoidome further includes many other overlapping receptor/signaling pathways such as transient receptor potential cation channel subfamily V member 1 (TRPV1), peroxisome proliferator-activated nuclear receptors- (PPARα and PPARγ), T-type Ca^{2+} channels, and orphan G protein-coupled receptors like GPR18, or GPR55 (Cristino *et al.*, 2020).

In vertebrates (eg, chick, mouse, Xenopus, and zebrafish) the endocannabinoid system is expressed in the central nervous system prior to and during neurodevelopment (Berghuis *et al.*, 2007; Harkany *et al.*, 2007; Krug and Clark, 2015; Lam *et al.*, 2006; Psychoyos *et al.*, 2012; Sufian *et al.*, 2019). Exposure to high doses of THC and CBD during embryonic development causes disrupted brain development and other teratogenic effects in mice and fish (Carty *et al.*, 2018; Fish *et al.*, 2019). Furthermore, perinatal manipulation of the endocannabinoid system by administering cannabinoids or by maternal marijuana consumption alters neurotransmission and behavioral functions in offspring of humans (Fried *et al.*, 2003; Fried and Smith, 2001), mice (De Salas-Quiroga *et al.*, 2015), rats (Fride and Mechoulam, 1996; O'Shea and Mallet, 2005; Rubio *et al.*, 1995), and zebrafish (Ahmed *et al.*, 2018; Carty *et al.*, 2019). Previous research in our laboratory established that high doses of THC (≥4 μM) or CBD (≥0.5 μM) are teratogenic to zebrafish (Carty *et al.*, 2018). Importantly, lower concentrations that did not induce overt morphological effects and were within the human therapeutic range resulted in reproductive abnormalities and gene expression changes that persisted into adulthood and old age (Carty *et al.*, 2019; Pandelides *et al.*, 2020a, 2020b). Furthermore, sublethal concentrations of THC or CBD caused significant larval behavioral alterations (Carty *et al.*, 2019, 2018).

THC is a known agonist of Cnr1 and Cnr2 receptors, whereas CBD acts as an allosteric modulator to these receptors (Laprairie *et al.*, 2015; Martínez-Pinilla *et al.*, 2017; Tham *et al.*, 2019). Due to the expression of Cnr1 and Cnr2 receptors throughout the central nervous system, cannabinoid compounds have the potential to affect neural development. There is evidence from cannabinoid receptor knock out models that Cnr1 and Cnr2 play important roles in mediating behavior, organ development, size, and metabolism (De Azua *et al.*, 2017; Liu *et al.*, 2016; Ravinet Trillou *et al.*, 2004; Schmitz *et al.*, 2016; Varvel *et al.*, 2005). However, the role of Cnr1 and Cnr2 in THC or CBD developmental toxicity has not been fully elucidated.

In the current study, we investigated effects of THC and CBD on the larval zebrafish transcriptome at 96 hpf following developmental exposure to assess the effects of cannabinoids on the whole organism. Resulting pathway analysis informed further investigation into the mechanistic contributions of Cnr1, Cnr2, and PPARγ in THC and CBD early life stage toxicities. We hypothesized that the cannabinoid receptors would play a significant mechanistic role in THC's, but not CBD's, acute toxic effects.

MATERIALS AND METHODS

Experimental animals. The following strains of zebrafish were used in this study—the *Tg(fli1: egfp)*, *cnr1*$^{-/-}$ *[Tu(cnr1*$^{zf679/zf679}$*)]* and *cnr2*$^{-/-}$ *[Tu(cnr2*$^{zf680/zf680}$*)]*. *Tg(fli1: egfp)* zebrafish were obtained from the Zebrafish International Resource Center (ZFIN, Eugene, Oregon). The cannabinoid receptor mutants (*cnr1*$^{-/-}$ and *cnr2*$^{-/-}$) (Liu *et al.*, 2016) were kindly provided by Dr Wolfram Goessling (Genetics Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115). *Tg(fli1:egfp)* which is *cnr*$^{+/+}$ was used as a wildtype control in studies involving *cnr1*$^{-/-}$ and *cnr2*$^{-/-}$ mutants. We have used *Tg(fli1:egfp)* strain to assess the impact of developmental exposure to both THC and CBD (Carty *et al.*, 2018, 2019; Pandelides *et al.*, 2020a, 2020b). Healthy adult zebrafish were maintained in Aquatic Habitats Zebrafish Flow-through System (Aquatic Habitats, Apopka, Florida) under ambient conditions (pH 7.5–8.0, dissolved oxygen 7.2–7.8 mg/l, conductivity 730–770 mS, and temperature 27–29°C). The *cnr1*$^{-/-}$ and *cnr2*$^{-/-}$ adults were genotyped prior to conducting the experiments. The primer sequences used for genotyping are provided in Supplementary Table 1. All the experiments and exposure protocols were in accordance with approved Institutional Animal Care and Use Committee guidelines and recommendations.

Embryos for exposure studies were obtained by setting up pairwise breeding of adult fish overnight. The next morning, eggs were collected, debris removed, and randomly sorted into scintillation vials (10 embryos per vial) containing embryo water (6 ml volume, sterilized deionized water; pH 7.4–7.7; 60 ppm Instant Ocean, Cincinnati, Ohio), and maintained at 28°C in an incubator. Exposed embryos were screened daily to assess overall health of the embryos.

Δ^{9}*-Tetrahydrocannabinol and CBD exposures*. The rationale for the exposure paradigm used in this study included several considerations. Previously, a dosing regimen of $1-4\,\mu$M THC and $0.25-0.5\,\mu$M CBD in zebrafish water resulted in the accumulation of $0.28-0.71$ mg/kg THC and $1.2-8.61$ mg/kg CBD in the whole larval zebrafish, respectively (Carty *et al.*, 2018, 2019). Because CBD bioconcentrates more than THC in zebrafish, there is higher acute toxicity in CBD-exposed zebrafish and a lower CBD concentration range was used. Representative concentrations used in this study (eg, 4 μM THC [3.75 mg/l] and 0.5 μM CBD [0.15 mg/l]), were lower than the typical 5 mg/kg THC dose used in prenatal and perinatal mice/rats studies (range $0.15-150$ mg/kg; Grant *et al.*, 2018). Furthermore, a 2.5 mg/kg THC rodent dose has been related to a human exposure from a single joint (approximately 120–220 mg THC) (Leishman *et al.*, 2018; Rubino *et al.*, 2008). Clinically, CBD in Epidiolex is FDA approved for dosing from 2.5 to 10 mg/kg twice daily, with the goal of maintenance dosing of $10-20$ mg/kg/day (Arzimanoglou *et al.*, 2020). In humans, THC and CBD can cross the placenta. Reported meconium and umbilical cord concentrations for THC were 0.016 and 0.0012 mg/kg, respectively, and 0.33 mg/kg CBD in meconium (Grant *et al.*, 2018; Jensen *et al.*, 2019; Kim *et al.*, 2018), which supports the observation of higher bioconcentration of CBD relative to THC. The exact dose a developing child is exposed to maternally of THC or CBD is unknown. THC is known to be quickly

metabolized within 24 h from many tissues and serum, but persists in fat (Brunet *et al.*, 2006).

Based on previous dose response studies conducted in our laboratory (Carty *et al.*, 2018; 2019), 4 μM THC and 0.5 μM CBD were chosen to investigate the effects of exposure on gene expression changes. Dimethyl sulfoxide (DMSO; final concentration 0.05%) was used as a carrier control. Δ^9-Tetrahydrocannabinol and CBD were procured from the NIDA Drug Supply Program (Research Triangle Park, North Carolina). *Tg(fli1:egfp)* embryos were exposed under static conditions from 6 h postfertilization (hpf) to 96 hpf. Every 24 h, embryos were observed for any developmental defects and mortalities. Any debris (sloughed chorions) were removed from vials during observation. Each treatment consisted of 3 biological replicates with 10 embryos per replicate.

RNA sequencing analysis. RNA sequencing (RNAseq) was conducted on the 3 biological replicates at the University of Mississippi Medical Center (Jackson, Mississippi). Library construction was done using TruSeq Illumina library preparation kit. Paired end 100 bp sequencing was done on an HiSeq2000 Illumina platform. Raw data files were assessed for quality using FastQC (Andrews, 2010) prior to preprocessing. Trimmomatic was used for preprocessing, to remove any remaining adaptor sequences and reads with low-sequence quality (Phred score less than 20). Trimmed sequence reads were mapped to the zebrafish genome using the STAR aligner (Dobin *et al.*, 2016). The number of reads mapped to annotated regions of the genome were obtained using HTSeq-count (Anders *et al.*, 2015). Ensembl version 84 (GRCz10) of the zebrafish genome and annotations were used in this analysis (Yates *et al.*, 2016) and statistical analysis was conducted using edgeR, a Bioconductor package (Robinson *et al.*, 2010). The quasi-likelihood model in edgeR (glmQLFTest) was used to perform differential gene expression analysis. Only genes with false discovery rate (FDR) of less than 5% were considered to be differentially expressed. Annotation of the differentially expressed genes was done using BioMart (Smedley *et al.*, 2015).

Gene ontology classification, KEGG pathway, and human phenotype analysis. Annotated zebrafish genes found to be differentially expressed (FDR < 0.05) were classified based on gene ontology (molecular function) using a gProfiler package g:GOSt (Reimand *et al.*, 2016). The up- and downregulated datasets for each treatment were processed individually. We then compared the GO terms between the 3 treatments using the g:Cocoa, a package of gProfiler (Reimand *et al.*, 2016). We visualized the GO terms using GOView and generated hierarchical DAG (directed acyclic graphs) graphs to highlight relationships between GOterms (child and parent terms) (Wang *et al.*, 2017). Only unique GO terms with a distinct set of genes were considered for further analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Phenology (HP) term analysis of the DEGs was done using gProfiler. Genes represented under each enriched KEGG and HP pathway were manually screened, and the pathways with unique lists of genes were selected.

Determining the developmental toxicity to THC and CBD exposure in Cnr *mutants*. Beginning at 6 hpf, zebrafish embryos from the $cnr^{+/+}$, $cnr1^{-/-}$, and $cnr2^{-/-}$ strains were exposed to 5 different concentrations of THC (2, 4, 8, 9.5, and 12 μM or 0.65, 1.25, 2.4, 3, 3.75 mg/l) or CBD (0.25, 0.5, 1, 2, and 4 μM or 0.075, 0.15, 0.3, 0.6, 1.2 mg/l). A 0.05% DMSO (control) group was included as a carrier control. Exposures were continued under static conditions until 96 hpf. Each treatment consisted of 5 biological replicates with 10 embryos per replicate.

Role of PPARγ in THC- and CBD-induced effects. To determine the role of PPARγ in THC- and CBD-induced effects, we exposed $cnr^{+/+}$, $cnr1^{-/-}$, and $cnr2^{-/-}$ strains to either a PPARγ antagonist GW9662 (0.5 μM; Jin *et al.*, 2020) alone or in combination with 4 μM THC or 2 μM CBD. A 0.05% DMSO (control) group was included as a carrier control. These embryos were exposed 6 – 96 hpf under static exposure conditions. Each treatment consisted of 5 biological replicates with 10 embryos per replicate.

Quantification of THC and CBD using GC/MS. Δ^9-Tetrahydrocannabinol and CBD in exposure medium (embryo water) was measured by GC/MS as described previously (Carty *et al.*, 2018). Briefly, deuterated THC-d3 (Sigma Aldrich, St Louis, Missouri) was added to the samples at 0 h post-treatment along with 2 M sodium hydroxide and extracted using hexane:ethyl acetate (9:1 vol:vol). Samples were then derivatized in N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (ThermoScientific) at 90°C for 1 h. Samples were then evaporated to dryness, reconstituted in iso-octane, and run on the GC/MS (Agilent Technologies 6890N; Mass Spectrometer 5973) with DB-5MS column (Agilent Technologies, Santa Clara, California) for analysis in selected ion monitoring mode. Retention times and ions [quantitative; qualitative] for quantifying THC-d3, THC, and CBD were as follows: 8.142 min [374; 389 m/z], 8.167 min [386; 371 m/z], and 6.936 min [390; 458 m/z]. Concentrations were calculated based on a 5-point standard curve (0.0625–1 mg/l). Percent recovery was 87% ± 20% for water samples. Measured water concentrations are listed in Table 1.

Larval behavioral assays. Larval locomotion behavior in response to light was measured following established methods using a ViewPoint ZebraBox (ViewPoint, Montreal, Canada) (Kirla *et al.*, 2016). At the end of the exposure (96 hpf), larvae were transferred into individual wells of a 96-well plate (300 μl embryo water/well) and acclimated for 5 min under ambient light and temperature. Locomotory assay conditions include initial 10 min under 100% light [8000 lux] followed by 10 min in the dark [0% light; 0 lux]; and 10 min in 100% light (Kirla *et al.*, 2016). The total distance travelled during the light and the dark phases was also calculated per larvae. Larvae that were unable to swim due to gross malformation were excluded from the behavior analysis. Behavior was assessed in all fish except those used for RNA sequencing. Each treatment condition consisted of 50 individual larvae.

Morphological phenotypes. After behavioral assessments, photographs were taken of all surviving larval fish (50 larval fish per treatment, 10 per replicate, $n = 5$ replicates) per treatment group to assess developmental deformities. Larvae were anesthetized in tricaine methanesulfonate (300 mg/l MS-222) buffered with 600 mg/l sodium bicarbonate. They were immediately placed on a microscope slide with a chamber containing 3% methyl cellulose and a lateral image was captured with a MicroFire camera (Optronics, Goleta, California) attached to a Zeiss Stemi 2000-C Stereo Microscope (Jena, Germany) using Picture Frame Application 2.3 software (Optronics). The phenotypes were scored blindly using ImageJ software (Schneider *et al.*, 2012). Total body length, diameter, and area of the eye, presence or absence of developmental abnormalities (yolk sac edema, pericardial edema, spinal curvature, swim bladder inflation failure) were recorded by 3 double-blinded reviewers. Pooled larval

Table 1. Cannabinoid Exposure Water Concentrations

Compound	Nominal Water Concentration (mg/l)	Measured Water Concentration (mg/l)
Control	0	ND
THC	0.65	0.22 ± 0.004
	1.25	0.32 ± 0.007
	2.5	0.50 ± 0.05
	3	0.68 ± 0.01
	3.75	0.77 ± 0.03
CBD	0.075	0.16 ± 0.006
	0.15	0.19 ± 0.008
	0.3	0.24 ± 0.005
	0.6	0.46 ± 0.03

Data presented as mean measured concentration ± SD of 3 – 9 replicates for the dose response and PPARγ-antagonist exposures.
Abbreviation: ND, not detected.

zebrafish were placed in RNA later (10 per replicate), frozen and stored at -80°C until further analysis.

RNA isolation, cDNA synthesis, and real time-quantitative PCR. RNA extraction was done using an RNeasy mini-kit (Qiagen, California) in conjunction with gDNA removal via RNase-Free DNase set (Qiagen) following the manufacturer's recommended protocol. RNA was quantified and evaluated for purity (260/280 ratio = 1.9 – 2.1) on a NanoDrop 2000 spectrophotometer (ThermoFisher, Massachusetts). RNA (250 ng) was then reverse transcribed to cDNA following the manufacture's protocol (Invitrogen, California). The RT-qPCR was performed on PPARγ (*pparγ*), PPARα (*pparαa*), and 18S ribosomal RNA (reference gene), using an Applied Biosystems 7500 real-time cycler with SYBR Green detection chemistry (Applied Biosystems, California) following the manufacture's protocol in a 25 μl reaction volume. Parameters for RT-qPCR were as follows: 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by 95°C for 15 s–60°C for 1 min–95°C for 15 s dissociation curve. Primers were optimized as described previously (Corrales *et al.*, 2014) and primer sequences are provided in Supplementary Table 1. Samples were run in duplicate followed by $2^{-\Delta\Delta CT}$ method evaluation (Livak and Schmittgen, 2001).

Statistical analysis. All data were assessed for normality and homogeneity of variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. The incidence of developmental deformities (%) was calculated per biological replicate ($n = 5$). Larval length, eye diameter, and eye area was recorded per fish and then averaged per replicate ($n = 5$). The differences in strain were assessed by comparing the 3 unexposed solvent controls (1-way analysis of variance [ANOVA], Tukey's posthoc test, $p \leq .05$) in order to make all pairwise comparisons between the 3 strains. Differences in treatment within each strain were assessed by ANOVA, Dunnett's posthoc ($p \leq .05$), in order to assess the difference between treated and the untreated control fish.

RT-qPCR was assessed using (ANOVA) on the ΔCT followed by Dunnett's posthoc test compared with the solvent controls ($p \leq .05$). The gene expression data were summarized in tables displaying the average log(2)ΔΔCt ± standard error of the mean. The differences in strain were assessed by comparing the ΔCt of the 3 unexposed solvent controls (ANOVA, Tukey's posthoc, $p \leq .05$).

Statistical analysis was conducted on the total distance travelled during the light and dark phases separately. First, the 3 DMSO controls were compared to assess the difference between the strains (ANOVA on ranks, Tukey's posthoc, $p \leq .05$). Next within each strain differences in concentration were assessed (ANOVA on ranks, Dunn's posthoc, $p \leq .05$). All graphing and statistical analyses were conducted using Sigmaplot 14.0 software.

RESULTS

Δ^9-Tetrahydrocannabinol- and CBD-Induced Transcriptional Responses

Transcriptomic analysis revealed differential expression of 904 and 1095 genes in the THC- and CBD-exposed groups, respectively, in comparison to the DMSO control (5% FDR). Among the 904 DEGs in response to THC, 744 genes were upregulated, and 160 genes were downregulated. Whereas with CBD, 774 genes were upregulated, and 321 genes were downregulated. Comparison of these 2 datasets revealed a total of 360 genes were differentially expressed in common in response to both THC and CBD. The entire list of differentially expressed genes as well as shared genes and their fold changes is provided in Supplementary Data (THC_CBD_DEGs.xlsx). Raw files have been deposited in the GEO database (accession number GSE164128).

The KEGG pathways enriched in the THC dataset included drug metabolism, metabolic pathways (glutathione, tyrosine, arachidonic acid, glycine, serine, and threonine metabolism), steroid hormone biosynthesis, retinol metabolism, and PPAR signaling. Some of the same KEGG pathways enriched in CBD dataset were PPAR signaling, steroid hormone biosynthesis, metabolic pathways, and retinol metabolism. The differentially expressed genes associated with the PPAR signaling pathway and retinol metabolism are shown in Figure 1 and 2, respectively. The human phenology term analysis revealed enrichment of complement deficiency in both THC and CBD datasets. The detailed list of enriched KEGG and human phenology pathways is shown in Table 2.

Role of Cnr1 and Cnr2 in THC- and CBD-Induced Developmental Toxicity

There was a dose-dependent effect of THC exposure on mortality in all 3 strains of fish (*cnr*$^{+/+}$, *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$). In controls (*cnr*$^{+/+}$), 60% mortality was observed with 8 μM THC (Figure 3A). In contrast, *cnr1* and *cnr2* null mutants were more sensitive to THC exposure and had significant mortality at 4 μM THC. Similarly, increased sensitivity to THC in the *cnr1* and *cnr2* null mutants was observed for developmental deformities (pericardial and yolk sac edemas, Supplementary Figure 1). Constitutive differences between the 3 strains are shown in Supplementary Figure 2.

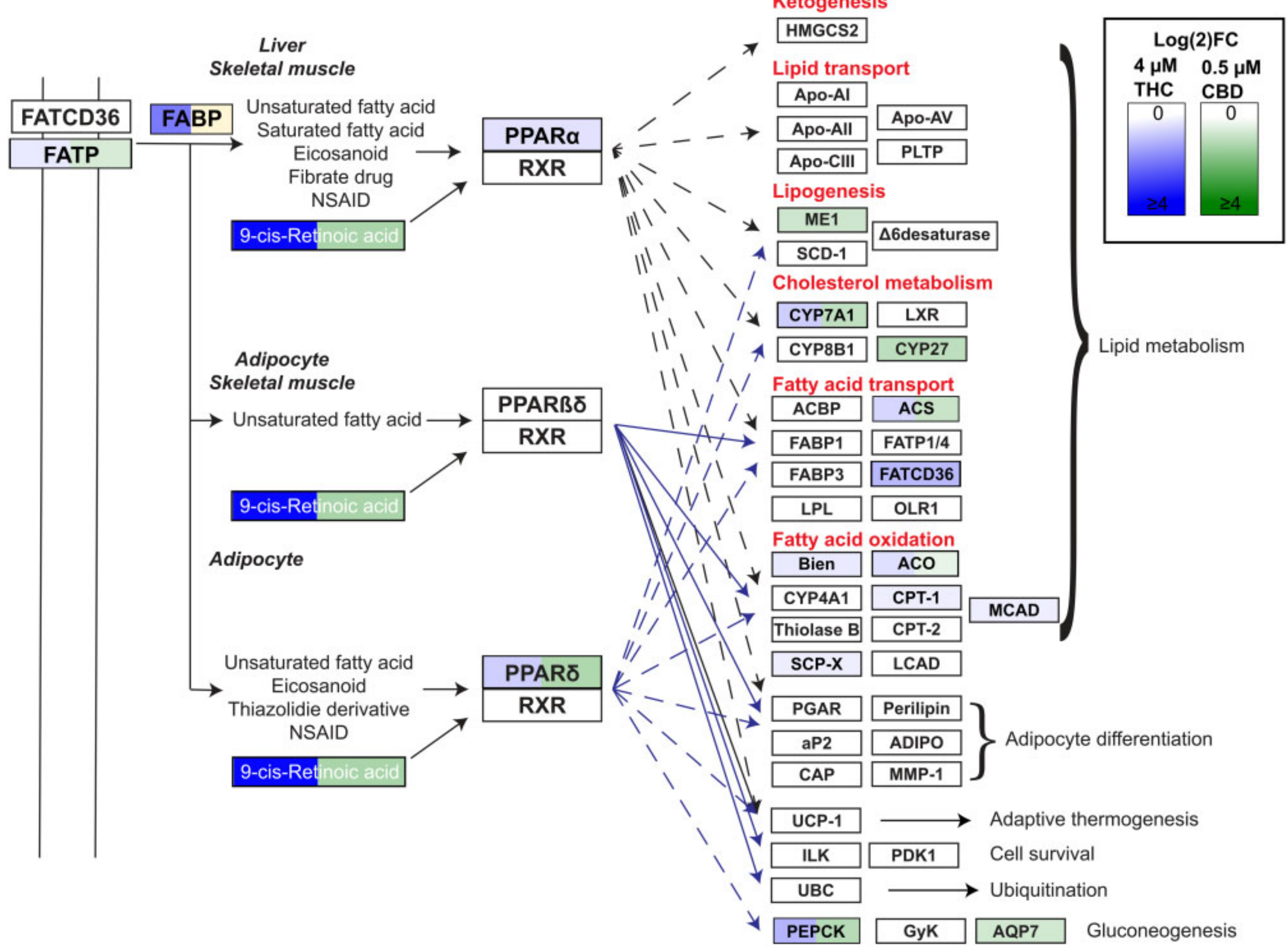

Figure 1. Δ9-Tetrahydrocannabinol (THC) and cannabidiol (CBD) altered PPARα,γ associated pathways. PPARs are known to dimerize with RXR and their roles in metabolism are well established. Significant upregulation (FDR ≤ 0.05) of genes within this KEGG signaling pathway are highlighted in gradients of blue (THC), green (CBD), or both blue and green (both THC and CBD). Note that 1 gene in the data (FABP, logFC = −0.61, yellow) set was significantly downregulated for CBD.

Developmental exposure to CBD did not show a dose-dependent effect on mortality in any of the 3 strains up to 2 μM CBD (Fig. 3B). However, at the highest concentration of CBD (4 μM), 90%–100% of the $cnr^{+/+}$ and $cnr2^{-/-}$ larvae and 40% of the $cnr1^{-/-}$ larvae died. The *cnr1* and *cnr2* null mutants were less sensitive than $cnr^{+/+}$ for developmental edemas (Supplementary Figure 1).

Exposure to THC and CBD caused a dose-dependent reduction in the total length in all 3 strains compared with the unexposed groups (Figs. 3C and D). In addition, exposure to both THC and CBD resulted in dose-dependent reduction in the eye area of larval fish in all 3 strains (Figs. 3E and F).

Role of cnr1 *and* cnr2 *Receptors in THC- and CBD-Induced Behavioral Deficits*

In $cnr^{+/+}$ fish, THC exposure significantly decreased larval locomotor activity particularly in the dark phase when compared with the vehicle control similar to previous in laboratory studies (Carty *et al.*, 2019, 2018) (Figure 4A; Supplementary Figs. 3A–C). Similarly, CBD exposure significantly reduced larval locomotion in the $cnr^{+/+}$ strain (Figure 4B; Supplementary Figs. 4A–C).

In $cnr1^{-/-}$ mutants, THC exposure at all concentrations significantly decreased larval locomotor activity in the dark phase (Figure 4C; Supplementary Figs. 3D–F). CBD caused a significant reduction in $cnr1^{-/-}$ larval locomotion during both light and dark phases, but effects were not dose-dependent (Figure 4D; Supplementary Figs. 4D–F). All concentrations of THC and CBD (except for 0.25 μM CBD) exposure decreased larval locomotory activity in the dark phase in $cnr2^{-/-}$ mutants (Figs. 4E and F; Supplementary Figs. 3 and 4G–I).

Role of PPARγ in THC- and CBD-Exposure Induced Effects

RNAseq results revealed that PPARγ was a significant upstream regulator of many of the differentially expressed genes. In order to elucidate the role PPARγ in the adverse outcomes associated with cannabinoid exposure, all 3 strains of fish embryos were exposed to either 4 μM THC or 2 μM CBD alone or in combination with the PPARγ antagonist (GW9662, 0.5 μM; Jin *et al.*, 2020). Larval exposure to 0.5 μM GW9662 alone did not cause any significant effects on any endpoints measured with the exception of larval locomotion in $cnr1^{-/-}$ mutants. However, a mixture of CBD and the PPAR antagonist caused significantly reduced survival in the $cnr^{+/+}$ strain compared with CBD alone. Although the opposite effect (increased survival) was observed in THC+GW9662 mixture for $cnr1^{-/-}$ fish and no significant change found in the $cnr2^{-/-}$ strain (Figs. 5A–J). Δ^9-Tetrahydrocannabinol and CBD either alone or in combination with PPARγ antagonist significantly reduced the total length of the fish in all 3 strains of fish (Figs. 5C–L). Significantly worse outcomes were observed in $cnr^{+/+}$ and $cnr2^{-/-}$ mutants, where the PPARγ antagonist in combination with CBD significantly decreased the total length of larvae in comparison to CBD alone. Although

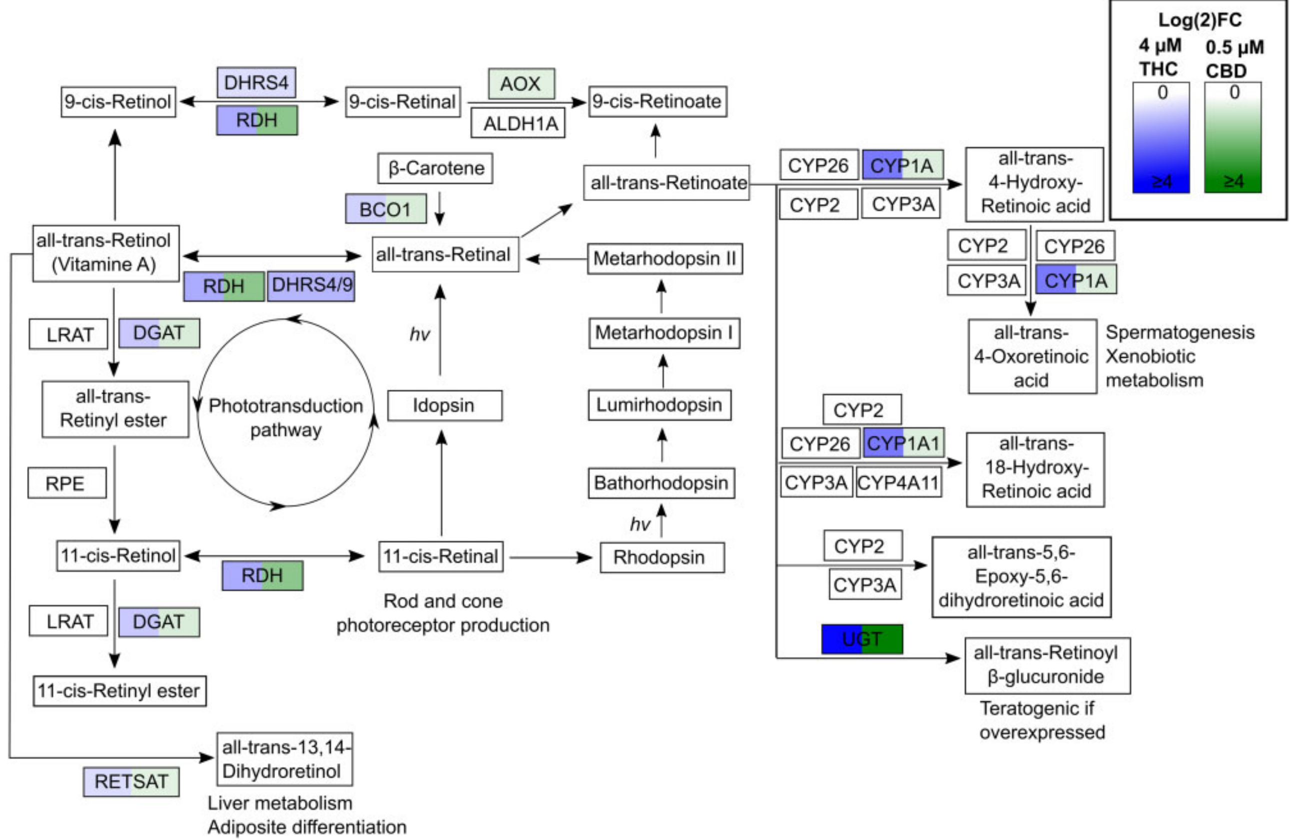

Figure 2. Δ9-Tetrahydrocannabinol (THC) and cannabidiol (CBD) altered retinol metabolism pathway. The retinol pathway plays an important role in important physiological functions including photoreceptor development, metabolism, etc. The KEGG retinol signaling pathway with genes significantly upregulated (FDR ≤ 0.05) in our data set are highlighted in gradients of blue (THC), green (CBD), or both blue and green (both THC and CBD). No genes were significantly downregulated in this pathway.

Table 2. Top HP, GO, and KEGG Pathway Analysis Terms Enriched in the Differentially Expressed Genes in Zebrafish Developmentally Exposed to 4 μM THC or 0.5 μM CBD

		4 μM THC		0.5 μM CBD	
		No. DEG	p-Value	No. DEG	p-value
GO ID	GO Pathway				
GO:0016491	Oxidoreductase activity	91	9.29E−21	67	1.76E−05
GO:0046906	Tetrapyrrole binding	28	5.39E−09	28	2.19E−07
GO:0061134	Peptidase activity	60	7.15E−08	58	1.71E−04
GO:0016787	Hydrolase activity	140	3.86E−05	N/A	
GO:0016936	Galactoside binding	5	5.07E−04	N/A	
GO:0004497	Monooxygenase activity	N/A		27	4.16E−08
HP ID	HP pathway				
HP:0004431	Complement deficiency	11	1.48E−05	13	5.19E−07
HP:0001937	Microangiopathic hemolytic anemia	7	1.92E−03	8	3.86E−04
HP:0011036	Abnormality of renal excretion	N/A		14	5.88E−03
HP:0005575	Hemolytic-uremic syndrome	7	1.92E−03	7	7.03E−03
HP:0001919	Acute kidney injury	N/A		9	1.55E−02
KEGG ID	KEGG pathway				
KEGG:00980	Metabolism of xenobiotics by CYP450	19	6.34E−12	N/A	
KEGG:00983	Drug metabolism—other enzymes	21	7.97E−11	N/A	
KEGG:00830	Retinol metabolism	18	1.44E−09	10	3.74E−02
KEGG:01100	Metabolic pathways	106	3.36E−09	95	2.59E−02
KEGG:00140	Steroid hormone biosynthesis	16	1.18E−08	10	1.09E−02
KEGG:03320	PPAR signaling pathway	13	2.62E−03	13	1.23E−02

Each treatment consisted of 3 biological replicates with 10 embryos per replicate. No. DEG. = N/A.

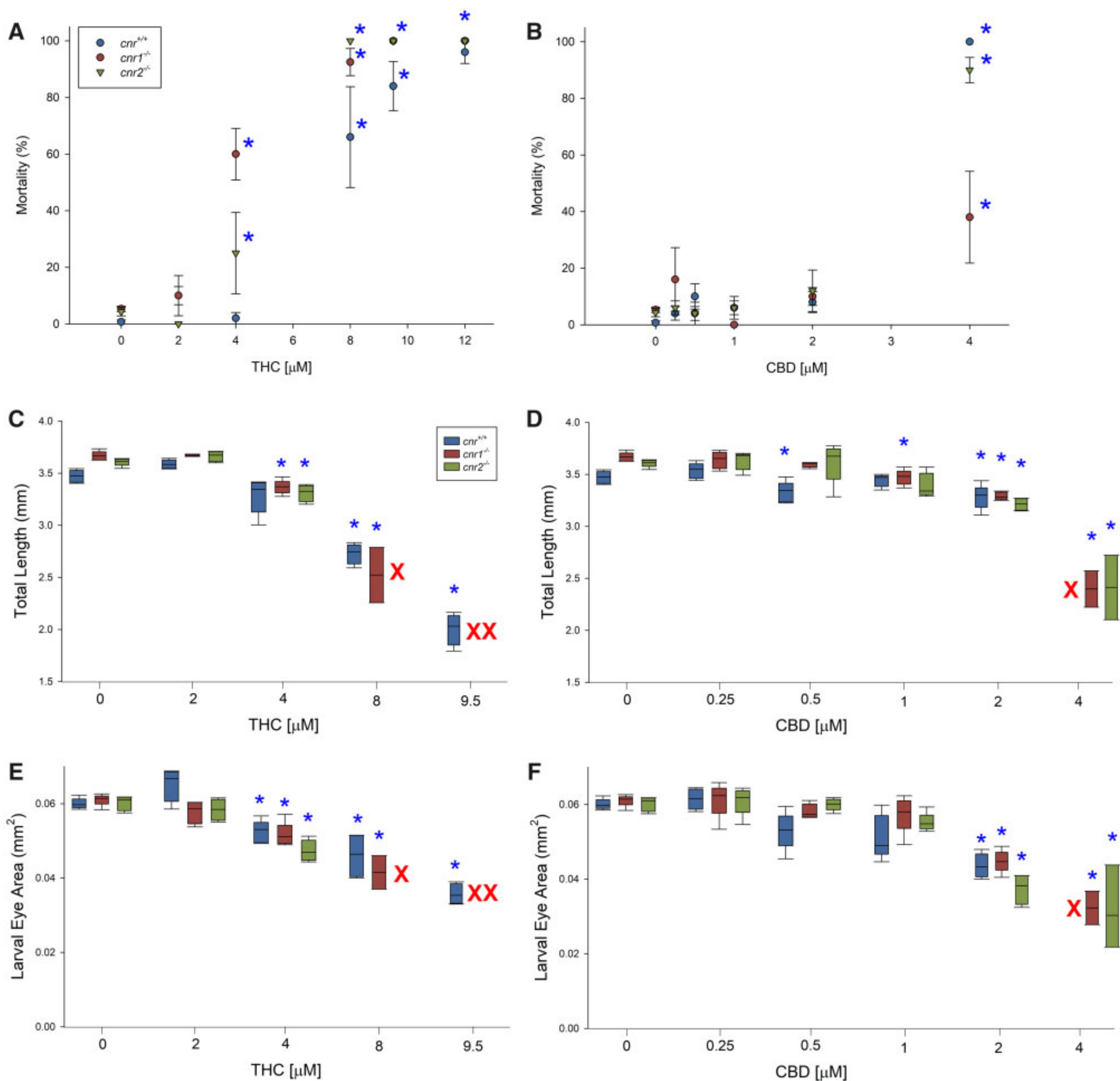

Figure 3. Mortality (%), total length (mm), and larval eye area (mm^2) of 96 hpf larval zebrafish developmentally exposed to Δ9-tetrahydrocannabinol (A, C, E) or cannabidiol (B, D, F) presented as box and whisker plots ($n = 5$). Asterisk indicates a significant difference compared with the within-strain solvent control (ANOVA, Dunnett's posthoc, $p \leq .05$). Red X's indicate concentration/strains with no survival.

exposure to the PPARγ inhibitor alone did not cause any significant increase in the incidence of yolk sac or pericardial edema, cotreatment with THC or CBD generally caused a significant increase in the incidence of malformations (pericardial and yolk sac edemas) in all 3 strains of fish, which was significantly higher than THC or CBD alone in the $cnr^{+/+}$ and $cnr2^{-/-}$ strains (except for pericardial edema in THC treated $cnr2^{-/-}$ fish) (Supplementary Figs. 5 and 6). There was no significant mixture effect for larval eye area (Supplementary Figure 7) and larval locomotor activity (Supplementary Figs. 8 and 9) in any of the 3 fish strains.

The Effect of THC, CBD, and/or a PPARγ Antagonist Exposure on PPARα and PPARγ Expression

Exposure to all treatments resulted in significant upregulation of *PPARα* in the $cnr^{+/+}$ fish (Figure 6). There was no significant effect on *PPARα* expression in the $cnr1^{-/-}$ fish, but in the $cnr2^{-/-}$ fish exposure to 2 μM CBD caused significant upregulation of *PPARα*. Furthermore, in the $cnr2^{-/-}$ fish exposure to GW9662 alone or in combination with THC or CBD did not significantly affect *PPARα* expression.

Exposure to 4 μM THC, GW9662 (a PPARγ inhibitor), and a combination of both resulted in a significant upregulation of *PPARγ* expression measured at 96 hpf in the $cnr^{+/+}$ fish (Figure 6). In contrast, the $cnr1^{-/-}$ fish did not differentially express *PPARγ* following THC or GW9662 exposure, and the $cnr2^{-/-}$ fish exhibited significant downregulation of *PPARγ* following THC or GW9662 exposure. The $cnr^{+/+}$ fish exposed to CBD alone or in combination with GW9662 did not significantly affect *PPARγ*. Unlike the $cnr^{+/+}$ fish, both GW9662 and the mixture of 2 μM CBD + GW9662 in the $cnr2^{-/-}$ resulted in significant

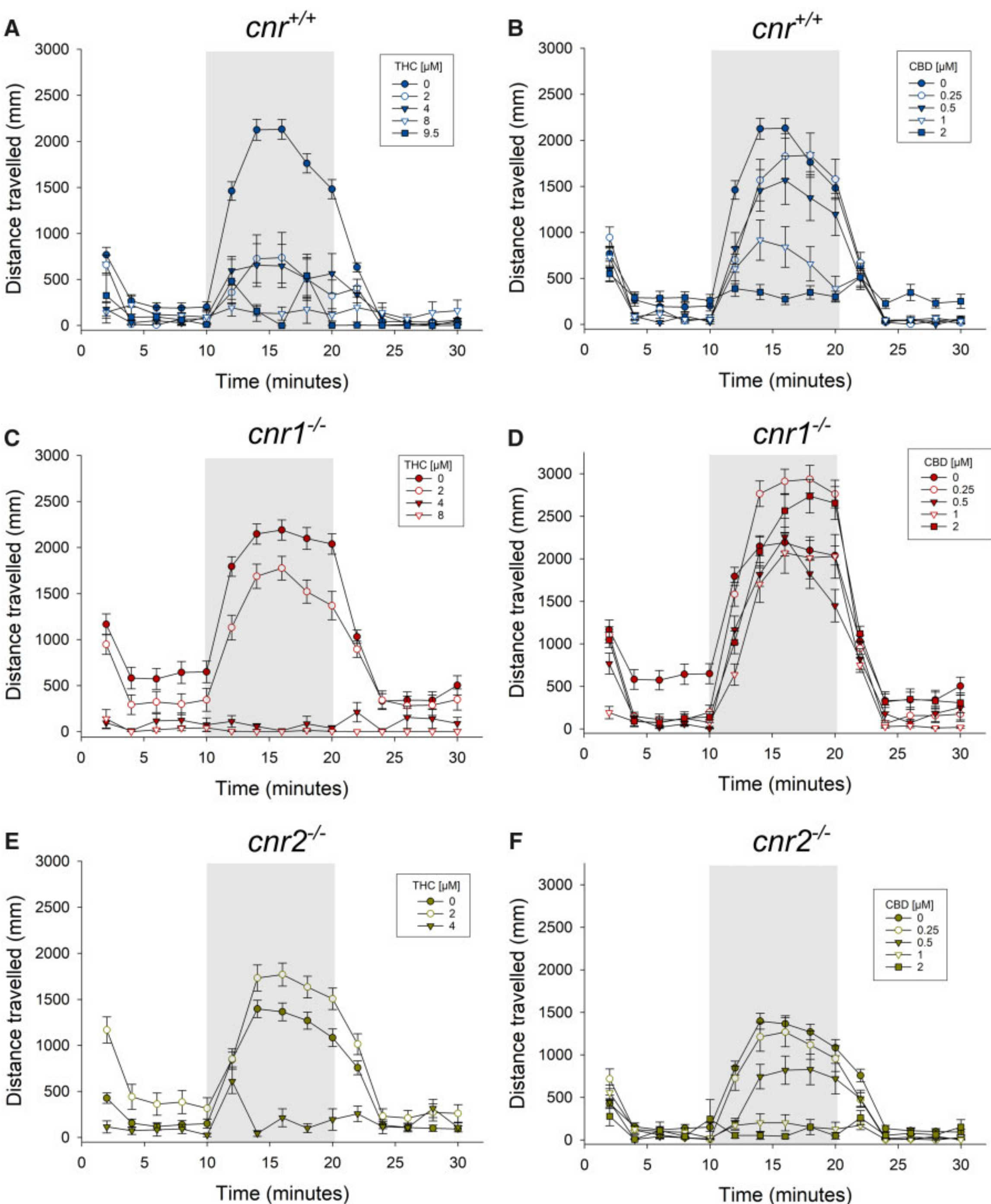

Figure 4. Total distance travelled (mean ± SE, $n = 50$) over the 30 min during the light:dark: Light test of the $cnr^{+/+}$, $cnr1^{-/-}$, and $cnr2^{-/-}$ strains of zebrafish used in this study exposed to THC (A, C, E) or CBD (B, D, F).

downregulation of *PPAR*γ. Both of the unexposed control *cnr* null strains had significantly higher relative expression of PPARα and PPARγ than the unexposed control $cnr^{+/+}$ when comparing expression (Supplementary Figure 10).

DISCUSSION

This study found that developmental exposure to THC or CBD in zebrafish results in significant effects on the transcriptome, larval behavior, and developmental abnormalities, consistent with previous studies. Furthermore, THC and CBD effects were ameliorated in *cnr* mutants, and by a PPARγ antagonist, suggesting these receptors play distinct roles in modulating the developmental effects of cannabinoid exposure. Collectively, these data demonstrate that exposure to THC or CBD during development causes significant adverse outcomes at both the cellular and organismal level. Based on the molecular changes observed in this study, we have proposed an adverse outcome pathway framework for THC and CBD developmental toxicity, beginning

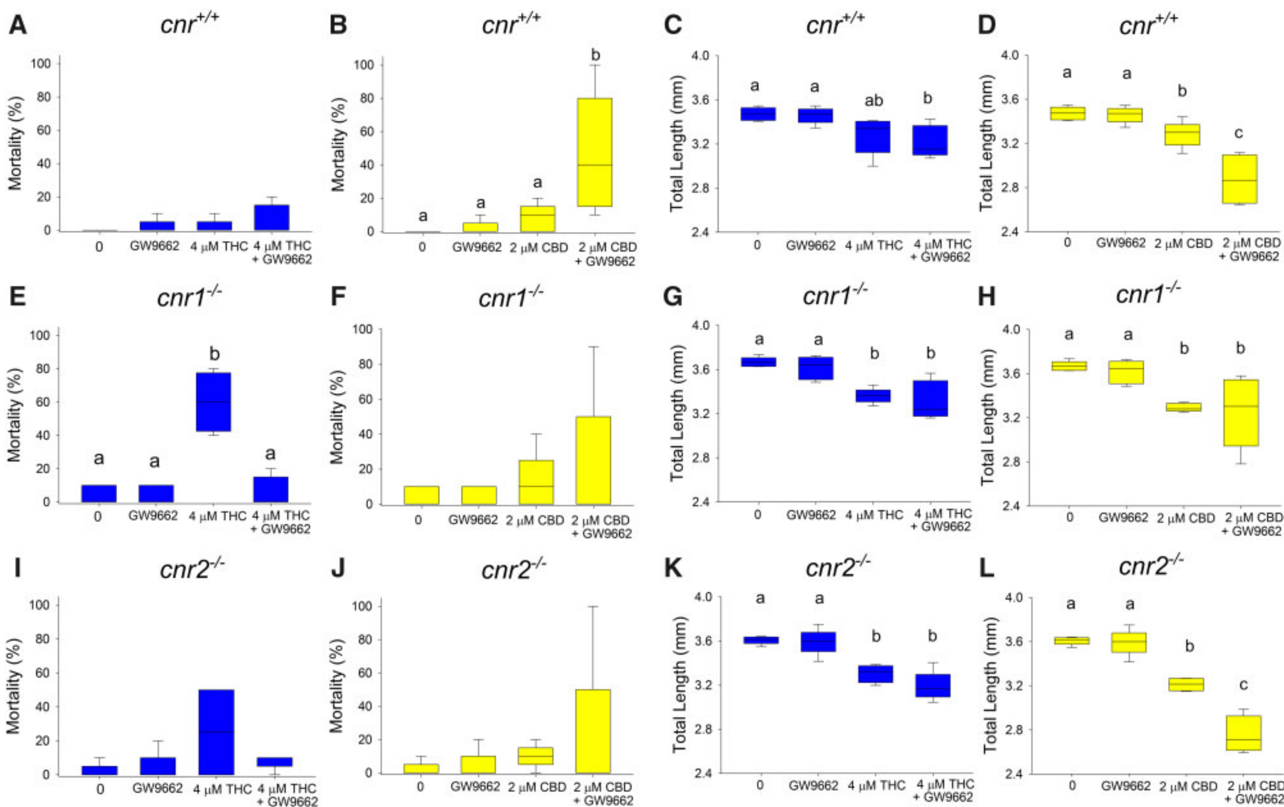

Figure 5. Survival (%, A:J) and total length (mm, C:L) of 96 hpf larval zebrafish developmentally exposed to THC, CBD, GW9662 (PPARγ antagonist) or a mixture ($n = 5$). Different letters indicate a significant difference between groups (ANOVA, Tukey's posthoc, $p \leq .05$).

with receptor binding to Cnr1, Cnr2, and/or PPARs, in turn altering metabolic pathways (eg, retinol), and resulting in adverse developmental and behavioral outcomes (Figure 7).

To our knowledge, this is the first study describing the transcriptional responses to THC and CBD exposure in a developing vertebrate. One of the predominant groups of differentially expressed genes were those related to diverse metabolic pathways, including cytochrome P450 genes involved in the breakdown of THC and CBD and genes associated with energy metabolism. These results are not surprising given the fact that developing zebrafish are metabolically active and metabolize a wide range of xenobiotic compounds. Both THC and CBD serve as substrates for cytochrome P450s, particularly *CYP3A4* and *CYP2C9*, significant contributors of THC and CBD metabolism (Hryhorowicz *et al.*, 2018). Our analysis revealed upregulation of a number of cyp2 (*cyp2c9, 2aa12, 2p6, 2p7, 2p8, 2k6, 2k16, 2k18, 2k19, 2x9, 2x10.2, 2ad6, 2ad3, 2aa7, 2aa12, 2y3*) and two cyp3 family member (cyp3c1 and cyp3c4) genes. Due to genome duplication, zebrafish have a total of 94 CYP genes, distributed among 18 gene families found also in mammals (Goldstone *et al.*, 2010). The relationship between human and zebrafish cyp2 and cyp3 gene families is more complex, with more than 1 ortholog to each human gene. Irrespective of the complexity, upregulation of numerous cyp2 and cyp3 family members suggests that THC and CBD are metabolized in zebrafish embryos.

Another group of metabolic genes differentially expressed in response to cannabinoid exposure included those associated with energy metabolism. The majority of these genes were upregulated in response to THC and CBD exposure, supporting well-established effects on energy homeostasis (Kunos *et al.*, 2008). One previous study (Liu *et al.*, 2016) showed that deletion of cannabinoid receptor in zebrafish leads to liver abnormalities, including delayed development, suggesting that the role of cannabinoids on energy metabolism are evolutionarily conserved. We also observed decreased growth (standard length) in the larvae at the end of the exposure period as well as decreased locomotory behavior, suggesting that effects on metabolic gene expression could lead to phenotypic changes. Furthermore, we have previously shown that this decrease in size due to developmental THC or CBD exposure persists into old age (2.5 years) for female zebrafish (Pandelides *et al.*, 2020a, 2020b). Together, these data suggest that metabolic disruption due to developmental THC or CBD exposure can result in long-term changes in growth.

A key group of transcriptional factors involved in energy homeostasis is peroxisome proliferator-activated receptors (PPARs). Our results showed enrichment of genes associated with PPAR signaling including differential expression of several lipid and carbohydrate metabolism genes. All 3 PPAR subtypes are ubiquitously expressed during zebrafish development (Den Broeder *et al.*, 2015) and their roles in development, adipocyte differentiation, neurodevelopment, and immune response are well established (Tyagi *et al.*, 2011). We observed upregulation of PPARα and PPARγ gene expression in response to THC and CBD exposure and differential expression of a number of downstream pathways.

There are multiple lines of evidence for THC and CBD interaction with PPARs including binding studies (Granja *et al.*, 2012) and increased transcriptional activity measured following exposure to THC or CBD (Hegde *et al.*, 2015; O'Sullivan *et al.*, 2009, 2005; Takeda *et al.*, 2014). Two hypotheses associated with cannabinoid/PPAR interactions (O'Sullivan, 2016) suggest that

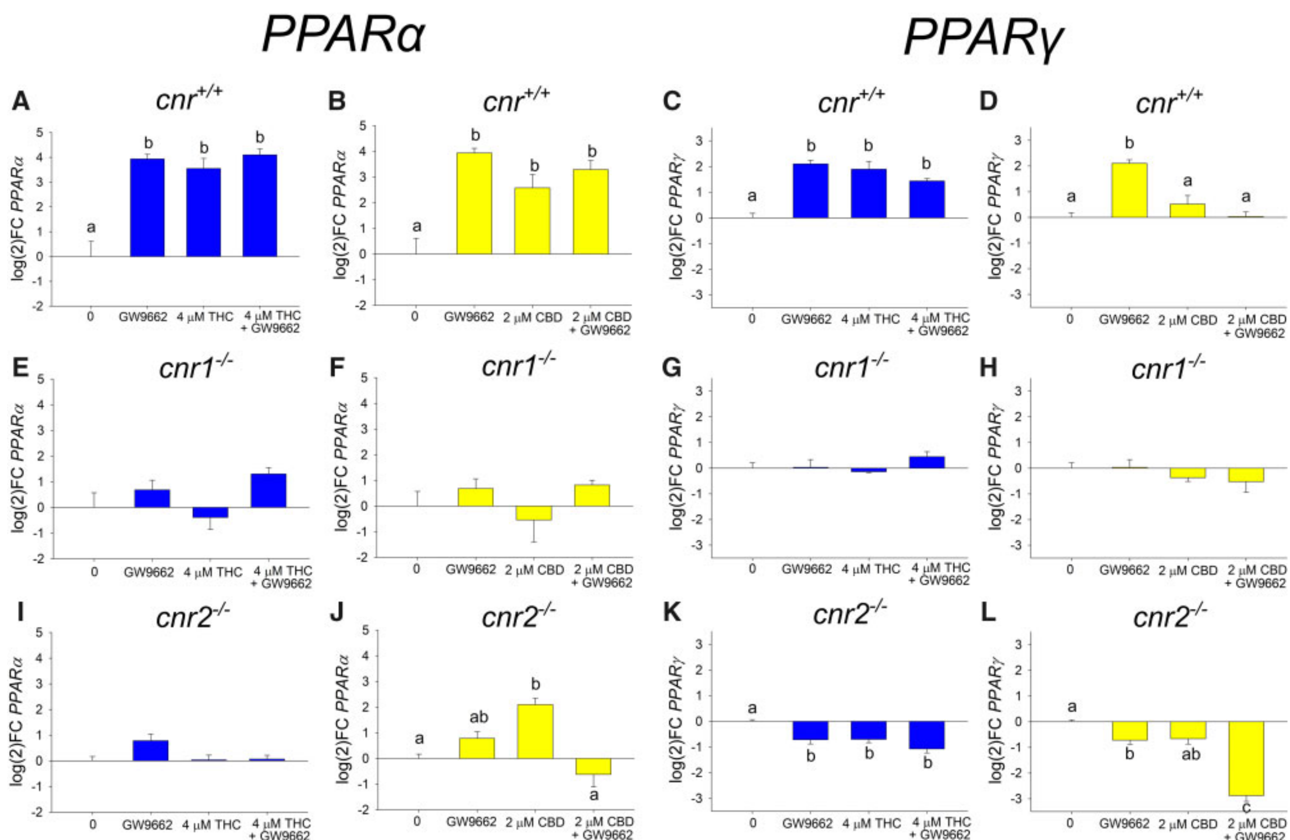

Figure 6. Expression log(2)fold change of *PPARα* (A:J) and *PPARγ* (C:L) of 96 hpf larval zebrafish developmentally exposed (6 – 96 hpf) to THC, CBD, GW9662 (PPARγ antagonist), or a mixture ($n = 3 - 5$). Different letters indicate a significant difference between groups (ANOVA, Tukey's posthoc, $p \leq .05$).

cannabinoids bind directly to PPARs and/or activation at the cell surface of cannabinoid receptors initiates intracellular signaling cascades that lead to the activation of PPARs indirectly.

Both THC and CBD are actively transported intracellularly by fatty acid binding proteins (FABPs) and this may be how they are transported into the nucleus (Elmes *et al.*, 2015). It is likely that FABPs direct cannabinoids either to FAAHs for enzymatic degradation, or to the nucleus for PPAR activation; but it is not yet known what is driving one pathway over another. Further, alteration of FABPs may drive increased gene expression of PPARα and γ (Wolfrum *et al.*, 2001). We observed significant upregulation of *fabp7a* and *fabp11a/fabp4* in only the CBD-treated fish (RNAseq data), suggesting CBD dysregulates FABPs. Increased adverse outcomes were observed in the mixture of CBD and the PPAR antagonist for both $cnr^{+/+}$ and $cnr2^{-/-}$ strains. Thus, PPARγ may play a role in the metabolism of CBD, counteracting some of the toxic effects at higher doses, but this needs further investigation.

Both *cnr* null strains had significantly higher expression of PPARγ than $cnr^{+/+}$ when comparing the expression of the unexposed controls, suggesting both Cnr1 and Cnr2 regulate endogenous PPAR expression. Further, the effects of THC or CBD on transcription of PPARs was not blocked by PPARγ antagonism. Additionally, the effect of exposure to THC or CBD on PPAR expression was either muted in the $cnr1^{-/-}$ fish or downregulated in the $cnr2^{-/-}$. It should be noted that whereas the concentration of THC or CBD did not cause significant toxicity in the $cnr^{+/+}$ fish, these concentrations did cause toxicity to the Cnr1 and Cnr2 knock out strains, which could have impacted their expression of PPARα and γ. Collectively, the results on PPARα and γ expression of our 3 strains suggest that Cnr1 and Cnr2 act upstream of the action of THC or CBD on PPARs.

PPARs play an important role in a number of pathways, dysregulation of these receptors by agonists/antagonists can lead to several adverse outcomes, including pulmonary fibrosis, edema, renal effects, liver and gall bladder disease, disruption of glucose metabolism, and changes in weight (Bortolini *et al.*, 2013; Brunmeir and Xu, 2018; Jeong *et al.*, 2019). In previous studies, THC caused time and PPARγ-dependent vasorelaxation in rat isolated arteries (O'Sullivan *et al.*, 2009, 2005). It is possible that when PPARγ was blocked in the $Cnr^{+/+}$ and $Cnr2^{-/-}$ cotreated with THC or CBD, the increased adverse outcomes such as pericardial edema were caused by PPARγ-dependent cardiovascular effects.

The PPARs also heterodimerize with other nuclear receptors such as retinoid X receptor (RXR) (Dawson and Xia, 2012; Qi *et al.*, 2000). Retinoids such as retinal, retinaldehyde, and apo14 modulate RXR and PPAR both in vitro and in vivo (Ziouzenkova *et al.*, 2007a, 2007b; Ziouzenkova and Plutzky, 2008). We observed significant upregulation of genes involved in retinol metabolism, including *rdh12l*, *rdh20*, *retsat*, *dhrs4*, *dhrs9*, *bco1*, *dgat1a* suggesting that PPAR-RXR crosstalk is affected. The PPARγ activates retinoic acid synthesis by inducing the expression of retinol metabolizing enzymes, including retinol dehydrogenase 10, DHRS9, and retinal metabolizing enzymes such as retinaldehyde dehydrogenase type 2 (RALDH2) in dendritic cells (Szatmari *et al.*, 2006). The PPARα-deficient mice exhibit disturbed retinoic acid homeostasis, with significantly reduced and increased expression of *dhrs4* and *raldh2*, respectively (Lin *et al.*, 2017). In the current study, we observed significant upregulation of both *pparα* and *pparγ*, which could

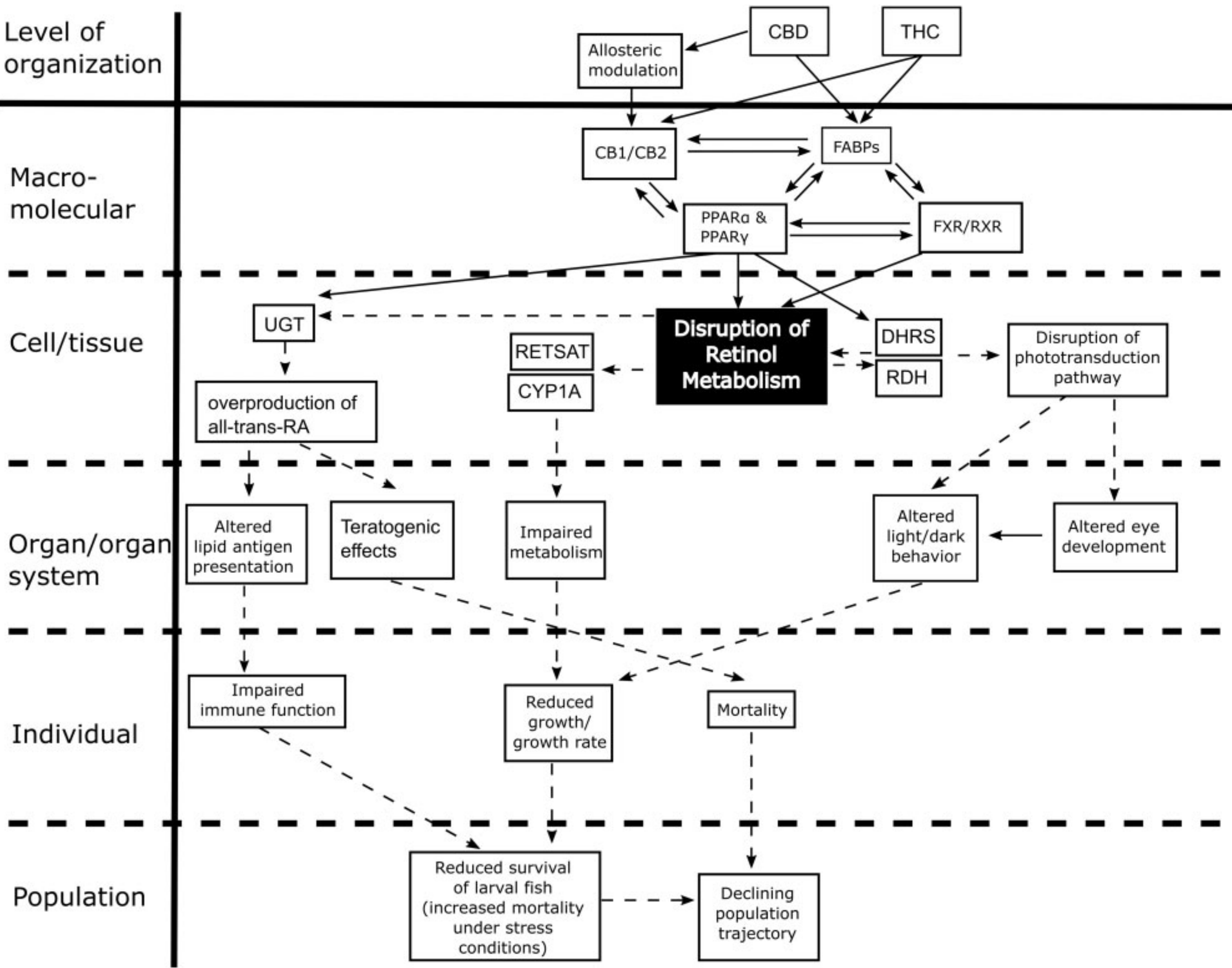

Figure 7. Proposed potential adverse outcome pathway for cannabinoid developmental toxicity focused on retinol metabolism and downstream adverse outcomes. Solid lines with arrows indicate known links, whereas dashed lines with arrows represent proposed adverse outcome pathways.

contribute to the disruption of retinol metabolism. Retinol is critical for both eye development and photoreception in vertebrate species (Luo *et al.*, 2006), including zebrafish (Marsh-Armstrong *et al.*, 1994). Our observations of eye size differences in THC- and CBD-exposed fish supports that disruption of retinol metabolism during development could be responsible for morphological defects.

In addition, we observed differential expression of genes associated with immune response particularly the complement system, a key innate immune response pathway. These effects were expected because both THC and CBD have well established anti-inflammatory properties (Hegde *et al.*, 2008; Klein *et al.*, 1998; Malfait *et al.*, 2000; Nagarkatti *et al.*, 2009; Stančić *et al.*, 2015). We have previously shown that developmental exposure to THC or CBD caused a significant reduction in the expression of many anti-inflammatory genes such as *tnfα*, *il-6*, and *il-1b* (Pandelides *et al.*, 2020a, 2020b). The anti-inflammatory properties of cannabinoids, such as THC, are mediated in part by cannabinoid receptors as well as other receptors including PPARα and γ (O'Sullivan, 2016). Together, these data suggest that immune changes caused by developmental THC or CBD exposure have the potential to have long-term consequences.

It is well established that THC and CBD effects are mediated in part by CNRs (Cristino *et al.*, 2020). The $cnr1^{-/-}$ and $cnr2^{-/-}$ strains in the current study exhibited significantly increased mortality and developmental abnormalities following THC exposure consistent with the fact that cnr1 and cnr2 null mutants have disrupted liver development and metabolic function (Liu *et al.*, 2016). Even before the liver is completely developed, zebrafish are capable of metabolic activity and metabolizing compounds such as caffeine or diclofenac as early as 25 – 50 hpf (Nawaji *et al.*, 2020). The diclofenac metabolites produced by the embryonic zebrafish in Nawaji *et al.* (2020) study are metabolized by CYP3A4 and CYP2C9 in humans, which as discussed above are important isozymes for THC and CBD metabolism. Consequently, impaired liver metabolism and clearance of THC and its active metabolites could contribute to the increased toxicity detected in the THC exposed knockout strains.

In contrast, CBD exposure did not have a pronounced effect in $cnr1^{-/-}$ and $cnr2^{-/-}$ mutants. Only the highest concentration of CBD caused significant mortality and affected growth reflecting the likelihood of different mechanisms of action between the cannabinoids. For example, THC has high affinity to Cnr1 (K_i = 5.05–80.3 nM) and Cnr2 (K_i = 3.13–75.3 nM), whereas CBD's is much lower (Cnr1 K_i = 4350–27 500 nM and Cnr2 K_i = 2400 to >10 000 nM). Cannabidiol is also a weak Cnr1 antagonist (IC_{50} = 3350 nM) and Cnr2 inverse agonist (IC_{50} = 27 500 nM) (Howlett *et al.*, 2002; Pertwee, 2008). Further, CBD acts as a negative

allosteric modulator for Cnr1 and Cnr2 at concentrations well below its reported K_i values, so it is possible that some of CBD's effects are still mediated through these 2 receptors (Laprairie *et al.*, 2015; Martínez-Pinilla *et al.*, 2017; Tham *et al.*, 2019). Further, the actions of CBD could be mediated through direct agonism with other receptors like PPARγ (Esposito *et al.*, 2011; O'Sullivan and Kendall, 2010), 5-hydroxytryptamine (serotonin) 1A (5-HT_{1A}) receptors (Sales *et al.*, 2018; Zanelati *et al.*, 2010), and transient receptor potential cation channel subfamily V member 1 (TRPV1) (De Petrocellis *et al.*, 2011).

Exposure to cannabinoids significantly impacts behavior in animal models, generally causing a reduction in motor activity as well as decreased anxiety-like symptoms (Carty *et al.*, 2019; Hasumi *et al.*, 2020; Hložek *et al.*, 2017; Luchtenburg *et al.*, 2019; Martin *et al.*, 1991; Sufian *et al.*, 2019; Varvel *et al.*, 2005). In zebrafish, the endocannabinoid system is critical to the development of the locomotor system (Luchtenburg *et al.*, 2019; Sufian *et al.*, 2019). Behavior was the most sensitive endpoint tested in this study, with significant effects observed at all concentrations tested in the $cnr^{+/+}$ strain except for the lowest CBD concentration. Exposure to THC or CBD generally caused reduced activity in a dose-dependent manner. It should be noted that the effects of cannabinoids on behavior can be biphasic with increased activity at low doses (lower than used in the present study) and decreased activity at higher doses (Viveros *et al.*, 2005). As expected, the $cnr1^{-/-}$ fish were more tolerant to THC exposure than the other 2 strains because CB1 receptor normally mediates many of the behavioral effects of THC (Varvel *et al.*, 2005). In addition to the Cnr1 and Cnr2 receptors, other endocannabinoid receptors could influence the effects of THC or CBD on behavior. Behavior especially in the context of development is a complex endpoint mediated by signaling across multiple cell types. For example, both proper eye development and photoreception is critical for zebrafish to respond to light: dark stimuli. It is possible that cannabinoid activation of other receptors such as TRPV1 could modulate retinal output as they are expressed in retinal ganglion and microglial cells (Ryskamp *et al.*, 2014). Another important gene that is part of the endocannabinoid pathway and mediates stress response in zebrafish is *faah2a* (Krug *et al.*, 2018). This gene is absent from rodents but is present in humans and fish (Wei *et al.*, 2006). We measured significant upregulation of *faah2a* in the THC- or CBD-exposed fish (RNAseq data). Disruption of this enzyme could cause a disruption in endocannabinoid levels, ultimately resulting in behavioral effects.

The endocannabinoid signaling system is critical during early development and alterations of it by cannabinoids are of concern. Understanding the relative risk of maternal or early life stage exposure to THC or CBD is essential. Developmental exposure to THC ($2-12\,\mu$M) or CBD ($0.25-4\,\mu$M) resulted in severe consequences for the development and gene expression of zebrafish embryos and larvae. Transcriptional responses observed in this study suggest that exposure to cannabinoids affects diverse physiological pathways ranging from metabolism to immune responses. Furthermore, retinol metabolism and PPAR signaling were significantly enriched following exposure to THC or CBD which could contribute to the developmental and behavioral abnormalities detected. We propose an AOP framework for THC and CBD developmental toxicity, beginning with receptor binding to Cnr1, Cnr2, and/or PPARs leading to alterations in metabolism and ultimately adverse outcomes at the organismal level (Figure 7). Specifically, THC exposure caused increased mortality and deformities (pericardial and yolk sac edemas, reduction in size) in $cnr1^{-/-}$ and $cnr2^{-/-}$ fish compared with $cnr^{+/+}$ suggesting Cnr receptors are involved in protective pathways. Conversely, the $cnr1^{-/-}$ larvae were more resistant to CBD-induced malformations, mortality, and behavioral alteration implicating Cnr1 in CBD-mediated toxicity. Behavior was the most sensitive endpoint to THC and CBD exposure with dose-dependent decreased larval distance travelled (96 hpf) at all concentrations tested in the $cnr^{+/+}$ fish (except $0.25\,\mu$M CBD). Differences in strain expression levels suggest that PPARγ is regulated by Cnr1 and Cnr2. Further, blocking PPARγ in addition to THC or CBD exposure resulted in an increase in developmental toxicity for the $cnr^{+/+}$ and $cnr2^{-/-}$ strains, but not the $cnr1^{-/-}$ strain, suggesting PPARγ plays an important protective role in THC/CBD metabolism. Collectively, these results indicate that PPARγ, Cnr1, and Cnr2 all play important roles in the developmental toxicity of THC and CBD with Cnr1 being the most critical.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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